PRODUCTION AND PURIFICATION OF GLUCOSAMINE AND ANGIOTENSIN-I CONVERTING ENZYME (ACE) INHIBITORY PEPTIDES FROM FIVE MUSHROOMS

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

Suranaree University of Technology

Academic Year 2016

กระบวนการผลิตและทำบริสุทธิ์กลูโคซามีนและเปปไทด์ที่มีฤทธิ์ยับยั้งแองจิโอเทนซินวันคอนเวอร์ติงเอนไซม์จากเห็ดห้าชนิด

นางผิง จาง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2559

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ผิง จาง : กระบวนการผลิตและทำบริสุทธิ์กลูโคซามีนและเปปไทด์ที่มีฤทธิ์ยับยั้งแองจิโอเทนซินวันคอนเวอร์ติงเอนไซม์จากเห็ดห้าชนิด (PRODUCTION AND PURIFICATION OF GLUCOSAMINE AND ANGIOTENSIN-I CONVERTING ENZYME (ACE) INHIBITORY PEPTIDES FROM FIVE MUSHROOMS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.มาโนชญ์ สุธีรวัฒนานนท์, 285 หน้า.

เห็ดเป็นอาหารที่มีการบริโภคมานานหลายศตวรรษ วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อผลิตทำบริสุทธิ์ และยืนยันสารกลูโคซามีนและเปปไทด์ที่มีฤทธิ์ยับยั้งเอนไซม์เอซีอีจากเห็ด 5 ชนิด คือ เห็ดเข็มทอง (*Flammulina velutipes*) เห็ดนางฟ้า (*Pleurotus ostreatus*) เห็ดฟาง (*Volvariella volvacea*) เห็ดชิตาเกะ (*Lentinus edodes*) และเห็ดหูหนูดำ (*Auricularia auricular*) ในการศึกษาพบว่ากรดซัลฟูริกมีประสิทธิภาพสูงกว่ากรดเกลือในการผลิตกลูโคซามีน โดยสภาวะที่เหมาะสมในการผลิตกลูโคซามีนคือสภาวะทีอัตราส่วนของเห็ดต่อกรดซัลฟูริกเข้มข้น 5.67 โมลาร์ เท่ากับ 1:10 โดยมวลต่อปริมาตร ที่อุณหภูมิ 100 องศาเซลเซียสและเวลา 4.03 ชั่วโมง ปริมาณกลูโคซามีนที่ผลิตได้ขึ้นอยู่กับปริมาณไคตินที่อยู่ในเห็ดและชนิดของกรดที่ใช้ในการย่อย โดยจลนพลศาสตร์ของการย่อยเป็นจัดเป็นปฏิกิริยาอันดับหนึ่ง ที่มีพลังงานกระตุ้นอยู่ระหว่าง 15.91-203.55 กิโลจูลส์ต่อโมล จากการศึกษาพบว่า เห็ดฟางให้ร้อยละผลได้กลูโคซามีนมากที่สุด (56.81±3.57 มิลลิกรัมต่อกรัม) และพลังงานกระตุ้นต่ำที่สุด (42.76 กิโลจูลส์ต่อโมล) หลังจากนำเห็ดที่ย่อยด้วยกรดแล้วมาตกตะกอนเปปไทด์ด้วยเอธานอล แล้วนำมาผ่านกระบวนการกรองอัลตราฟิลเทรชัน ปริมาณกลูโคซามีนเพิ่มขึ้น 2 เท่า ไฮโดรไลเซทจากเห็ดนางฟ้าแสดงกิจกรรมการยับยั้งเอนไซม์เอซีอีสูงที่สุด (IC_{50} 64.111 มิลลิกรัมต่อมิลลิลิตร) และประสิทธิภาพการยับยั้งเพิ่มขึ้น 69-175 เท่า หลังจากการทำบริสุทธิ์เปปไทด์ด้วยการทำบริสุทธิ์แบบ 4 ขั้นตอน ประกอบด้วยการกำจัดสี การตกตะกอน การแยกด้วยโครมาโตกราฟีแบบไอออนและเจลฟิลเทรชัน เปปไทด์ที่ได้คงตัวต่อความร้อน สภาพกรด/ด่าง และกระบวนการย่อยด้วยเอมไซม์ gastrointestinal proteases ทั้งนี้พบเปปไทด์ชนิด ASPYAFGL MLCSTTF และ LASLFGNDP ซึ่งมีประสิทธิภาพในการยับยั้งเอนไซม์เอซีอีสูงด้วยค่า IC_{50} เท่ากับ 0.1080 0.1524 และ 0.2491 ไมโครโมลาร์ ตามลำดับ การศึกษานี้แสดงให้เห็นว่าไฮโดรไลเซทจากเห็ดที่ผ่านการย่อยด้วยกรดมีกลูโคซามีนและเปปไทด์ที่มีฤทธิ์ในการยับยั้งเอนไซม์เอซีอี สามารถนำมาใช้เป็นสารตั้งต้นสำหรับอาหารสุขภาพสำหรับป้องกันและรักษาโรคข้อเข่าเสื่อมและโรคความดันโลหิตสูงได้

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา Pin Zhang

ปีการศึกษา 2559 ลายมือชื่ออาจารย์ที่ปรึกษา

PIN ZHANG : PRODUCTION AND PURIFICATION OF GLUCOSAMINE AND ANGIOTENSIN-I CONVERTING ENZYME (ACE) INHIBITORY PEPTIDES FROM FIVE MUSHROOMS. THESIS ADVISOR : ASSOC. PROF. MANOTE SUTHEERAWATTANANONDA, Ph.D., 285 PP.

GLUCOSAMINE/ACE INHIBITORY PEPTIDES/MUSHROOM/ACID HYDROLYSIS/PRODUCTION/PURIFICATION

Mushrooms have been consumed as nutritional foods for many centuries. The objectives of this study were to produce, purify and identify glucosamine and ACE inhibitory peptides from five mushrooms, including the enoki mushroom (*Flammulina velutipes*), oyster mushroom (*Pleurotus ostreatus*), straw mushroom (*Volvariella volvacea*), shiitake (*Lentinus edodes*) and wood ear mushroom (*Auricularia auricular*). Sulfuric acid was more effective than hydrochloric acid for glucosamine production. The optimum conditions were identified as follows: ratio of raw material to acid volume, 1:10 (w/v); sulfuric acid, 5.67 M; hydrolysis temperature, 100°C; and time, 4.03 h. In addition, the glucosamine yield was found to be dependent on the chitin contents of the raw materials and the type of acid used. Hydrolysis kinetics were fitted with a first-order reaction model with activation energies ranging from 15.91 to 203.55 kJ/mol. Enoki mushroom was the easiest to hydrolyze, while the wood ear mushroom needed a longer time for glucosamine to be liberated. Among the five mushrooms, straw mushroom had the highest glucosamine yield (56.81±3.57 mg/g) and the relatively low activation energy (42.76 kJ/mol) suggested that sulfuric acid hydrolysis of the straw mushroom could be suitable for future industrial application

for glucosamine production. Glucosamine was enriched 2-fold in the ethanol-soluble fraction after ultrafiltration and ethanol precipitation. Oyster mushroom hydrolysate showed the maximum ACE inhibitory activity (IC_{50}, 64.111 mg/mL). The potent ACE inhibitory peptides after purification were increased 69-175 fold by a four-step purification procedure. All of the purified peptides showed satisfactory stability against heat, pH and in vitro gastrointestinal digestion. Thirteen novel peptides were identified, of which ASPYAFGL, MLCSTTF, and LASLFGNDP were found to exhibit high inhibition potency with IC_{50} values of 0.1080, 0.1524, and 0.2491 μM, respectively. This study suggested that mushroom hydrolysates containing glucosamine and ACE inhibitory peptides could be exploited as multifunctional ingredients for functional foods against osteoarthritis and hypertension.

School of Food Technology

Academic Year 2016

Student's Signature Pin Zhang

Advisor's Signature

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LIST OF ABBREVIATIONS

AA	=	Adjuvant arthritis
ACE	=	Angiotensin-I converting enzyme
ACLT	=	Anterior cruciate ligament transection
ACR	=	American College of Rheumatology
Ang-I	=	Angiotensin-I
Ang-II	=	Angiotensin-II
ANN	=	Artificial neural network
ANOVA	=	Analysis of variance
AOAC	=	Association of Official Analytical Chemists
AP	=	Activating protein
AR	=	Amphiregulin
BBD	=	Box-Behnken design
BSA	=	Bovine serum albumin
°C	=	Degree Celsius
CA	=	Cellulose acetate
CAD	=	Canadian dollar
CAM	=	Complementary alternative medicine
CE	=	Capillary electrophoresis
CIA	=	Collagen-induced-arthritis
COs	=	Chitooligosaccharides

LIST OF ABBREVIATIONS (Continued)

COX	=	Cyclooxygenase
CTGF	=	Connective tissue growth factor
CV	=	Coefficient of variation
CVDs	=	Cardiovascular diseases
Da	=	Dalton
DA	=	Degree of acetylation
DF	=	Degrees of freedom
DI	=	De-Ionized
DM	=	Dry matter
DNA	=	Deoxyribonucleic acid
DO	=	Dissolved oxygen
DW	=	Dry weight
DWGP	=	Defatted wheat germ protein
E_a	=	Activation energy
EDTA	=	Ethylenediaminetetraacetic acid
EGFR	=	Epidermal growth factor receptor
ES	=	Effect size
ESI-QUAD-TOF	=	Electrospray ionization quadrupole-time-of-flight mass spectrometer
FAO	=	Food and Agriculture Organization of the United Nations
FAP	=	Furylacryloyl-L-phenylalanine
FAPGG	=	N-3-(2-furyl acryloyl)-phenylalanylglycylglycine

LIST OF ABBREVIATIONS (Continued)

Fmoc-Cl	=	9-fluorenylmethoxycarbonyl chloride
FMOC-Su	=	N-(9H-Fluoren-2-ylmethoxycarbonyloxy) succinimide
FPLC	=	Fast protein liquid chromatography
GAGs	=	Glycosaminoglycans
GAIT	=	Glucosamine/chondroitin Arthritis Intervention Trial
GG	=	Glycylglycine
GI	=	Gastrointestinal
GIT	=	Gastrointestinal tract
GlcN	=	Glucosamine
GlcNAc	=	N-acetyl-glucosamine
GlcN-HCl	=	Glucosamine hydrochloride
GlcN-S	=	Glucosamine sulfate
GLUT	=	Glucose transporter
HA	=	Hyaluronic acid
HHL	=	Hippuryl-histidyl-leucine
HPLC	=	High performance liquid chromatography
HS	=	Heparan sulfates
iNOS	=	Inducible nitric oxide synthase
IL	=	Interleukin
JNK	=	c-Jun NH2-protein kinase
JSN	=	Joint space narrowing
JSW	=	Joint space width

LIST OF ABBREVIATIONS (Continued)

k	=	Reaction rate
KKS	=	Kallikrein-kinin system
LC	=	Liquid chromatography
LC-MS/ESI	=	Liquid chromatography-electrospray ionization mass spectrometry
LMWC	=	Low molecular weight chitosan
mPGES1	=	Microsomal prostaglandin E synthase 1
MAs	=	Meta-analyses
MAPK	=	Mitogenactivated protein kinases
MDA	=	Malondialdehyde
MMPs	=	Matrix metalloproteinases
MS	=	Mean square
MW	=	Molecular weight
MWCO	=	Molecular weight cut-off
NF-κB	=	Nuclear factor-kappa B
NO	=	Nitric oxide
NOS	=	Nitric oxide synthase
NSAIDs	=	Nonsteroidal anti-inflammatory drugs
OA	=	Osteoarthritis
OARSI	=	Osteoarthritis Research Society International
OPA	=	*o*-phtaldialdehyde

CHAPTER I

INTRODUCTION

1.1 Background of the selected topics in this study

1.1.1 Glucosamine and osteoarthritis

Osteoarthritis (OA) is the most common type of arthritis. It is characterized by the progressive degeneration of articular cartilage, subchondral bone sclerosis, osteophyte formation, synovial inflammation, synovial angiogenesis and bone remodeling (Dieppe and Lohmander, 2005, Xie et al., 2015). Pain and loss of function are the main clinical features of OA (Haugen et al., 2011, Haugen et al., 2013).

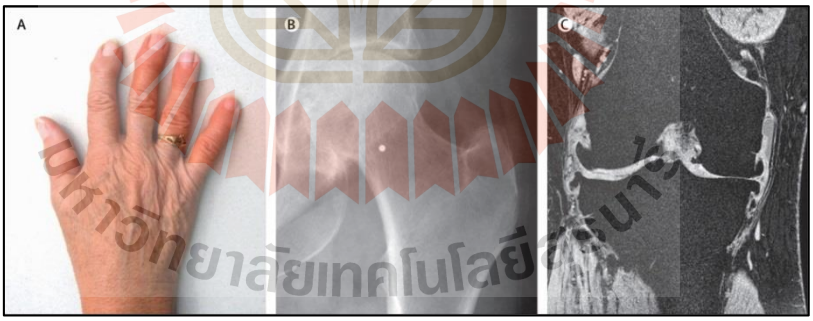


Figure 1.1 Osteoarthritic joints of the hand, hip, and knee (Bijlsma, Berenbaum and Lafeber, 2011). (A) OA is predominantly identified in the distal interphalangeal and proximal interphalangeal joints-deformations of the distal interphalangeal joints are clearly visible. (B) Plain radiograph of an osteoarthritic hip joint showing the narrowing of the joint space and clearly visible osteophytes. (C) Magnetic Resonance Imaging of an

osteoarthritic knee with clear medial cartilage loss and osteophyte formation, with minor synovial swelling.

Worldwide estimation reveals that 10% to 15% of all adults aged over 60 have some degree of OA, with prevalence higher among women (20%) than men (10%) (Veronese et al., 2016, WHO, 2014). OA may develop in any joint if it is used extensively or damaged from fractures or other injuries. However, OA most commonly affects the weight-bearing joints such as the knee, hip, hand, spine, and feet than in the wrist, elbow, and shoulder joints (Litwic, Edwards, Dennison and Cooper, 2013). In people older than 80 years, 53% of women and 33% of men had radiographic OA of the knee. The age-standardized and sex-standardized incidence rate of OA of the hand is 100 per 100,000 person-years, for the hip is 88 per 100,000 person-years, and for the knee is 240 per 100,000 person-years (Oliveria et al., 1995).

It is projected that 130 million people will suffer from OA worldwide, of whom 40 million will be severely disabled by the disease by 2050 (Kohlhof et al., 2016). The prevalence of OA is increasing because of several concomitant factors: population growth and aging due to increasing life expectancy, increasing overweight and obesity prevalence, and lack of disease-modifying OA drugs able to modify the progression of the disease (Salmon et al., 2016). Age is the strongest predictor of OA and therefore, increasing age and extended life expectancy will result in a greater occurrence of OA. OA most commonly affects the middle-aged and elderly, although it may begin earlier as a result of injury or overuse. OA becomes more common with age, and after age 50 years more women than men are affected. For example, 67% of women and 55% of men had radiographic OA of the hand were reported from the

Rotterdam study of a population-based cohort of 3906 people 55 years or older reported (Dahaghin et al., 2005). OA is thought to be a leading cause of physical disability and morbidity that affecting half of the individuals over age 65 (Joseph et al., 2015, Loeser, 2010, Lv et al., 2015).

OA's burden is escalating at a fast rate due to the high prevalence of disease and high use of associated resources (Murray et al., 2013, Woolf and Pfleger, 2003, Yelin et al., 2007). The direct costs associated with OA are significant compared to other musculoskeletal diseases (Bitton, 2009, Yelin, 1998). A patient with OA, even in its early stages, consumes health care resources at almost double the rate of the general population without OA (DiBonaventura et al., 2012). Sharif et al. (2015) estimated the direct cost burden of OA in Canada using a microsimulation model. From 2010 to 2031, as the prevalence of OA is projected to increase from 13.8% to 18.6%, the total direct cost of OA is projected to increase from $2.9 billion to $7.6 billion, an almost 2.6-fold increase (in 2010 $CAD). From the highest to the lowest, the cost components that will constitute the total direct cost of OA in 2031 are hospitalization cost ($2.9 billion), outpatient services ($1.2 billion), alternative care, and out-of-pocket cost categories ($1.2 billion), drugs ($1 billion), rehabilitation ($0.7 billion) and side-effect of drugs ($0.6 billion). By 2040, the associated economic burden is projected to rise 6-fold from close to $200 billion in 2015 (Bombardier, Hawker and Mosher, 2011).

The etiology of OA is multifactorial, with inflammatory, metabolic, and mechanical causes (Beyreuther, Callizot and Stohr, 2007). Despite the fact that OA is a widespread and debilitating disease, the principal treatment objectives are limited to control pain adequately, improve function, and reduce disability with analgesics,

physiotherapy and, when symptoms are severe, joint replacement (Larkin et al., 2015, Wluka et al., 2014). For decades, the traditional pharmacologic management of OA includes mainly analgesics, nonsteroidal anti-inflammatory drugs (NSAIDs) and visco-supplements (Arunkumar, Indulekha, Vijayalakshmi and Srivastava, 2016). These drugs are mainly symptomatic without well-documented influence on the duration of the disease and its progression. They are also associated with long-term adverse effects such as gastrointestinal bleeding and cardiovascular events (Wieland, Michaelis, Kirschbaum and Rudolphi, 2005). Thus the need for interventions to prevent and reduce disease progression is important.

Recently, dietary supplements like glucosamine (GlcN, 2-amino-2-deoxy-D-glucose) have become mainstream products in the management of OA (Reginster, Bruyere, Lecart and Henrotin, 2003). GlcN is an amino sugar that each molecule contains a glucose and an amine. Structurally, it is a modified glucose molecule in which the hydroxyl group of the C2 atom is replaced by an amino group so it has a small molecule (molecular weight = 179.71 g/mol) that can be absorbed easily *in vivo*. In the human body, it is a constituent of glycosaminoglycan in the cartilage matrix and synovial fluid and has been used orally for the treatment of OA since the early 1980s. After oral administration, GlcN is bioavailable and reaches the articular cartilage. It is preferentially incorporated by the chondrocytes into the components of the glycosaminoglycan chains in the intact cartilage, stimulates the synthesis of physiological proteoglycans, and decreases the activity of catabolic enzymes, including metalloproteases (Piperno et al., 2000). In addition, the compound may reverse some of the negative effects of interleukin-1 on cartilage metabolism (Fenton, Chlebek-Brown, Caron and Orth, 2002). Also, there is a mild anti-inflammatory effect

exerted by the suppression of superoxide radical generation or the inhibition of inducible nitric oxide synthesis and selectively, of the cyclooxygenase-2 pathway (Azuma et al., 2015, Nagaoka et al., 2011).

1.1.2 ACE inhibitory peptides and cardiovascular diseases

Cardiovascular diseases (CVDs) are a group of diseases involving the heart, blood vessels, or the sequelae of poor blood supply due to a diseased vascular supply (Bloom et al., 2011). Over the past decade, CVDs have become the number one leading cause of death worldwide, representing nearly 30% of all deaths. In 2012, CVDs were responsible for 17.5 million deaths and the annual CVDs-related mortality is projected to increase to 22.2 million in 2030 (WHO, 2014). In 2010, the global cost of CVDs is estimated at US$ 863 billion (an average per capita cost of US$ 125), and it is estimated to rise to US$ 1,044 billion in 2030 - an increase of 22% (Bloom et al., 2011). Hypertension also referred to as high blood pressure, is a medical condition in which the blood pressure level in the arteries is consistently above the normal range (> 140/90 mmHg) (Ibadallah, Abdullah and Shuib, 2015). It represents a leading risk factor for CVDs (Geng et al., 2016). Due to the prevalence of hypertension and its great impact on the health status of human populations, it is important to maintain a healthy lifestyle to reduce the risk of hypertension. Thus, the prevention of hypertension is concomitantly required to combat the enormous burden of CVDs and to improve the quality and length of life of the global population.

Although hypertension is influenced by factors such as cigarette smoking, alcohol consumption, obesity, and environmental pollutants, it is well recognized that angiotensin-I converting enzyme (ACE, dipeptidyl carboxypeptidase I, Kininase II, EC 3.4.15.1) plays an important role in the regulation of peripheral blood pressure

mainly through the renin-angiotensin (RAS) and kallikrein-kinin systems (KKS) (Choi et al., 2001, Lee et al., 2004). ACE is a zinc-containing enzyme located in the endothelial lining of the vasculature of the lung, is an exopeptidase that cleaves dipeptides from the C-terminus of various oligopeptides (Choi et al., 2001). ACE catalyzes the conversion of inactive Angiotensin-I (Ang-I, Asp-Arg-Val-Tyr-Ile- His- Pro-Phe-His-Leu) to Angiotensin-II (Ang-II, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) (Mao and Li, 2015). Ang-II, a potent vasoconstrictor, stimulates the secretion of aldosterone, which enhances sodium and water reabsorption in the nephron, and therefore increases the arterial pressure by bringing about a rise in the intravascular fluid volume. Furthermore, ACE regulates the inactivation of a vasodilator, bradykinin (Toopcham, Roytrakul and Yongsawatdigul, 2015). Thus, a constant blood pressure is normally maintained by the hypertensive peptide, Ang-II, and the hypotensive peptide, bradykinin. These conversion actions endow ACE with a positive contribution to the regulation of blood pressure and therefore, inhibition of ACE has been considered as a target for the prevention and treatment of hypertension.

To date, a series of ACE inhibitory drugs including captopril (D-3-mercapto-2-methylpranory-l-proline), enalapril, lisinopril and so on has been synthesized and currently used clinically as antihypertensive drugs in addition to other beneficial effects on glucose and lipid metabolism, such as decreasing insulin requirements in diabetes and increasing exercise tolerance (Lee et al., 2004). However, these synthetic drugs are believed to have disadvantages, such as easy digestion by the protease in the body, and various undesirable side effects such as dizziness, cough, taste disturbances, skin rashes and more serious complications such as proteinuria and disturbance of kidney function (Jang et al., 2011). For this reason, safer, novel and

economical ACE inhibitors that have resistance to digestion by various proteases are necessary for the prevention and remedy of hypertension.

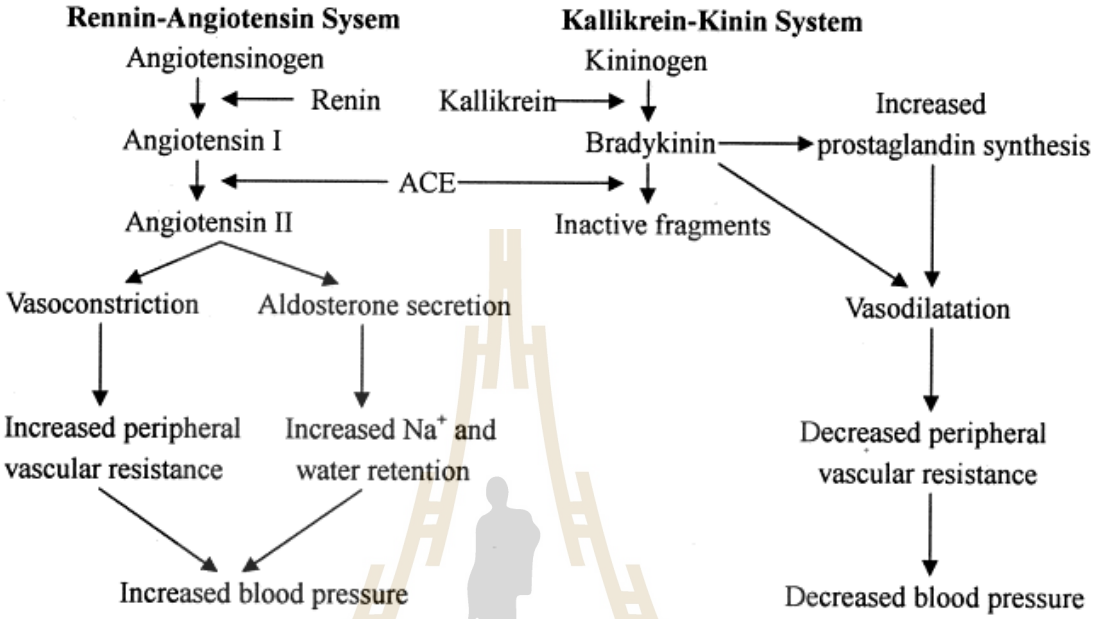


Figure 1.2 Role of ACE in blood pressure regulation (Li, Le, Shi and Shrestha, 2004).

The ACE inhibitory peptides derived from food proteins are considered to be milder and safer compared with synthetic drugs; furthermore, these peptides usually have multifunctional properties and are easily absorbed. Therefore, researchers are interested in research for inhibitors derived from food protein. ACE inhibitory peptides produced from food protein was first reported by Oshima, Shimabukuro and Nagasawa (1979). Afterward, many other ACE inhibitory peptides have been discovered from different food proteins and processed food (Ahn, Jeon, Kim and Je, 2012, Ashar and Chand, 2004, Balti et al., 2010, Banerjee and Shanthi, 2012, Byun and Kim, 2001, Chen et al., 2012, Escudero, Sentandreu, Arihara and Toldrá, 2010, Gibbs, Zougman, Masse and Mulligan, 2004, Hatanaka, Miyahara, Suzuki and Sato,

2009, Himaya, Ngo, Ryu and Kim, 2012, Je, Park, Kwon and Kim, 2004, Jung et al., 2006, Kim et al., 2016, Ko et al., 2012a, Ko et al., 2012b, Lee, Qian and Kim, 2010, Lin, Lv and Li, 2012, Pihlanto, Virtanen and Korhonen, 2010, Puchalska, García and Marina, 2014, Quirós et al., 2007, Rho et al., 2009, Ruiz, Ramos and Recio, 2004, Sheih, Fang and Wu, 2009). In Japan, the thermolysin-digested dried bonito (Katsuobushi oligopeptide) has been officially approved as "Foods for Specified Health Use" by the Ministry of Health and Welfare and marketed for hypertension treatment (Fujita, Yamagami and Ohshima, 2001, Nakashima et al., 2002).

In recent years, the research on mushrooms and their production have received increased attention because of the recognition that mushrooms are nutritious food with health-stimulating properties and medicinal effects (Kalač, 2013, Lavanya and Subhashini, 2013). Some research papers have reported ACE inhibitory peptides from water extracts of some mushrooms (Choi et al., 2001, Jang et al., 2011, Kang et al., 2013, Lau, Abdullah, Shuib and Aminudin, 2014). However, no study has been conducted on the production of GlcN and ACE inhibitory peptides from mushrooms by acid hydrolysis.

1.1.3 Chitin, protein, and peptides from mushrooms

Chitin and its deacetylated form, chitosan are natural polysaccharides. Chitin is the dominant component of arthropods' exoskeletons and fungal cell walls. It is characterized by β-(1→4)-branched N-acetyl-glucosamine (GlcNAc) units (Nitschke, Altenbach, Malolepszy and Mölleken, 2011). These chitin molecules are cross-linked via hydrogen bonds to each other, giving enormous strength to the chitin fibers. Therefore, chitin is typically an amorphous solid that is insoluble in water, dilute acids and alkali (Mario, Rapanà, Tomati and Galli, 2008). In organisms, chitin

fibrils embedded in a matrix of highly cross-linked, insoluble proteins make up a significant fraction of the cuticles of arthropods, annelids, molluscs, and the cell walls of many fungi and yeasts (Roseman, Li and Comb, 2010). Every year about 1,011 metric tons of chitin are produced from the oceans (LeCleir, Buchan and Hollibaugh, 2004). On the other hand, chitosan which is characterized by β-(1→4)-branched GlcN units has improved solubility because of its deacetylation (Kumar, 2000). There is only a small percentage of GlcN in native chitin, but the quantity of GlcN will be increased after chitin isolation (Roseman, Li and Comb, 2010). Due to the harsh chemical treatment conditions during chitin isolation, mainly severe alkaline treatment, chitin deacetylation has occurred (Aranaz et al., 2009). Chitin and chitosan have various commercial applications, but a greater interest can be found by converting them into its monomer components of GlcNAc and GlcN with improved properties and more applications. As it is well known, the hydrolysis of chitin and chitosan in concentrated acids proceeds with the formation of GlcN (Shabrukova, Shestakova, Zainetdinova and Gamayurova, 2002).

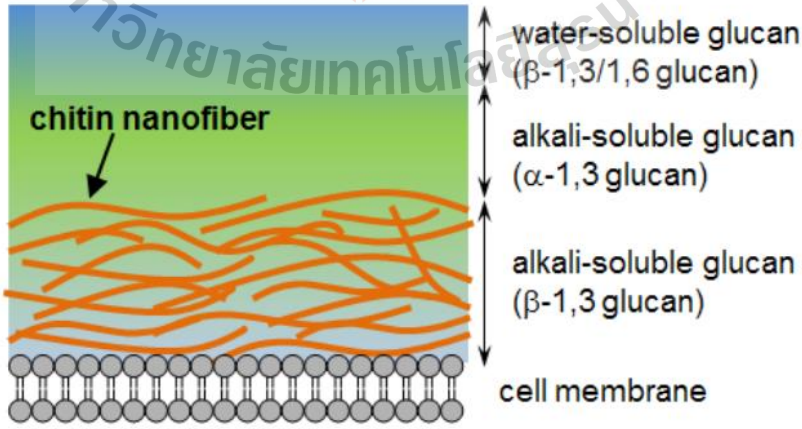


Figure 1.3 Schematic presentation of the cell-wall structure of a typical mushroom (Ifuku, Nomura, Morimoto and Saimoto, 2011).

Mushrooms, as the well-known fungi, have been consumed as popular foods throughout the world for many centuries (Phat, Moon and Lee, 2016). Nowadays there is an increasing public interest in the secondary metabolites from mushrooms for discovering new drugs or lead compounds. A number of bioactive constituents have been isolated from mushrooms including small molecule compounds, polysaccharides, proteins, polysaccharide-protein complexes, etc. (Ferreira, Vaz, Vasconcelos and Martins, 2010, Quang, Hashimoto and Asakawa, 2006, Wasser, 2010). These bioactive components have become popular sources of natural antioxidative, antitumor, antiviral, antimicrobial, and immunomodulatory agents. Among all the bioactive constituents of mushroom, the most extensively investigated compounds are mushroom polysaccharides. However, bioactive proteins constitute another important part of functional components in mushrooms, which also have increasing interests due to their pharmaceutical potential (Wong et al., 2010). Mushrooms produce a large number of proteins and peptides with interesting biological activities, such as ribosome inactivating proteins, antifungal proteins, ribonucleases, ubiquitin-like proteins and peptides, lectins, cellulases, xylanases, laccases, invertases and trehalose phosphorylases. Several proteins with antifungal property and other medicinal applications have been isolated from mushrooms such as a few *Pleurotus* species (*Pleurotus eryngii*, *Pleurotus ostreatus*, and *Pleurotus sajor-caju*), *Hypsizigus marmoreus* and *Lyophyllum shimeiji*, the medicinal mushroom (*Ganoderma lucidum*), the wild mushroom (*Polyporus alveolaris*) and two edible mushrooms (*Tricholoma giganteum* and *Agrocybe cylindracea*) (Wong et al., 2010). Kalač (2009) reviewed the chemical compositions and nutritional value of European species of wild growing mushrooms. Dry matter content of mushrooms was usually 100 g/kg. The main

components of dry matter were structural polysaccharides and proteins. The main representative carbohydrates included chitin, glycogen, mannitol, and trehalose. Forty-seven species of Macedonian edible mushrooms were analyzed by Petrovska (2001). The mean crude protein content was about 32.6% in dry matter. The protein content of most mushrooms is relatively higher than most vegetables. On the other hand, the chitin content of mushrooms is in the range of 0.3-19.6% DW (Cheung, 1996, Crestini, Kovac and Giovannozzi-Sermanni, 1996, Manzi, Aguzzi and Pizzoferrato, 2001, Mario, Rapanà, Tomati and Galli, 2008, Ofenbeher-Miletić, Stanimirović and Stanimirović, 1984, Tshinyangu and Hennebert, 1996). Additionally, the cultivation of mushrooms is inexpensive and not time-consuming (Petrovska, 2001). Therefore, mushrooms have the potential to be used as a raw material for the production of protein, chitin, and their oligomers including GlcN, peptide, and amino acid.

1.2 Research objectives of this study

(1) To optimize the conditions of GlcN production by acid hydrolysis from the oyster mushroom.

(2) To study the kinetics of GlcN production from five mushrooms.

(3) To purify and characterize GlcN and ACE inhibitory peptides from five mushroom hydrolysates.

(4) To evaluate the storage stability of mushroom hydrolysates.

1.3 Hypotheses of this study

(1) Under the optimized conditions, the chitin in mushroom is converted into GlcN efficiently. The factors affecting the GlcN production are acid type and concentration, hydrolysis temperature and time, dried or fresh mushroom, and the ratio of raw material to acid volume.

(2) Different raw materials have different chitin contents. Thus, under the same acid hydrolysis conditions, the yield of GlcN will be different.

(3) For different material, the existing state of chitin is different. As the converted products, the production of GlcN from different materials has different trend and kinetic parameters.

(4) The mushrooms have high protein content. After acid hydrolysis, the amino acid content and peptide content are high in the mushroom hydrolysate. Among the various peptides, the ACE inhibitory peptides may be present.

1.4 Significance and design routes of this study

1.4.1 Significance of this study

In industry, GlcN is mainly produced by concentrated hydrochloric acid hydrolysis of chitin extracted from exoskeletons of shellfish or other marine resources (lobster, shrimp, krill, crab, and prawn). However, this method has limitations such as low yield (below 65%) through consulting literature (Sashiwa et al., 2002). Another concern is the high content of sodium chloride in the final product. That may be increased the process cost and the risk of stroke, heart failure, osteoporosis, stomach cancer and kidney disease (Appel et al., 2011). On the other hand, the GlcN production from marine resources becomes limited by raw material supply as the

demand continues to increase (Deng et al., 2005). Moreover, GlcN from shellfish may not be suitable for consumers with shellfish allergies (Chen et al., 2012, Sitanggang, Wu and Wang, 2009). Considering all these conditions, in this study, five kinds of mushrooms were selected as new materials to avoid the shellfish allergies. Then, the GlcN yield from hydrochloric acid and sulfuric acid was compared to find a novel, efficient method to produce GlcN with high conversion rate and without sodium chloride and potential shellfish allergies in final samples.

1.4.2 The design route of this study

In this study, five kinds of mushrooms were used to produce GlcN and ACE inhibitory peptides, including enoki mushroom (*Flammulina velutipes*), oyster mushroom (*Pleurotus ostreatus*), straw mushroom (*Volvariella volvacea*), shiitake (*Lentinus edodes*), and wood ear mushroom (*Auricularia auricular*).

The study containing 6 continuous experimental studies was shown as the following procedures.

(1) Chemical composition analysis. The moisture, crude fat, ash, and total nitrogen content were analyzed according to AOAC Official Method (AOAC, 2000). The chitin content was determined according to the previous study (Ifuku, Nomura, Morimoto and Saimoto, 2011).

(2) Optimization of GlcN production from the oyster mushroom. The Box-Behnken design (BBD) was used to determine the optimum hydrolysis temperature, time, and acid concentrations. A second order transferred polynomial model (as an inverse model) was also obtained, which defines the GlcN yield as a function of hydrolysis temperature, time, and acid concentration.

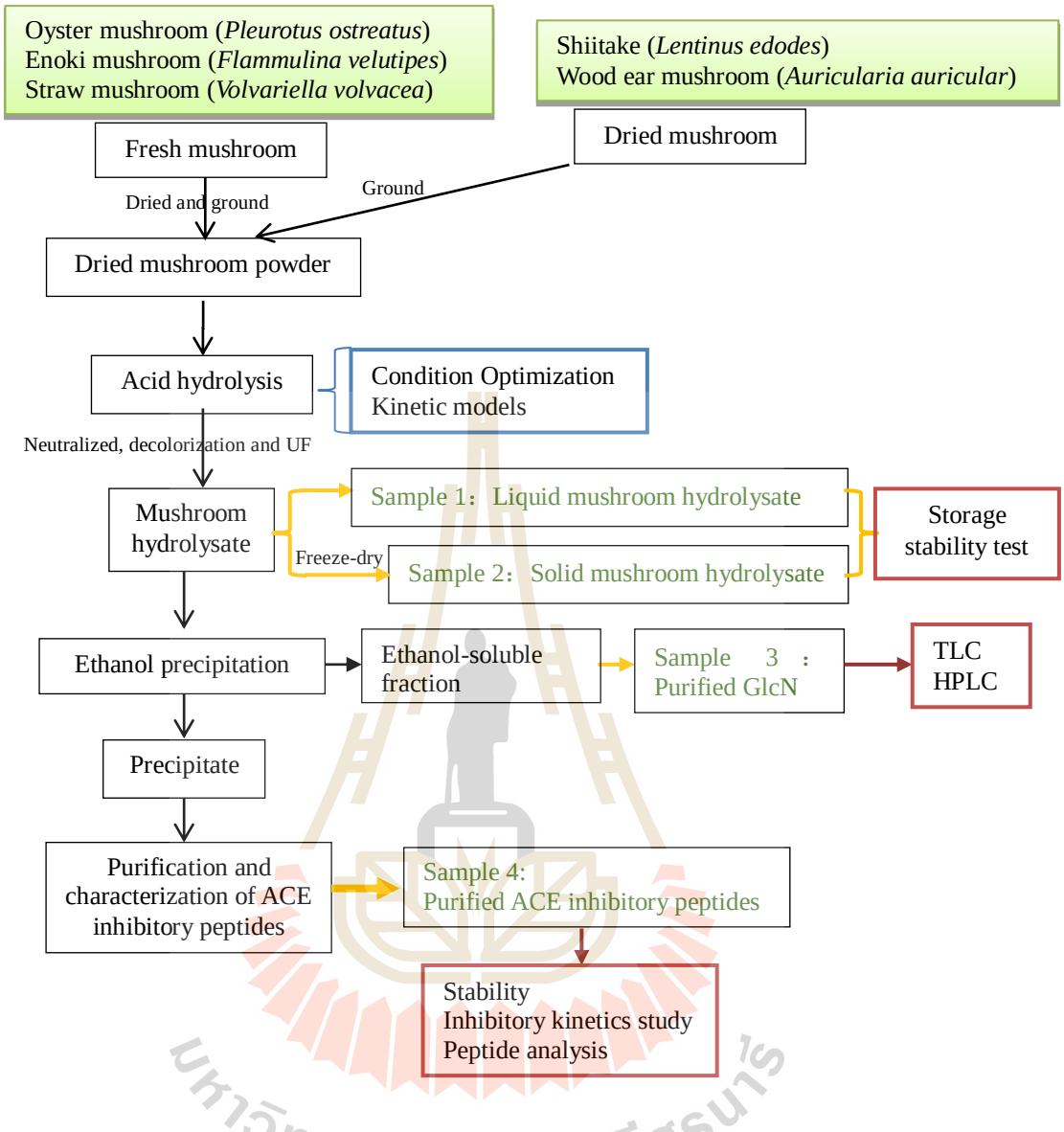


Figure 1.4 Flow diagram of the main steps carried out in this study.

(3) Kinetics of GlcN production from different mushrooms. Under the optimum sulfuric acid concentration and the ratio of raw material to acid volume for mushroom hydrolysis, the influence of temperature and time on GlcN production kinetics will be monitored within 6 h for the five mushrooms. The GlcN concentration was determined by high performance liquid chromatography (HPLC) according to the

AOAC official method 2005 at 30 min intervals (Zhou, Waszkuc and Mohammed, 2005).

(4) Purification and characterization of GlcN. The GlcN was separated from mushroom hydrolysates by decolorization, ultrafiltration, and ethanol-assisted precipitation.

(**5) Purification and characterization of ACE inhibitory peptides.** The solid part (precipitate) after GlcN purification will be vacuum-dried and used for the purification of ACE inhibitory peptides through ion exchange and gel filtration chromatography. Then, the stability and inhibitory pattern of the purified peptides were studied. Peptide analysis of the purified peptides were also investigated.

(6) The storage stability of mushroom hydrolysates. Five kinds of mushroom hydrolysates, including liquid form after neutralization, decolorization, and ultrafiltration, and solid form after freeze-drying, were tested for their stability. The samples were stored for 60 days at different temperature (-20, 4, 26°C). The GlcN content and ACE inhibitory activity were determined at 0, 15, 30, 45, and 60th day.

1.5 Expected results of this study

The expected results were:

(1) To obtain the information about optimized sulfuric acid hydrolysis conditions for GlcN production from different mushrooms;

(2) To understand the kinetics of GlcN production from different mushrooms;

(3) To separate and characterize GlcN from mushroom hydrolysate;

(4) To isolate and identify ACE inhibitory peptides as byproducts from mushroom hydrolysate;

(5) To estimate the storage stability of mushroom hydrolysate (liquid and powder form) which contains GlcN and ACE inhibitory peptides.

The results of used mushrooms for the production of GlcN and ACE inhibitory peptides can expand the usage of mushrooms as the nutraceutical and pharmaceutical supplements. Mushrooms have advantages over the traditional crustacean shells. 1) GlcN from mushrooms is suitable for vegetarians and shellfish-allergic consumers. 2) Mushrooms can be cultivated throughout the year without geographical and seasonal restrictions. 3) The cultivation of mushroom is economical, low production cost, time-saving, high economic efficiency, and easy production technologies (Rosmiza, Davies, Jabil and Mazdi, 2016). 4) The composition of mushrooms are relatively consistent and is not associated with inorganic materials, thus, no demineralization treatment is required and the heavy metal hazard can be avoided. The optimized sulfuric acid hydrolysis also can be used for other raw starting materials to produce GlcN. This method is a simple and efficiency method that can be used as an alternative method for hydrochloric acid hydrolysis.

CHAPTER II

LITERATURE REVIEWS

2.1 Mushrooms

A mushroom is the fleshy, spore-bearing fruiting body of a fungus, typically produced above ground on soil or on its food source. More than 2300 species of mushrooms exist in nature, but for commercial purposes, only 22 species are intensively cultivated on ground or wood with particular environmental and nutritional conditions (Manzi, Aguzzi and Pizzoferrato, 2001). World production of mushroom is growing and exceeds ten million tons in 2014. China is the largest producer with a market share of 73.57%. Italy (5.78%) and United States of America (4.16%) are the other major countries. The preferred cultivated mushrooms worldwide are *Agaricus bisporus* (button mushroom), *Lentinus edodes* (shiitake), *Pleurotus* spp. (oyster mushrooms), *Auricula auricula* (wood ear mushroom), *Flamulina velutipes* (winter mushroom) and *Volvariella volvacea* (straw mushroom) (Muzzarelli et al., 2012).

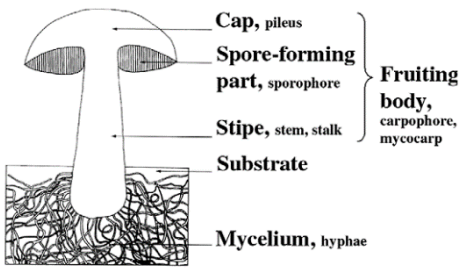


Figure 2.1 A sketch of a mushroom and used mycological terms (Kalač, 2009).

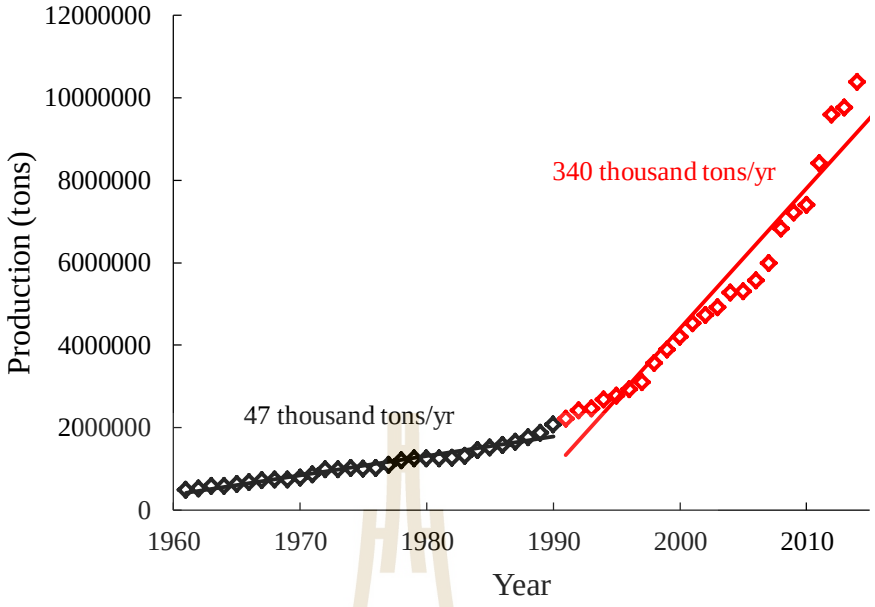


Figure 2.2 World production of mushrooms from 1961 to 2014. Source: FAOSTAT Database

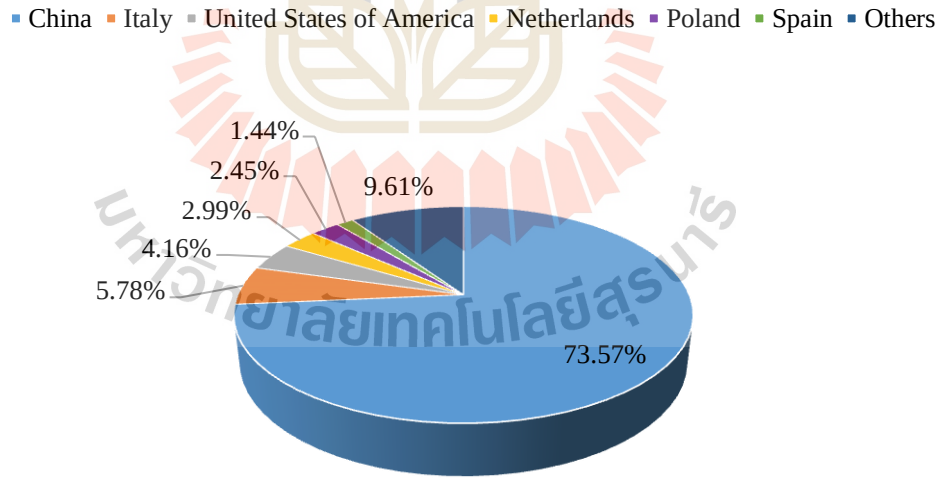


Figure 2.3 World production of mushrooms in 2014. Source: FAOSTAT Database

Mushrooms have been consumed as popular foods throughout the world for many centuries not only for their texture and flavor but also for their nutritional

properties (Liu et al., 2015, Phat, Moon and Lee, 2016). The edible cultivated mushrooms are valuable and important foods. They also have some valuable properties including remarkable quantity and high quality of proteins, low energy level, some important elements such as potassium and phosphorus, some odorous and taste materials (Vetter, 2007). The contents of these favorable components have been reported in several publications (Manzi, Aguzzi and Pizzoferrato, 2001, Manzi, Marconi, Aguzzi and Pizzoferrato, 2004, Mario, Rapanà, Tomati and Galli, 2008, Vetter, 2007). Generally, mushrooms contain about 57% carbohydrates, 25% protein, 5.7% fat, and 12.5% ash (Kalač, 2009). The carbohydrates in the mushrooms are in the form of glycogen, chitin, and hemicellulose instead of starch. Mushrooms are rich in good quality proteins with lysine and tryptophan that are normally deficient in cereals. They are recommended as the alternative source of proteins for bringing the protein malnutrition gap in the developing countries of the world (Mehta et al., 2013). Besides their nutritive value, mushrooms also exhibit certain medicinal properties. Mushrooms have been reported to be therapeutic foods that are useful in preventing diseases such as hypertension (Yahaya, Rahman and Abdullah, 2014), hypercholesterolemia (Ismail, Soliman, Nassan and Mohamed, 2015) and several types of cancer (Konno et al., 2015). The ingredients which are responsible for these functional characteristics are dietary fiber, chitin, the structural polysaccharide of cellular walls, β-glucans, homo- and hetero-glucans with β (1→3), β (1→4) and β (1→6) glucosidic linkages (Manzi, Aguzzi and Pizzoferrato, 2001). On the other hand, the chitin content of mushrooms is in the range of 0.3-19.6% DW (Manzi, Aguzzi and Pizzoferrato, 2001, Mario, Rapanà, Tomati and Galli, 2008). Additionally, the cultivation of mushrooms is inexpensive and not time-consuming (Petrovska, 2001).

Therefore, mushrooms have the potential to be used as a raw material for the production of chitin, chitosan and their oligomers such as GlcN.

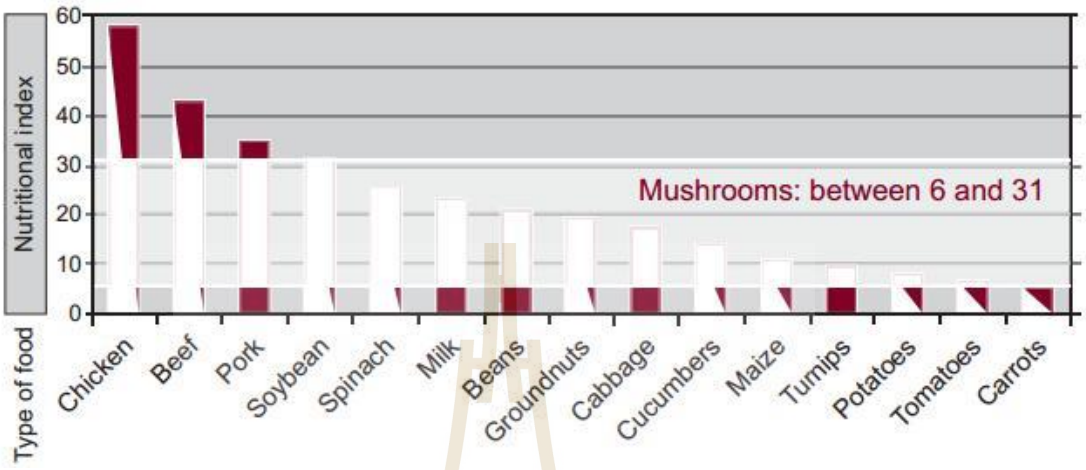


Figure 2.4 A comparison of the nutritional index (essential amino acids, vitamins and minerals) of different foods compared to mushrooms (Boa, 2004).

2.1.1 The chitin and chitosan content of mushrooms

Chitin is a linear cationic polysaccharide consisting of GlcNAc units through a β (1→4) linkage (Nitschke, Altenbach, Malolepszy and Mölleken, 2011, Zhang, Haga, Sekiguchi and Hirano, 2000). It may be regarded as cellulose with hydroxyl at position C-2 replaced by an acetamido group. It is the most abundant natural amino polysaccharide and is estimated to be produced annually almost as much as cellulose (Baba, Noma, Nakayama and Matsushita, 2002). It is the main component of the exoskeleton of Arthropoda, such as crabs, lobsters, shrimp, crustaceans, insects, etc. It is also an important component of the fungal cell wall (Ifuku, Nomura, Morimoto and Saimoto, 2011). In the exoskeletons of arthropod, chitin is always cross-linked with protein via aspartate and/or histidine moieties (Stankiewicz et al., 1998). In the fungal cell walls, chitin is responsible for the rigidity

and shape of the cell walls (Vetter, 2007). α-, β-, and γ- forms of chitin are identified with different chitin microfibril orientations. The α-form which has antiparallel chains is the most abundant in nature. It is found in the shells of crustaceans (crabs, shrimp, lobsters, etc.), in the shells and skeletons of mollusks and krill, insects, etc. and in the cell walls of fungi (mushrooms, bakers' yeast, etc.). The β-form which is rare in nature has parallel chains. It can be found in squid pens, in the extracellular spines of the euryhaline diatom, and in pogonophore tubes. The γ- form has a mixture of antiparallel and parallel chains found in the cocoons of insects (Zhang, Haga, Sekiguchi and Hirano, 2000).

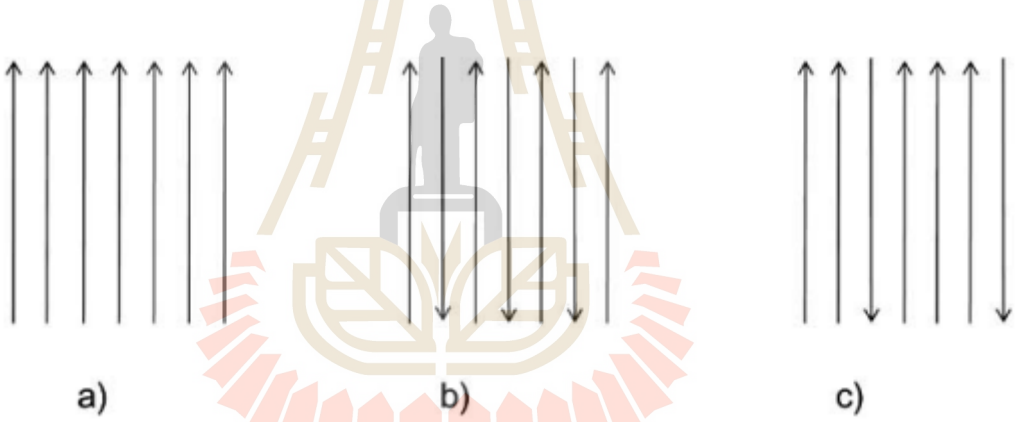


Figure 2.5 Three polymorphic configurations of chitin: a) α-chitin, b) β-chitin, and c) γ-chitin (Aranaz et al., 2009).

Chitin is insoluble in aqueous solutions and organic solvents because of its high degree of crystallinity (Mario, Rapanà, Tomati and Galli, 2008). Thus, to improve its solubility and optimize its use, chitosan is obtained from chitin by homogeneous or heterogeneous alkaline N-deacetylation or by enzymatic deacetylation. Chitosan, the uncompleted *N*-deacetylated derivative of chitin, consisting of GlcN and GlcNAc units and has a mean molecular mass of over 1 mDa,

which corresponds to a chain length of approximately 5000 monomeric units (Kumar and Tharanathan, 2004). Chitosan has been found quite a number of biomedical and biotechnological applications recently because of its improved solubility (Nitschke, Altenbach, Malolepszy and Mölleken, 2011). Its functions and biological activities are influenced by its chain length and degree of acetylation (DA). The structures of GlcN, GlcNAc, cellulose, chitin and chitosan are shown in Figure 2.6 and Figure 2.7.

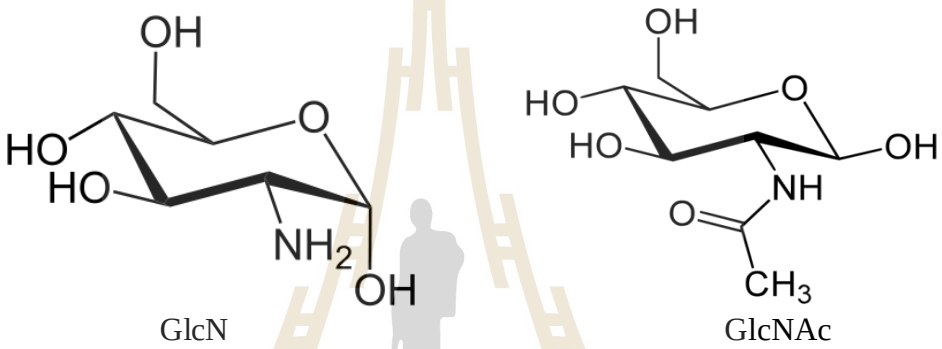


Figure 2.6 Molecular structure of GlcN (left) and GlcNAc (right).

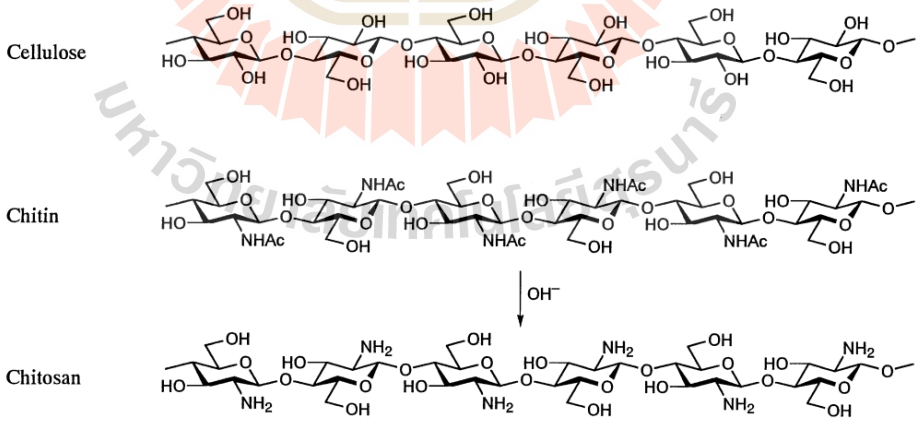


Figure 2.7 Structure of cellulose, chitin and 100% acetylated chitosan (Kumar, 2000).

Chitin and chitosan have become of great interest not only as an underutilized resource but also as a new functional material of high potential in various fields. Most

polymers used in present-day are synthetic materials, their biocompatibility and biodegradability are much more limited than those of natural polymers such as cellulose, chitin, chitosan and their derivatives. In this respect, chitin and chitosan are recommended as suitable functional materials, because these natural polymers have excellent properties such as biocompatibility, biodegradability, non-toxicity, adsorption properties, etc. Chitin and chitosan are useful chelating agent due to their high percentage of nitrogen (6.89%) compared to synthetically substituted cellulose (1.25%). They can selectively bind trace metals and thereby inhibits the production of toxins and microbial growth (Shahidi, Arachchi and Jeon, 1999). Both chitin and chitosan have been studied as dietary fiber. The studies suggest that chitin and chitosan are able to lower cholesterolemia, modulate the immune system and inhibit tumoral growth (Esteban, Cuesta, Ortuno and Meseguer, 2001, Gopalakannan and Arul, 2006, Koide, 1998, Lee et al., 2008, Manzi, Aguzzi and Pizzoferrato, 2001, Manzi et al., 1999, Zhang, Cheung and Zhang, 2001). They are responsible for decreasing the physiological cholesterol pool (Razdan and Pettersson, 1994, Sugano et al., 1980, Trautwein, Jürgensen and Erbersdobler, 1997, Ylitalo et al., 2001, Zacour et al., 1992).

Like cellulose, chitin and chitosan function naturally as a structural polysaccharide. In native as well as modified forms both chitin and chitosan are used in a wide range of applications, such as in food, biotechnology, material science, drugs and pharmaceuticals, and recently in gene therapy too. *In vivo*, the ambiguous effects because of their low absorption on organism owing to the high MW, high viscosity, low solubility and low chemical reactivity (Kumar and Tharanathan, 2004, Kumar, 2000). In another word, the very high molecular weight (MW) and therefore a very

high viscosity of chitin and chitosan precluded their use in several biological applications. For some specific applications, more than chitin and chitosan themselves, their degradation products, namely water-soluble low molecular weight chitosan (LMWC), chitooligosaccharides (COs) and monomers, were found to be much more useful. Hence, its depolymerization into various low MW products, especially the chitooligomers, is required for efficient utilization. The latter overcome these drawbacks and are useful as antitumor and immune-stimulating agents. A variety of degradation methods, viz. chemical, physical and enzymatic, are being worked out to generate these degradation products (Allan and Peyron, 1995, Choi et al., 2002, Grigolon, Azevedo, Santos and Franco, 2001, Jia and Shen, 2002, Kumar, Gowda and Tharanathan, 2004, Kumar, Varadaraj, Lalitha and Tharanathan, 2004, Xing et al., 2005). Both chitin and chitosan oligomers possess additional functional properties such as antitumor activity (Liang, Chen, Yen and Wang, 2007, Qin et al., 2002, Seo et al., 2000), immuno-enhancing effects, and enhancing protective effects against infection with some pathogens in mice (Howling et al., 2001, Prashanth and Tharanathan, 2007, Suzuki et al., 1984, Wang and Chen, 2005), antifungal (Li et al., 2008, Zhang, Tan, Yuan and Rui, 2003) and antimicrobial (Helander et al., 2001, Lee et al., 2009, No et al., 2002, Vishu, Varadaraj, Gowda and Tharanathan, 2005, Zivanovic et al., 2004) activities. Additionally, they have lower viscosity, low MWs and short chain lengths and are soluble in the neutral aqueous medium. Subsequently, they seem to be readily absorbed *in vivo*.

As mentioned above, chitin and chitosan exist in the fungal cell wall. Thus, the cell wall of mushrooms could be an important source for chitin and chitosan production. Mushrooms are well-known for their application in various medical

domains. In particular, β-glucans have been proposed as the active immune-stimulating agents. Furthermore, after isolation of the glucans, the remaining dietary fiber, consisting mainly of chitin, could be used for chitin and chitosan preparation as well (Kurtzman Jr, 2005).

To evaluate the possibility of using fungal biomass as a source of chitin and chitosan, chitin and chitosan were isolated from the mycelium of seven species of Basidiomycetes by alkaline treatment. Such material was characterized by its purity, DA and crystallinity. Chitin yields ranged between 8.5 and 19.6% DW and the chitosan yield was approximately 1% DW. The characteristics of the fungal chitin were similar to those of commercial chitin. Chitosan, with a low DA, comparable with that of commercial chitosan, were obtained by the chemical deacetylation of fungal chitins (Mario, Rapanà, Tomati and Galli, 2008). Recently, Erdogan, Kaya and Akata (2017) determined the chitin contents of the two different mushroom species were 11.4% DW for *Lactarius vellereus* and 7.9% DW for *Phyllophora ribis*, respectively, suggesting that these two mushroom species can be used as an alternative chitin source.

Vetter (2007) determined and compared the chitin contents of pileus (cap) and stipes (stem) of fruiting bodies of *Agaricus bisporus*, *Pleurotus ostreatus* and *Lentinula edodes* (shiitake). Based on the experimental data, chitin content of the cultivated mushrooms was considered as a characteristic index of the species and seems to be independent of the cultivars (varieties). Generally, the chitin level of the pileus was higher than stipes. The presented data also confirmed that a mushroom saprotrophic (*A. bisporus*) had higher chitin level than the wood-rotting ones (*P. ostreatus*, *L. edodes*).

To extend the knowledge on chemical and nutritional characteristics of commercial mushrooms widely consumed in Italy, fresh and processed mushrooms (*Agaricus bisporus*, *Pleurotus ostreatus* and *Boletus* spp.) were analyzed by Manzi , Aguzzi and Pizzoferrato in 2001. The chitin content ranged between 0.3 and 3.9 g/100g of edible portion, *Boletus* spp. was the richest source. The differences between cooked and raw samples exclusively depended on the water content. In fact, if the value were reported on DW basis, no differences can be observed. In the later research, *Boletus* spp., *Agrocybe aegerita* and *Pleurotus eryngii* were examined. The chitin content ranged from 0.5 to 3.3 g/100 g of edible weight. The chitin level of dried and rehydrated mushrooms (from 1.8 to 3.3 g/100 g of edible weight) was higher than in the frozen and fresh samples because of the incomplete rehydration (Manzi, Marconi, Aguzzi and Pizzoferrato, 2004).

In the study of Cheung (1996), the mycelia, caps and stalks of four edible mushrooms (*Lentinus edodes*, *Lycophyllum shimeji*, *Pleurotus sajor-caju*, and *Volvariella volvacea*) were analyzed. The chitin contents of the fruiting bodies and mycelia within each mushroom species were similar. But *Lycophyllum shimeji* was the exception in which the mycelia (5.51%) had only half the amount of chitin compared to its fruiting bodies. The chitin contents in *L. edodes* and *V. volvacea* were higher than those of *L. shimeji* and *P. sajor-caju*. Ospina Álvarez et al. (2014) demonstrated that *Ganoderma lucidum* fungus may be used as a potential raw material for chitin production. Chitin was isolated from the *Ganoderma lucidum* submerged cultures mycelium and the chitin contents extracted from 9-day mycelia was 413 mg/g DW.

Table 2.1 Chitin content of preferred cultivated mushrooms.

Items	Chitin content (% DW)	Reference
Agaricus bisporus (common mushroom)	[a]2.78	(Bano, Srinivasan and Srivastava, 1963)
	8.5 ± 1.4	(Mario, Rapanà, Tomati and Galli, 2008)
	6.67 ± 1.04 (pileus); 7.31 ± 1.43 (stipes)	(Vetter, 2007)
	[a]0.60 ± 0.04 (raw); 0.70 ± 0.04 (cooked)	(Manzi, Aguzzi and Pizzoferrato, 2001)
	Deep frozen: 0.34 ± 0.01 (raw); 0.52 ± 0.02 (cooked)	
	Canned: 0.61 ± 0.05 (raw); 0.74 ± 0.06 (cooked)	
	9.60 ± 0.15 (mycelia); 4.69 ± 0.90 (fruiting body)	(Nitschke, Altenbach, Malolepszy and Mölleken, 2011)
Auricularia auricula-judae (wood ear mushroom)	19.6 ± 1.1	(Mario, Rapanà, Tomati and Galli, 2008)
	5.4	(Kadnikova et al., 2015)
Flammulina velutipes (enoki mushroom)	1.21 ± 0.18 (mycelia); 9.83 ± 0.45 (fruiting body)	(Nitschke, Altenbach, Malolepszy and Mölleken, 2011)
Lentinula edodes (shiitake)	10.1 ± 1.0	(Mario, Rapanà, Tomati and Galli, 2008)
	8.07 ± 0.19 (pileus)	(Vetter, 2007)
	6.55 ± 0.18 (stipes)	
	19.0 (caps); 17.3 (stalks); 21.8 (mycelia)	(Cheung, 1996)
	2.49 ± 0.19 (mycelia); 1.87 ± 0.20 (fruiting body)	(Nitschke, Altenbach, Malolepszy and Mölleken, 2011)
	[a]0.5 ± 0.1 (raw); 0.6 ± 0.1(cooked)	(Manzi, Marconi, Aguzzi and Pizzoferrato, 2004)
Pleurotus ostreatus (oyster mushrooms)	15.3 ± 2.2	(Mario, Rapanà, Tomati and Galli, 2008)
	3.78 ± 0.97 (pileus)	(Vetter, 2007)
	2.8 ± 0.75 (stipes)	
	[a]0.32 ± 0.05 (raw); 0.63 ± 0.07 (cooked)	(Manzi, Aguzzi and Pizzoferrato, 2001)
	0.82 ± 0.08 (mycelia); 0.76 ± 0.22 (fruiting body)	(Nitschke, Altenbach, Malolepszy and Mölleken, 2011)
	0.34%	(Tshinyangu and Hennebert, 1996)
Volvariella volvacea (straw mushroom)	17.3 (caps); 17.0 (stalks); 17.1 (mycelia)	(Cheung, 1996)

[a] % fresh weight

Zivanovic, Busher and Kim (2000) determined the ultra-structural and compositional changes in fresh mushrooms during 9 days of post-harvest storage at 12°C. The chitin content increased during mushroom storage and appeared to be associated with toughening (r^2=0.95). Due to the protease activity, protein degradation occurred and the liberated amino acids were probably used in cell metabolism and chitin synthesis since no amino-acid accumulation during storage had been reported. Most polysaccharides were extracted after deacetylation and depolymerization of chitin, indicating that structural glucans should be mainly bound with chitin.

In 2004, Wu, Zivanovic, Draughon and Sams determined the accumulation of chitinous material in *Agaricus bisporus* stalks during postharvest storage at 4 and 25°C. The chitinous material was extracted after alkali treatment and acid reflux of alkali insoluble material and analyzed for yield, purity, DA, and crystallinity. The yield of crude chitin isolated from stalks was 27.00% DW after storage at 25°C for 5. The DA of fungal chitin was from 75.8 to 87.6%, which is similar to commercially available crustacean chitin. The yield of crude fungal chitin of 0.65-1.15% on a fresh basis indicates the potential for the utilization of these mushroom byproducts.

The solid-state fermentation of *Lentinus edodes* was found to be a flexible and easily scaled-up approach to low acetylation degree chitosan production (Crestini, Kovac and Giovannozzi-Sermanni, 1996). The results showed that the chitosan yield was 6.18 g/kg and the acetylation degree was 12.5% at 12th day after inoculation. These data were of particular significance: the solid-state fermentation gave yields 50 times higher than other chitosan production methods from fungi, and the chitosan had a lower DA than crustacean commercial chitosan.

Chitin was extracted and purified from dried fruitbodies of *P. ostreatus* var. *columbinus* grown on wheat straw and grass hay. The yield of chitin was about 0.34% DW of fruitbodies. The purified chitin had similar infrared spectrum as the commercial chitin and contained 5.9% nitrogen (Tshinyangu and Hennebert, 1996).

2.1.2 The protein content of mushrooms

Due to low-fat content and absence of cholesterol, edible mushrooms have an established history of use in the Orient as a highly nutritious foodstuff. They have received increasing attention as food and pharmaceuticals because of their bioactive components which display antitumor, antioxidant, antiviral, antimicrobial, and immunomodulatory activities (Chang, Chien, Tong and Sheu, 2007, Chen, Y. et al., 2012, Li et al., 2013, Ye et al., 2005). A number of bioactive constituents have been isolated from mushrooms including small molecule compounds, polysaccharides, proteins, polysaccharide-protein complexes, etc. (Ferreira, Vaz, Vasconcelos and Martins, 2010, Quang, Hashimoto and Asakawa, 2006, Wasser, 2010). Among all the bioactive constituents of mushrooms, polysaccharides have been investigated most extensively (Kozarski et al., 2011, Pan et al., 2010, Zhang, Yan, Chen and He, 2012, Zhou and Chen, 2011). However, bioactive proteins constitute another important type of functional components in mushrooms, which also has increasing interests due to their potential pharmaceutical value (Wong et al., 2010, Xu, Yan, Chen and Zhang, 2011).

The protein content of mushrooms is influenced by the composition, flush number, harvest time and strain. Most mushrooms have high protein content, usually around 20-30% DW (Kalač, 2009, Petrovska, 2001). Approximate analyses of mushroom protein (*Pleurotus* sp.) revealed that it contains 2.78% protein and 0.14%

non-protein nitrogen on a fresh-weight basis. Compared with fruits and vegetables, mushroom is a better source of protein, containing lysine, arginine, histidine, and threonine in high concentrations. However, mushroom is primarily deficient in phenylalanine and methionine when compared with egg protein, (Bano, Srinivasan and Srivastava, 1963).

The common mushroom (*Agaricus bisporus*) has high protein content. The crude protein content has been reported between 19-38% DW, but the results are overestimated because of the non-protein nitrogen-containing compounds. To verify protein determination methods, the protein content, total nitrogen content, free amino acids and ammonia/urea content in different fraction of mushroom extracts were compared (Braaksma and Schaap, 1996). The Kjeldahl analysis in combination with the adopted factor 4.38 resulted in a protein content of 28% DW. A generally accepted protein determination method based on protein-dye binding was compared with amino acid analyses on the same samples and proved to be reliable. The exact protein content of mushroom examined was 0.5% of fresh weight and 7% DW, which was four times lower than the usually reported value.

Tshinyangu and Hennebert (1996) analyzed the protein content of *P. ostreatus* var. *columbinus* grown on wheat and grass hay. Protein was extracted by water and sodium chloride and then purified by dialysis and 10% TCA precipitate. Based on this method, the protein content was 17.1% DW and 23.5% DW of fruitbodies, respectively. The extracted protein contained 15.2% nitrogen. The calculated protein/nitrogen ratio, namely the new conversion factor was 6.58.

In order to assess their role in human nutrition, the proximate and mineral composition of four cultivars of *Pleurotus* mushrooms were tested (Shah, Khalil and

Jabeen, 1997). Mushroom protein was deficient in the sulfur containing amino acids, methionine and cystine. It was found that mushrooms can serve as a good source of protein, dietary fiber and mineral matter. The protein content of mushrooms was much higher than cereals (wheat and maize), and comparable to that of food legumes (peas and lentil). The study conducted by Hung and Nhi (2012) evaluated the nutritive values of five popular Vietnamese edible mushrooms (*Pleurotus ostreatus*, *Volvariella volvacea*, *Lentinula edodes*, *Auricularia polytricha* and *Ganoderma lucidum*). Protein contents ranged from 7.2-36.6%.

In the study of Manzi, Aguzzi and Pizzoferrato (2001), the protein content of *Agaricus bisporus* samples were significantly affected by the technological process such as deep frozen and canned. The protein content of raw, frozen and canned *Agaricus bisporus* was 1.63±0.06 g/100 g edible weight, 1.30±0.01 g/100g edible weight, and 1.53±0.02 g/100g edible weight, respectively. Cooking procedure significantly increased the protein content by decreasing the moisture content.

Mushrooms are useful for different practical applications and productions. Based on the present review of contents of chitin and protein in mushrooms, mushrooms can be considered as a good source of chitin and protein and are the potential starting materials for chitin and protein isolation, or other bioactive ingredients such as GlcN and peptide production.

2.2 Glucosamine

GlcN is a cationic monosaccharide product generated from chitin or chitosan by hydrolysis and is categorized into hexosamine and is a water-soluble substance. GlcN, an amino monosaccharide, is a natural component of glycoproteins found in

connective tissues and gastrointestinal (GI) mucosal membranes (Aghazadeh-Habashi, Carran, Anastassiades and Jamali, 2005, Chen, Shen and Liu, 2010, Sharma and Arora, 2013). It is a precursor of the disaccharide unit of glycosaminoglycans (GAGs) which are the building-blocks of the articular cartilage, the proteoglycans (Xing et al., 2006). GlcN is a naturally occurring sugar that is synthesized from glucose by virtually all cells and is an essential component of glycoproteins and proteoglycans. Healthy men have serum GlcN concentrations of ~40 μmol/L (Monauni et al., 2000). Exogenous GlcN is taken into cells by glucose transporters (GLUT-2 and GLUT-4) where it is phosphorylated and further metabolized (Baron et al., 1995).

2.2.1 Biological, nutrimental and pharmaceutical effects of glucosamine

Nowadays, chitin and chitosan are produced by many companies on a commercial scale for various applications, for example, cosmetics, pharmaceuticals, agriculture, water treatment and absorption of heavy metals (Mario, Rapanà, Tomati and Galli, 2008). However, the present researches have been focused on the conversion of chitin and chitosan into their oligomers because these oligomers not only have the effects as chitin and chitosan but also have some specific properties that are different from chitin and chitosan. During the last few decades, many biological, nutrimental and pharmaceutical effects of GlcN, such as membrane stabilizing activity and liver protective effect (Sal'nikova, Drogovoz and Zupanets, 1990), wound healing promoting by enhancing hyaluronic acid synthesis (McCarty, 1996), and application in osteoporosis treatment (Hochberg et al., 2015, Rovati, Girolami, D'Amato and Giacovelli, 2015, Runhaar et al., 2015) have been widely reported and GlcN is receiving more attention recently because of its easy absorption and excellent properties.

2.2.1.1 Anti-osteoarthritis effect

OA is currently defined by the American College of Rheumatology (ACR) as a heterogeneous group of conditions that leads to joint signs and symptoms which are associated with the defective integrity of articular cartilage, in addition to related changes in the underlying bone at the joint margins (Altman et al., 1986). It is destined to become one of the most prevalent and costly diseases in our society that affects all joint tissues such as the knee, hand, hip and spine in elderly population (Kloppenburg et al., 2015, Loeser, Goldring, Scanzello and Goldring, 2012, Samuels, Krasnokutsky and Abramson, 2008). It causes pain, aching, stiffness, limitation of motion and deformity. It is associated with degeneration of articular cartilage and of the menisci, and sclerosis of subchondral bone accompanied by inflammation of the synovial membrane (Henrotin and Lambert, 2013). OA exacts a heavy economic toll due to its high prevalence in the general population and potential for causing progressive disability (Guillemin et al., 2012). Its prevalence after the age of 65 years is about 60% in men and 70% in women (Sarzi-Puttini et al., 2005). More than 13% of Americans aged 55 to 64 years, and more than 17% of Americans aged 65 to 74 years, have pain and functional limitations related to knee OA (Spahn, Kirschbaum and Kahl, 2006). Only 135 of the 1040 subjects aged 55 to 65 participating in the Rotterdam study were free of radiographic OA in the hands, knees, hips, and spine (Racine and Aaron, 2013). In Italy, the prevalence of symptomatic OA in subjects older than 65 years is 30% for the knee, 15% for the hand, and 8% for the hip (Cimmino et al., 2001, Mannoni et al., 2000).

OA is a painful and disabling disease that affects millions of patients. Its etiology is complex and largely unknown but is most likely multi-factorial. It poses a

dilemma: it often begins attacking different joint tissues long before middle age, but cannot be diagnosed until it becomes symptomatic decades later, at which point structural alterations are already quite advanced. That is why OA already evolves over decades but still remains incurable. The treatment of OA mostly involves control of symptoms, i.e. reduction of pain and improvement of joint function, and relies on a combination of non-pharmacological and pharmacological approaches as recommended in recent clinical guidelines (Hochberg et al., 2012, Jordan et al., 2003, Zhang et al., 2005, Zhang et al., 2007a, Zhang et al., 2007b, Zhang et al., 2008, Zhang et al., 2010). To date, the management of OA is challenging and no specific drugs or therapy has yet been found to compensate the loss of cartilage and to stop the progression of OA (Zhang et al., 2010). Symptom control is important, but it is not the only objective for OA treatment. An ideal treatment would preserve articular structures, improve patient quality of life, and have a good safety profile (Hochberg et al., 2013).

The current treatment, such as analgesics and NSAIDs administration, merely provides pain reduction and improvement of function without structural recovery. Mild to moderate pain in OA patients can be relieved using simple analgesics such as acetaminophen. Bradley and coworkers demonstrated that acetaminophen 1000 mg 4 times a day had the same effect as ibuprofen 1200 or 2400 mg/day in patients with knee and hip OA (Bradley et al., 1991). Acetaminophen-induced toxicity includes hepatotoxicity and potential renal damage, and data from a previous study suggested that, although it has long been considered safe when used with anticoagulants such as warfarin, acetaminophen may affect prothrombin time (Hylek et al., 1998). NSAIDs are widely used to reduce pain and inflammation and improve function in OA patients

(Hochberg and Dougados, 2001). The ability of different NSAIDs to inhibit cyclooxygenase (COX), the enzyme that catalyzes the synthesis of cyclic endoperoxides from arachidonic acid to proinflammatory and other forms of prostaglandin (PG), varies: some seem to be potent inhibitors of PG synthesis, whereas others more prominently affect non-PG-mediated biological events (Furst, 1994). Two COX isoforms have been identified (COX-1 and COX-2), and NSAIDs are now classified as nonselective or non-COX-2 selective NSAIDs and Coxibs (COX-2 selective agents). GI intolerability problems, including dyspepsia, abdominal pain, and nausea, are the most frequent adverse events associated with nonselective NSAIDs (Gabriel, Jaakkimainen and Bombardier, 1991). GI mucosal damage, such as ulcers, bleeding, perforation, and obstruction, also is common (1 to 2% ulcer complications, 2 to 4% peptic ulcer symptoms with ulcer complications and symptomatic ulcers) (Griffin, Ray and Schaffner, 1988). Nephropathy can occur with both COX-2 selective and nonselective NSAIDs. The risk factors for adverse kidney effects include serious hemodynamic compromise such as hemorrhaging, dehydration, moderate/severe congestive heart failure, excessive diuresis, and cirrhosis with or without ascites (Simon et al., 1996). Older people with intrinsic renal disease are at greater risk of adverse renal effect due to NSAIDs (Hawkey, 1999). Overall, they are associated with long-term adverse effects such as GI bleeding and cardiovascular events (Wieland, Michaelis, Kirschbaum and Rudolphi, 2005). Therefore, people become to rely on complementary alternative medicine (CAM) that is seemingly safer and allegedly more effective than NSAIDs.

As early as 1969, GlcN was looked at for use in reducing the symptoms of OA since it is the major component of human inter-articular lubricant connective tissue.

Our joints are cushioned by cartilages and lubricated with synovial fluid such that we can move and twist any joint freely without pain. The principal lubricating substances in our cartilage, tendons, ligaments, synovial fluid and mucous membranes are proteoglycans and GAGs. GlcN which is naturally produced by the body is the main ingredient needed to produce GAGs. GlcN stimulates the chondrocytes to produce proteoglycans and increase the production of hyaluronic acid (HA) resupply of synovial fluid to act as a lubricant, while chondroitin sulfate attracts water into the cartilage and acts as a shock absorber. The proteoglycans are subjected to continuous metabolic turnover, undergoing constant breakdown and resynthesis. The imbalances in these processes that occur with aging or with other medical conditions are partially responsible for the development of arthritis. In old men, the body loses the capacity to produce sufficient GlcN causing thinning of the cartilage and leads to joint degeneration (Hammad, Magid and Sobhy, 2015).

Nowadays, GlcN or its salt forms have been intensively described in the patent as well as in the scientific literatures. GlcN supplements including GlcN sulfate (S) and hydrochloride (HCl) are widely used as symptomatic slow-acting drugs for OA treatment. GlcN is suspected to be the active compound on joint diseases as being capable of providing a good repair and rebuilding for human joints cartilage, decelerating the degenerative processes, decreasing pain and maintaining and improving joint function in OA. On the basis of joint-space narrowing measured on plain radiographs, GlcN-S and GlcN-HCl have also been revealed to have disease-modifying potential. *In vitro* and *in vivo* experiments, many of which have recently been reviewed, have provided much evidence for possible mechanisms of

action leading to structure-modifying effects of GlcN (Henrotin, Mobasheri and Marty, 2012).

***In vitro* Effect**

GlcN was first believed to affect cartilage by providing building blocks for synthesis of GAGs by the GLUT receptor, then by competing with glucose (Henrotin et al., 2013). Studies reported that GlcN could have anti-catabolic and anti-inflammatory effects: when GlcN-HCl (100 μg/mL) is applied to chondrocytes and synoviocytes it inhibits inflammatory mediators, PGE_2, nitric oxide (NO), and matrix metalloproteinases (MMPs) (Nakamura et al., 2003). This is the result of its effect on different cell-signaling intermediates: for example, it has been revealed to inhibit COX-2 and MMP-13 in human skin fibroblasts (10 mmol/L) by blocking p38 (Hong et al., 2009). These results support previously published data obtained from chondrosarcoma cell lines and mouse macrophages RAW 264.7 (Lin et al., 2008, Rafi, Yadav and Rossi, 2007). GlcN (2 mmol/L) blocks activation of the signaling intermediates p38 and Akt, thus inhibiting COX-2 expression, PGE_2 production and nitric oxide synthase (iNOS) expression and synthesis (p38), and MMP-3 inhibition and proteoglycan synthesis (Akt) (Lin et al., 2008). Namely, GlcN is preferentially incorporated by chondrocytes into the components of the GAGs chains in intact cartilage and stimulates the synthesis of physiological proteolglycans and it also can prevent the cartilage degeneration by inhibiting the production of MMPs such as MMP1, MMP3 and MMP13 and its anti-inflammation function (Thakral, Debnath and Dent, 2007). Inhibition by GlcN-HCl (2.5-10 mmol/L) of COX-2 and iNOS in mouse macrophages was also found to occur via inhibition of p38 (Rafi, Yadav and Rossi, 2007). Finally, a cationic derivative of GlcN (10-50 μg/mL) was proved to inhibit

production of cytokines and mitogenactivated protein kinases (MAPK), p38 and c-Jun NH_2-protein kinase (JNK) in RAW 264.7. The latest study revealed that GlcN inhibited MMP-2 and MMP-9, and confirmed its effect on nuclear factor-kappa B (NF-κB) and on activating protein (AP)-1 (Mendis, Kim, Rajapakse and Kim, 2008). It was also revealed that GlcN affected both NF-κB synthesis and its translocation to the nucleus. The inhibitory effect of GlcN on NF-κB was first observed in human OA chondrocytes with GlcN-S (10-1,000 μg/mL) (Largo et al., 2003). This effect was recently revealed to occur via an epigenetic mechanism: GlcN prevents demethylation of specific CpG sites in the interleukin (IL)-1β promoter, consequently preventing expression of IL-1β (Imagawa et al., 2011).

Inhibition of MMPs could be caused by the increased GAG production observed in synovial cells and chondrocytes with GlcN-HCl (0.06-1 mmo/L) (Igarashi et al., 2011). Another study suggested that the protective effect of GlcN-HCl in rat cartilage was brought about via induction of anabolic mediators, including transforming growth factor (TGF)-β1 and connective tissue growth factor (CTGF) (Ali et al., 2011). Kapoor et al. (2012) revealed that GlcN-S (1-2 mmo/L) reduced PGE_2 in human chondrocytes via inhibition of microsomal prostaglandin E synthase 1 (mPGES1). The same study revealed inhibition of COX-2 and increased levels of an anti-inflammatory transcription factor, peroxisome proliferator-activated receptor (PPAR)-λ.

Chiusaroli et al. (2011) revealed that low concentrations of GlcN-S (0.001-100 μmol/L) inhibited IL-1β-stimulated IL-6, tumor necrosis factor (TNF)-α, MMP-3 and a disintegrin and metalloproteinase with thrombospondin motifs-5 in a chondrosarcoma cell line (SW1353), via inhibition of NF-κB and AP-1. The same

authors continued the investigation with an *in vivo* experiment on the STR/ort mouse (which spontaneously develops OA), and reported that GlcN reduced all OA histology scores.

A recent pharmacoproteomic study by Calamia et al. (2010) revealed that GlcN-S inhibited proteins involved in signal transduction pathways, redox and stress response, and protein synthesis and folding processes. GlcN may also increase levels of chaperone GRP78, suggesting another possible mechanism for GlcN's anti-inflammatory effect.

In addition to cartilage and synovial membrane, it is now well recognized that subchondral bone has an important function in OA pathogenesis. GlcN (0.1-1 mmol/L) induced osteoblast differentiation and blocked differentiation of osteoclasts in mouse cells (MC3T3-E1), limiting the effect on bone resorption and enabling bone deposition (Igarashi, Sakamoto and Nagaoka, 2011). The effect that it produced on the osteoblastic differentiation of MG-63 cells was more than that associated with an anti-inflammatory action (Kim, Mendis, Rajapakse and Kim, 2007).

Animal Studies

In animal studies, GlcN had different protective effects. High doses of GlcN (300 mg/kg) given to rats with adjuvant arthritis (AA) reduced both arthritic score and synovitis (Hua et al., 2005). This effect was associated with reduction of PGE2 and NO concentrations in plasma. Different studies have illustrated the anabolic effect of GlcN. GlcN-HCl (100 mg daily) administered to rabbits after anterior cruciate ligament transection (ACLT) preserved cartilage (Tiraloche et al., 2005). The same effect was observed when GlcN-HCl (20 or 100 mg/day) was administered to rabbits after chymopapain injection (Oegema, Deloria, Sandy and Hart, 2002). Cartilage

preservation was believed to be caused by restoration of GAG content. Loss of GAG in cartilage, and bone erosion and osteophyte formation, were also inhibited by GlcN-HCl (20 mg/kg/day) administered to collagen-induced-arthritis (CIA) mice (Ivanovska and Dimitrova, 2011). Reduced soluble receptor activator of nuclear factor-kappa B ligand and IL-6, and increased IL-10, were believed to cause this protective effect. For rats, GlcN-HCl also reduced macroscopic changes induced by ACLT, and inhibited degradation and enhanced synthesis of type II collagen (Naito et al., 2010). For guinea pigs spontaneously developing OA, GlcN-HCl (200 mg/kg) reduced Mankin histology scores for OA via inhibition of MMP-3 mRNA (Taniguchi et al., 2012). For ACLT rabbits with early stage OA, GlcN-HCl (100 mg/day) preserved joint structure, possibly by attenuating bone changes (Wang et al., 2007). Finally, GlcN-S (250 mg kg^{-1} day^{-1}) affected rat nociception, protected cartilage and reduced synovitis, via inhibition of MAPK, p38, and JNK, and increasing extra-cellular Erk1/2 (Wen et al., 2010). It is noted that most experimental data concern GlcN-HCl.

Clinical Efficacy

Whether GlcN should be regarded as a SYSADOA and whether it has a structure-modifying effect remain controversial. However, numerous published clinical trials document a symptomatic effect, i.e. reduced pain and improved joint function. There have been several meta-analyses (MAs) of data from these trials (Bruyère et al., 2008, McAlindon, LaValley, Gulin and Felson, 2000, Poolsup, Suthisisang, Channark and Kittikulsuth, 2005, Towheed et al., 2000, Vlad, LaValley, McAlindon and Felson, 2007).

As early as 1969, Bohne reported that the intra-articular injection of GlcN was helpful to treat OA (Nakamura, 2011). Later, the first double-blind clinical trial of GlcN on OA was conducted (Pujalte, Llavore and Ylescupidez, 1980). The efficacy and tolerance of oral GlcN-S were tested against placebo in a prospective double-blind trial in 20 out-patients with established OA. Patients given GlcN-S experienced earlier alleviation of symptoms compared with those who had placebo. The use of GlcN-S also resulted in a significantly larger proportion of patients who experienced lessening or disappearance of symptoms within the trial period. No adverse reactions were reported by the patients treated with GlcN, and no variation in laboratory tests was recorded.

Thereafter, clinical use of GlcN received little attention as a potential therapy of OA. However, the situation significantly changed in the end of 20th century. GlcN allegedly became explosively popular among the general population who had joint pain or who wanted to protect joints in North America and then all over the world. The GlcN administration was recommended to take with chondroitin sulfate and other remedies such as exercise, weight management and antioxidant foods. The benefit of GlcN and chondroitin preparations for OA was evaluated by using meta-analysis combined with systematic quality assessment (McAlindon, LaValley, Gulin and Felson, 2000). The ES of GlcN was 0.44 which implied that GlcN had intermediate effects on OA. The effects of the specific drug GlcN-S on the long-term progression of OA joint structure changes and symptoms were assessed (Reginster et al., 2001). A randomized, double-blind placebo controlled trial, in which 212 patients with knee OA were randomly assigned 1500 mg sulfate oral GlcN or placebo once daily for 3 years, was done. As assessed by the Western Ontario and McMaster Universities

scores, symptoms worsened slightly in patients on placebo compared with the improvement observed after treatment with GlcN-S. This finding suggested that it could be a disease modifying agent in OA. Moreover, joint space narrowing was less in the GlcN group compared to that in the placebo group, which indicated that GlcN suppressed the progression of articular degradation.

Another positive trial, in which 1500 mg of GlcN-S was administered once daily for 6 months was issued (Herrero-Beaumont et al., 2007). Three hundred eighteen patients with symptomatic knee OA were assigned to receive GlcN-S, acetaminophen or placebo. The primary efficacy outcome measure was the change in the Lequesne index after 6 months. The secondary parameters outcome measures including the Western Ontario and McMaster Universities Osteoarthritis Index and response according to the Osteoarthritis Research Society International (OARSI) criteria were assessed using an intent-to-treat analysis. The findings indicated that GlcN-S at the oral once-daily dosage of 1,500 mg should be more effective than placebo in treating knee OA symptoms.

The disease-modifying effect of GlcN was analyzed by two MAs (Lee et al., 2010, Wandel et al., 2010). On the basis of an effect size (ES) for joint pain of -0.17 (-0.28 to -0.05) and for joint space width (JSW) of -0.16 (-0.25 to 0.00), Wandel et al. (2010) reported no clinically meaningful effect. However, this meta-analyses had several limitations and several OA experts have questioned the validity of the conclusions (Henrotin, 2012). Disadvantages of this MA were addressed in part by the report from the British Medical Journal post-publication review meeting, which states that the study data did not directly support the strong negative conclusion.

A second MA reported a small-to-moderate protective effect on the minimum joint space narrowing (JSN) after three years. However, it included only two trials (Lee et al., 2010). The Glucosamine/chondroitin Arthritis Intervention Trial (GAIT) study, the largest randomized controlled trial (RCT), did not report any significant effect of GlcN-HCl for knee OA patients (Clegg et al., 2006), and no effect of GlcN-S was observed for hip OA (Rozendaal et al., 2008). However, a recent trial suggested GlcN-S could prevent total knee replacement (TKR) (Bruyère et al., 2008).

When analyzing the symptomatic and structure-modifying effects of GlcN, it is important to consider the RCT analysis provided in OARSI's recommendations. OARSI guidelines committee members analyzed 19 RCTs (16 of GlcN-S and three of GlcN-HCl) (Zhang et al., 2010). They reported an ES for pain of 0.46 (0.23 to 0.69), which was reduced in size compared with the previous analysis 0.61 (0.28 to 0.95) but still equivalent to a moderate effect on symptoms (Zhang et al., 2008). Data analysis continues to emphasize the difference between different preparations of GlcN: the ES for pain of GlcN-S is 0.58 (0.30 to 0.87), whereas that of GlcN-HCl ES is -0.02 (-0.15 to 0.11). In addition, the ES for pain of GlcN-S tended to decrease when only high-quality clinical trials were included in analysis 0.29 (0.003 to 0.57). This analysis also reported a reduction of JSN ES for GlcN-S of 0.24 (0.04 to 0.43) for knee OA, but no effect on hip OA.

2.2.1.2 Antioxidant activity

The data from several studies suggested that GlcN could be effectively employed as an ingredient in health or functional food, to alleviate oxidative stress.

In 2006, Xing and other researchers investigated the antioxidant potency of chitin derivative-GlcN-HCl by employing various established *in vitro* systems, such as superoxide ($O_2^{\cdot-}$)/hydroxyl ($^{\cdot}OH$)-radical scavenging, reducing power, and ferrous ion chelating potency (Xing et al., 2006). The satisfying results were obtained as expected. First, GlcN-HCl had pronounced scavenging effect on superoxide radical. For example, the $O_2^{\cdot-}$ scavenging activity of GlcN-HCl was 83.74% at 0.8 mg/mL. Second, the $^{\cdot}OH$ scavenging activity of GlcN-HCl was also strong and was about 54.89% at 3.2 mg/mL. Third, the reducing power of GlcN-HCl was more pronounced. The reducing power of GlcN-HCl was 0.632 at 0.75 mg/mL. However, ferrous ion-chelating potency was soft. Furthermore, ferrous ion-chelating potency, the scavenging rate of radical, and the reducing power of GlcN-HCl increased with their increasing concentration, and they were concentration dependent. The multiple antioxidant activity of g GlcN-HCl was evident as it showed considerable reducing power, superoxide/hydroxyl-radical scavenging ability.

Later then, the antioxidant activity of GlcN *in vitro* and *in vivo* tests were investigated (Yan et al., 2007). Results showed that GlcN possessed excellent antioxidant activities as manifested by strong chelating effect on ferrous ions and protection of macromolecules such as protein, lipid, and deoxyribose from oxidative damage induced by hydroxyl radicals. The metal ion chelating ability of GlcN was evaluated by measuring its chelating ability on Fe^{2+}. Iron has the capacity of readily accepting or donating electrons, which makes it not only physiologically essential but also biochemically dangerous since it can cause oxidative damage by catalyzing the conversion of superoxide and hydrogen peroxide to free radical species. GlcN showed strong chelating effect on Fe^{2+} as EDTA, which implied that GlcN could bind iron

ions strongly enough to remove them from oxidizable substances. It was possible that the nitrogen atom of GlcN is involved in the formation of the metal complex since the analogues of GlcN such as glucose and GlcNAc showed very weak chelating effects as compared with GlcN. The Fenton reaction model system containing EDTA-$FeCl_3$, H_2O_2 and ascorbic acid was used as *in vitro* ·OH generating system. The ·OH that generated can attack deoxyribose, lipid and protein leading to oxidative damage to them as manifested by degradation of deoxyribose, malondialdehyde (MDA) formation of lipid and protein carbonyl formation. GlcN showed 75% inhibitory effects on deoxyribose degradation at 4 mg/mL. Lipid peroxidation was efficiently inhibited by GlcN which showed 85% inhibitory percent at 4 mg/mL. Carbonyl content in protein was also concentration-dependently decreased by GlcN.

2.2.1.3 Anti-inflammatory effect

GlcN is expected to exert an anti-inflammatory action, since GlcN suppresses inflammatory cell activation. To further extend the anti-inflammatory actions of GlcN, the effects of GlcN on synovial cells, endothelial cells and intestinal epithelial cells *in vitro* and *in vivo* systems were investigated (Nagaoka et al., 2011). Firstly, GlcN suppressed the IL-1β-induced activation of synovial cells *in vitro*. Furthermore, GlcN administration repressed synovial cell hyperplasia, cartilage destruction and inflammatory cell infiltration in rat AA. Secondly, GlcN suppressed the TNF-α-induced activation of intestinal epithelial cell HT-29 *in vitro*. In addition, GlcN administration improved the clinical symptoms, and colonic inflammation and tissue injury in dextran sulfate sodium-induced colitis in rats. Finally, GlcN suppressed the TNF-α-induced activation of endothelial cells *in vitro*. Moreover, GlcN administration repressed the formation of atherosclerotic lesion and infiltration of

inflammatory cells into the lesion in spontaneously hyperlipidemic mice B6 KOR Apo^{shl}. Together these observations support the idea that GlcN can function as not only a chondroprotective agent but also an anti-inflammatory molecule in the body.

2.2.1.4 Antimicrobial effect

Malik, Singh and Mathur (2013) assessed the antimicrobial activity of GlcN. The results obtained showed some degree of potential antimicrobial property of GlcN at all variable concentrations. It also concluded the probable antimicrobial activity of GlcN even at dilute concentration of 0.25 mg/mL. The maximum inhibitory zone i.e. 30±0.71 mm was observed against *Staphylococcus saprophyticus* (MTCC 6155) and minimum being against *Micrococcus luteus* (MTCC 106) i.e. 21±0.53 mm at 1 mg/mL concentration. GlcN was observed to be a strong inhibitory antimicrobial agent at 1 mg/mL concentration.

2.2.1.5 Anticancer effect

Many reports have confirmed the inhibition effect on tumor growth of GlcN *in vivo* and *in vitro*. However, the mechanism for the anticancer effect of GlcN is still not clearly understood. Several mechanisms proposed for anticancer action of GlcN are alterations of uracil and adenine nucleotide contents, disruption of the structure and function of cellular membrane system, and inhibition of protein, RNA and DNA synthesis. Since there are several reports suggesting GlcN inhibits protein synthesis, Oh et al. (2007) examined whether GlcN affected p70S6 K activity, an important signaling molecule involved in protein translation. The results showed that GlcN inhibited the activity of p70S6K and the proliferation of DU145 prostate cancer cells and MDA-MB-231 breast cancer cells. GlcN decreased phosphorylation of p70S6K, and its downstream substrates ribosomal protein S6, and eIF-4B, but not

mTOR and 4EBP1 in DU145 cells, suggesting that GlcN induced inhibition of p70S6K but not through the inhibition of mTOR. In addition, GlcN enhanced the growth inhibitory effects of rapamycin, a specific inhibitor of mTOR. These findings suggest that GlcN can inhibit growth of cancer cells through dephosphorylation of p70S6K.

The anti-proliferative, growth suppressive and/or pro-apoptotic effects of GlcN-HCl on YD-8 human oral squamous cell carcinoma cells was investigated (Jung et al., 2012). The results showed that GlcN-HCl down-regulated hypoxia-inducible factor-1α which is considered as a tumor angiogenic transcription factor. It is thus suggested that GlcN-HCl had ability for anti-cancer and may be used as a potential anti-cancer drug.

2.2.1.6 Immune regulatory capacity

The potential immune regulatory capacity of GlcN has also been suggested. Indeed, GlcN has been shown to suppress proinflammatory cytokine action in human chondrocytes (Shikhman, Kuhn, Alaaeddine and Lotz, 2001) to inhibit NF-κB activation and IL-1β bioactivity in rat chondrocytes (Gouze et al., 2002) and to suppress unprimed T cell response by interfering with functions of APCs and by a direct inhibitory effect on CD3-induced T cell proliferation (Forchhammer et al., 2003). Furthermore, the addition of GlcN to immune cells *in vitro* prevented both their activation and their ability to initiate the MLR. More importantly, a single daily i.v. injection of GlcN was able to prolong cardiac allograft survival in mice (Ma et al., 2002). GlcN also showed a therapeutic effect in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis (Zhang, Yu, Gran and Rostami, 2005).

2.2.1.7 Wound healing capacity

HA synthesized by fibroblasts is importance for rapid healing and reduced scarring since its promotion of mesenchymal and epithelial cells' migration and proliferation through interactions with membrane receptors-hyaladherins.

HA is a kind of polysaccharide with repeated disaccharide subunits consisting of glucuronic acid and GlcNAc. From the pathway of GAG (Figure 2.8), it seems likely that the rate of GlcN-6-phosphate synthesis is the pace-setting part and the availability of exogenous GlcN is a rate-limiting factor for HA and other GAGs biosynthesis. Thus, there was a hypothesis that the adequate intake of GlcN would increase the speed and quality of wound healing and reduce the risk of scarring and related complications, such as keloids, intraperitoneal adhesion and intestinal stricture (McCarty, 1996). For further research, the experimental verification and the formal double-blind study are required for this hypothesis. A systematic study can help make GlcN useful not only in the treatment of surgical or traumatic wounds, but also in the management of cutaneous or GI ulcers.

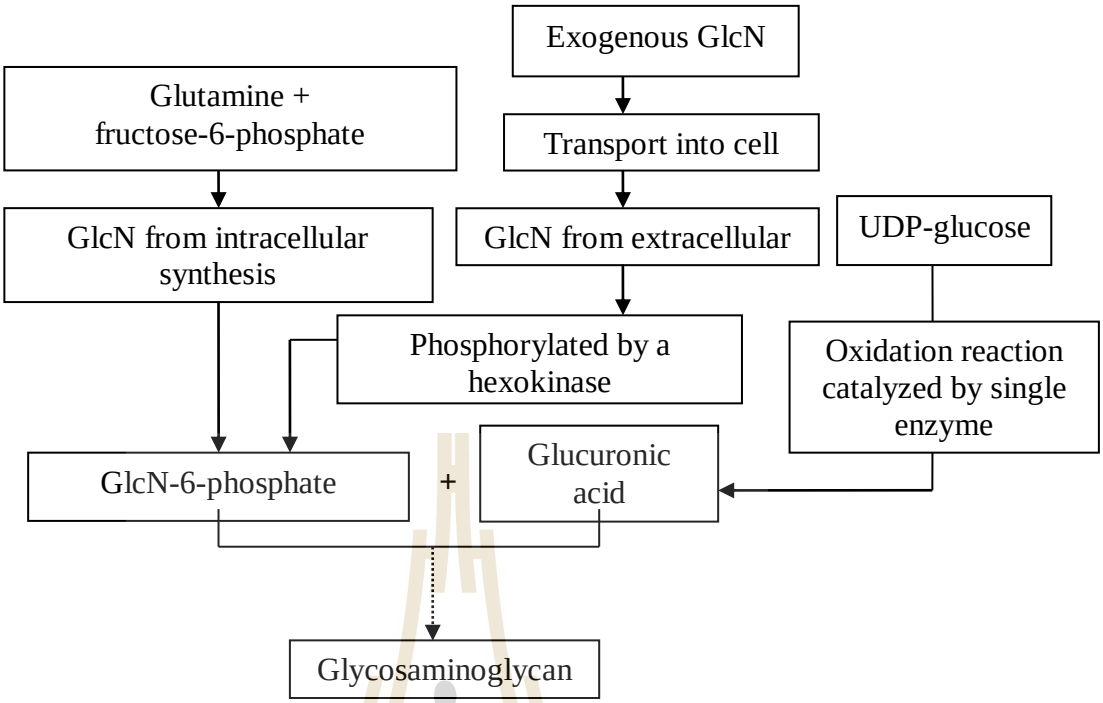


Figure 2.8 The pathway of glycosaminoglycan synthesis. GlcN: glucosamine; UDP: uridine diphosphate. (McCarty, 1996).

2.2.1.8 Psoriasis

Autocrine productions of amphiregulin (AR) and TGF-α can promote the proliferation of keratinocytes and activate the epidermal growth factor receptor (EGFR). In turn, the excessive activation of EGFR leads to the excessive production of AR and TGF-α. Thus, there is a vicious circle of the basal keratinocytes proliferation in psoriasis. In addition, when keratinocytes are cultured at high density, the growth is mainly dependent on AR and a lesser extent on TGF-α. To break the vicious circle and heal psoriasis, inhibition of AR activity is the most important point.

Generally, heparinoids such as heparin, heparan sulfates (HS) and other heavily sulfated mucopolysaccarides are used to treat psoriasis. It acts as antagonist of AR activity and disrupts the interaction of AR with EGFR through the binding of AR

and heparinoids. As reported in the previous papers, GlcN availability is rate-limiting for mucopolysaccharide and the GlcN supplement can enhance the biosynthesis of HS proteoglycans. According to these considerations, a feasible idea is given that oral intake of GlcN would provide therapeutic benefit in psoriasis due to the stimulation of endogenous HS biosynthesis (McCarty, 1997).

Furthermore, ω-3-rich fish oil may reduce the keratinocytes production of autocrine growth factors, including AR, by restraining the excessive activity of protein kinase C. It is suggested that intake GlcN with fish oil should increase the benefits for managing psoriasis.

2.2.1.9 Migraine prophylaxis

According to the study of Russell and McCarty (2000), ten patients with migraine or migraine-like vascular headaches have been treated with daily oral GlcN for 4-6 weeks, a substantial diminution in frequency and/or intensity of headache has been noted and the benefit seems to be dose-dependent. The reason is that GlcN is the rate-limiting precursor for GAG synthesis. Thus, GlcN administration could promote the production of endogenous heparin in dural mast cell and heparin has inhibition on the neurogenic inflammation which is responsible for migraine and other vascular headaches.

2.2.1.10 Skin aging

Interestingly, GlcN is also related to the skin aging process by inhibiting the polyglycan/collagen degradation, increasing the moisture content, improving the smoothness of the skin, and reducing the appearance of visible wrinkles and fine lines. The skin aging is characterized mainly by the continuous loss of elasticity and moisture, and reflected by major structural changes and variations in

compositions. Compared with young skins, aged skins have less collagen and GAGs which are produced by the skin cells.

GlcN is related to the inhibition of the collagen degradation by inhibiting MMP1. MMP1 is recognized as a marker of aged skin and can be induced by UV (Petiard, Michaux and Courtois, 2013).

HA is a GAG and has a good capacity of hydration. When young skin cells are exposed after exfoliation, it is produced in higher quantities by the skin cells. It is composed of a chain of alternating, repeating D-glucuronic acid and GlcNAc molecules. As it is known, GlcNAc is the rate-limiting factor in the HA production in living cells. Thus, the topical application of GlcN assists in the continued production of HA.

2.2.1.11 Side effects

The usual dose recommended for benefit oral administration of GlcN is 1500 mg. It is a very large doses and well tolerated without documented toxicity (Anderson, Nicolosi and Borzelleca, 2005, Thakral, Debnath and Dent, 2007). There are no reports of overdosage and allergic reactions. There are no known interactions with any other nutritional supplement, drug, herb or food. Biochemical, hemostatic and hematological measurements indicate that it is safe.

There are no known or reported contraindications to GlcN supplementation. Concerns have been expressed for the potential to increase insulin resistance if GlcN is given intravenously, as it has been shown to do so in both normal and experimentally diabetic animals. However, this effect is not seen with oral preparations. Some researchers, however, do suggest that it is contraindicated in diabetes, with concerns about its effect on insulin secretion. Individuals who are

diabetic or overweight should notice on the caution and carefully monitor blood sugar levels if supplements are taken. There is no data, children and pregnant or nursing women should avoid consumption (Wolinsky and Driskell, 2004).

Side effects are few and are usually mild GI problems such as upset stomach, dyspepsia, abdominal distension and cramps; constipation, diarrhea, nausea and heartburn. These suggest that it is better taken with food. Short-term adverse effects for GlcN use also include headache, drowsiness and skin rash or pruritis (Anderson, Nicolosi and Borzelleca, 2005).

Although side effects occur with the use of GlcN, it appears to be a generally safe compound when used in patients with OA. It favorably compares with the adverse effect profiles of other drugs used in the treatment of OA, such as analgesic agents or NSAIDs (Reginster et al., 2001, Thakral, Debnath and Dent, 2007).

2.2.2 Production of glucosamine

2.2.2.1 Acid hydrolysis

For industrial production, most of GlcN is derived from chitin or de-proteinized and de-mineralized exoskeletons of shellfish (lobster, shrimp, krill, crab and prawn) by acid hydrolysis (Ajavakom, Supsvetson, Somboot and Sukwattanasinitt, 2012). Concentrated hydrochloric acid is the most common one that used for hydrolyzing raw materials to GlcN completely (Ferrer et al., 1996).

The GlcN was obtained from acid hydrolysis of waste shrimp shell with high chitin content 22.6%. The concentrated hydrochloric acid was used in the acid hydrolysis process and gave a GlcN yield of 79-89% (Ferrer et al., 1996). From another study, the experimental results showed the GlcN contents range from 0-22.33% of DW cell wall in acid hydrolysates. Hydrolysis of cell wall samples with 6N HCl

was performed at 105°C for 1 h under N_2-atmosph. The determined GlcN may be a degradation product of biopolymers containing GlcNAc (Burczyk et al., 1999). U.S. Pat. No. 6486307 describes an improved method for production of GlcN-HCl from chitin acid hydrolysis. A very fine size chitin is obtained by grinding and then digested with concentrated hydrochloric acid at 95°C. Then, the GlcN-HCl was decolorized with activated charcoal and washed with ethanol (Gandhi and Laidler, 2002).

To improve the reaction rate and selectivity, the microwave and ultrasonic wave assisted acid hydrolysis of shrimp shell α-chitin were investigated (Ajavakom, Supsvetson, Somboot and Sukwattanasinitt, 2012). Microwave heating shortened the hydrolysis time from 120 min in the conventional heating process to 12 min with the GlcN-HCl yield of 57%. Sonication was used in assisting chitin dissolution in 38% HCl to produce GlcN-HCl in 62% yield.

Although these natural sources for GlcN production are acceptable for some applications, it also has some limitations. These limitations include the significant variations in the compositions of the wild shellfish because they grow under uncontrolled natural circumstance. The unpredictable size and compositions of the shellfish from different growing conditions and species may lead to inconsistent quality of GlcN. The price and availability of shellfish may vary significantly over time because of its seasonal harvests. Also, the wild shellfish is exposed to environmental contaminants, for example, heavy metals that may be concentrated in shellfish and retained in the final GlcN product. A further concern is that significant portions of the human population have shellfish allergies and are unable to consume products or ingredients derived from shellfish. Hyper allergenic individuals may be still allergic to highly processed GlcN which even minute traces of allergens present

from the original shellfish. Thus, whether or not all of the allergens have been removed, for the shellfish allergenic people, the consumption of shellfish-derived GlcN is a concern. An additional problem is that excessive harvest of shellfish from the seas and oceans may contribute to depletion of the world's marine resources and have a great negative environmental impact. Another concern is the high content of sodium chloride in the finally product may be increase the risk of stroke, heart failure, osteoporosis, stomach cancer and kidney disease.

Another more recent method is producing GlcN from microbial biomass and biomass waste generated from fermentation for example, *Aspergillus*, *Penicillium*, *Mucor* and mushroom, or the bio-waste from citric acid fermentation. As described in U.S. Pat. No. 7049433, No. 8034925 and No. 8222232 (Anderson et al., 2012, Fan et al., 2006, Fosdick, Bohlmann, Trinkle and Ray, 2011), the method generally includes cultivating the microorganisms by fermentation, and then the obtained biomass reacts with concentrated hydrochloric acid. In patents of US 8383808, a method to prepare GlcN-HCl from citric acid residue to alleviate the residue environment pollution was described (Jianguo, Degui and Wang, 2013). The citric acid residue was from citric acid production by means of fermentation. After acid hydrolysis, suction filtration and concentration, a vegetarian, safety and environment-friendly GlcN-HCl without fishy odor and heavy metal pollution was obtained.

Several studies have been reported on production of GlcN using wild-type fungi (Table 2.2). Most of these studies used wild-type fungi that belong to Ascomycotina (*Aspergillus* sp.) and Zygomycotina (*Rhizopus* sp., *Mucor* sp.) subdivision.

Several fermentation aspects such as pellet size, working volume, agitation rate and stimulating factor were studied to produce GlcN using a wild-type fungi, *Aspergillus* sp. BCRC 31742 cultivated under submerged fermentation (Sitanggang, Wu, Wang and Ho, 2010). The optimum conditions for culture cultivation was pellet diameter of 2.15 mm, 50 mL working volume (250 mL T-flask), incubation at 30°C, 200 rpm and pH 7.0 for 5 days, the biomass concentration was 33.82 g/L and GlcN concentration was 7.05 g/L. Methanol was the best stimulating factor in terms of GlcN concentration compared to glutamic acid, cycloheximide and ethanol. Addition of methanol (1.5% v/v) into fermentation medium led to maximum GlcN concentration of 7.48 g/L obtained.

Response surface methodology was employed for optimizing GlcN fermentation medium constituents (Chang, Sitanggang and Wu, 2011). Under the optimized medium constituents, the GlcN concentration was 5.48 g/L from cultivation of *Aspergillus* sp. BCRC 31742 in flask. Cultivation in fermenter resulted in GlcN concentration of 3.91 g/L with biomass of 14.6 g/L.

These methods have tried to remove the possibility of allergic reactions. However, the long cultivation periods increase costs and slow down the production rates significantly. Additional, the amount of chitin in biomass is low and the resulting GlcN may have low yield and unacceptable high ash content in many cases.

Table 2.2 GlcN fermentation profiles of different wild-type fungi.

Microbial	Medium	Biomass (g/L)	GlcN conc. (g/L)	Content (mg/g dw cells)	Productivity (mg/(L h))	GlcN yield (mg/g carbon)	Reference
Rhizopus oligosporus NRRL 2710	SDB[a]	---	---	0.11	---	---	(Sparringa and Owens, 1999)
Rhizopus oligosporus NRRL 2710	SDA[b]	---	---	0.11	---	---	(Sparringa and Owens, 1999)
Aspergillus sp.	WBS[c]	---	---	24.10	---	---	(Carter, Nokes and Crofcheck, 2004)
Monascus pilosus	RSA[d]	---	0.26	---	---	13.20	(Yu et al., 2005)
Aspergillus sp. BCRC31742	GP[e]	18.50	3.43	185	20.40	137	(Hsieh, Wu, Wei and Wang, 2007)
Monascus pilosus BCRC31527	RSA	17.70	0.72	40.40	4.28	35.90	(Hsieh, Wu, Wei and Wang, 2007)
Rhizopus oligosporus BCRC 31996	SDB	2.09	0.40	188	2.34	13.20	(Hsieh, Wu, Wei and Wang, 2007)
Aspergillus sp. BCRC31742	WF[f]	21.56	5.48	250	32.60	160	(Chang, 2008)
Rhizopus oryzae ATCC20344	Dairy manure	---	---	160	---	---	(Liao, Liu, Frear and Chen, 2008)
Aspergillus sp. BCRC31742	WF	33.82	7.05	210	58.73	210	(Sitanggang, Wu, Wang and Ho, 2010)
Aspergillus sp. BCRC31742	WF-M[g]	28.68	7.48	260	62.33	220	(Sitanggang, Wu, Wang and Ho, 2010)

[a]Medium: sabouraud dextrose broth.
[b]Medium: sabouraud dextrose agar.
[c]Medium: wheat bran and soy bean meal.
[d]Medium: rice bran + sucrose + ammonium chloride.
[e]Medium: glucose-peptone.
[f]Medium: white fine granulated sugar-peptone.
[g]Medium: white fine granulated sugar-peptone (addition of methanol)

2.2.2.2 Enzymatic hydrolysis

Several researches have tried to use enzymatic hydrolysis instead of acid hydrolysis. The production is usually GlcNAc. If the target production is GlcN, there is another step needed to convert GlcNAc to GlcN. There are two kinds of enzymes needed for the production of GlcNAc from chitin. Firstly, chitinases (EC 3.2. 1.14) which are a group of complex hydrolytic enzymes that catalyze depolymerisation of chitin into oligo-GlcNAc chains. Then the chitobiase (EC 3.2.1.52) also called β-N-acetylglucosaminidase is responsible for the conversion of oligomers to GlcNAc. Many chitinases and chitobiase were produced from *Aeromonas* sp. (Kuk et al., 2005a, Kuk et al., 2005b, Lien, Too, Wu and Yu, 2005, Sashiwa et al., 2002), *Acremonium* sp. (Sashiwa et al., 2003, Sukwattanasinitt, Zhu, Sashiwa and Aiba, 2002), *Trichoderma* sp. (Binod et al., 2007, Donzelli, Ostroff and Harman, 2003, Sashiwa et al., 2003, Sukwattanasinitt, Zhu, Sashiwa and Aiba, 2002), *Aspergillus* sp. (Jung, W.-J. et al., 2006, Sukwattanasinitt, Zhu, Sashiwa and Aiba, 2002), *Bacillus* sp. (Liang, Chen, Yen and Wang, 2007, Pichyangkura et al., 2002, Thamthiankul, Suan-Ngay, Tantimavanich and Panbangred, 2001), *Burkholderia* sp. (Pichyangkura et al., 2002), *Paenibacillus* sp. (Jung, Souleimanov, Park and Smith, 2007), *Penicillium* sp. (Binod et al., 2007), *Serratia* sp. (Carroad and Tom, 1978, Cosio, Fisher and Carroad, 1982, Donzelli, Ostroff and Harman, 2003, Tom and Carroad, 1981, Wang et al., 2009), *Streptomyses* sp. (Kapaun, Loos and Reisser, 1992) have been reported and applied to production of GlcNAc from chitin.

Aeromonas sp. DYU-Too 7 was screened to produce chitinases. The enzymes had an optimal reaction temperature and pH at 50°C and 7.0, respectively. The best concentration of chitin in the fermentation process was 4%. GlcNAc, the main product

in the hydrolysate, was 0.7 g/L at 36 h of cultivation (Lien, Too, Wu and Yu, 2005). A crude enzyme preparation from a bacterium *Aeromonas* sp. GJ-18 isolating from coastal soil was used to hydrolyze swollen chitin to GlcNAc. The optimum pH for enzyme activity was 5. The major product was GlcNAc when the temperature was below 45°C; while the major product was $(GlcNAc)_2$ when the temperature was above 50°C. When the crude enzyme preparations (10 U) was incubated with swollen chitin at 40°C, the yield of GlcNAc was 83.0% within 5 days. After 9 days, the yield was increased to 94.9% (Kuk et al., 2005b). Kuk et al. (2005a) developed a typical protocol using *Aeromonas* sp. GJ-18 crude enzyme preparation as a valuable biological process for a selective and efficient production of GlcNAc from chitin by simply controlling the enzymatic reaction temperature. The analyzed results indicated that a selective production of GlcNAc; a major hydrolytic product, was achieved at 45°C, and the yield of GlcNAc gradually increased with incubation time. By using the strong chitinolytic activity crude enzyme preparation from *Aeromonas* sp. GJ-18, swollen chitin was hydrolyzed with a yield of 74% GlcNAc within 5 days, and the relative composition of GlcNAc in total hydrolysate produced was 93.7%.

In the study of Sashiwa et al. (2002), the effective production of GlcNAc from by crude enzymes derived from *Aeromonas hydrophila* (A) H-2330 could be achieved with a good yield of 66%-77%. In the later research, more details were studied on the production of GlcNAc from various chitins and N-acetyl-chitooligosaccharides by enzymatic hydrolysis (Sashiwa et al., 2003). The usage of mixture of the crude enzyme preparations such as cellulases derived from *Trichoderma viride* (T) and *Acremonium cellulolyticus* (A) and lipase, hemicellulase, pectinase could enhance the production of GlcNAc from the hydrolysis of α-chitin and β-chitin. These

non-chitinolytic crude enzymes can degrade both chitin and chitosan due to the presence of endo- and exo-type chitinase in crude enzyme preparations. This method is economically feasible because these crude enzymes are cheap.

An extracellular 104 kDa exo-β-D-glucosaminidase with strong chitosanolytic enzyme activity was purified and characterized from the culture supernatant of *Aspergillus fumigatus* S-26. The enzyme cannot degrade chitin, cellulose, and starch, it only can liberate GlcN from chitosan (Jung, W.-J. et al., 2006).

Preparation of GlcNAc from chitin by inexpensive, commercially available, non-chitinase enzymes from *Aspergilus niger*, *Acremonium cellulolyticus* and *Trichoderma viride* were investigated. The results indicated that cellulase *A. cellulolyticus* exhibited highest chitinolytic activity at its optimum pH 3 and enzymes from *A. niger* displayed higher β-N-acetylhexosaminidase activity. The yield of GlcNAc was significantly increased when the enzymes from *A. niger* were mixed with cellulase *A. cellulolyticus*. The appropriate mixing ratio of cellulase *A. cellulolyticus* to enzymes from *A. niger* was 9:1 (w/w) and an optimum substrate concentration was 20 mg/ml (Sukwattanasinitt, Zhu, Sashiwa and Aiba, 2002).

Chitin, colloidal chitin and water-soluble chitosan were hydrolyzed by crude enzyme solution produced by *Bacillus amyloliquefaciens* V656 at 37°C for 1, 12, and 24 h. The amount of GlcN increased with the hydrolysis time increased. After hydrolyzed for 24 h, the GlcN concentrations were 0.22, 0.24, and 0.2 mg/ml, respectively (Liang, Chen, Yen and Wang, 2007). Colloidal chitin was hydrolyzed by the exochitinase from *Bacillus thuringiensis* subsp. *pakistani*. GlcNAc was generated from early stage (Thamthiankul, Suan-Ngay, Tantimavanich and Panbangred, 2001).

Finely α-chitin powder from crab shells and β-chitin from squid pens were digested by crude chitinase from *Burkholderia cepacia* TU09 and *Bacillus licheniformis* SK-1 for the production of GlcNAc. *B. cepacia* TU09 chitinase hydrolyzed β- and α-chitin within 1 and 7 days, respectively, giving a final GlcNAc yield greater than 85%. Chitinase from *B. licheniformis* SK-1 produce GlcNAc in the yield of 75% and 41% from β- and α-chitin within 6 days (Pichyangkura et al., 2002).

GlcNAc was produced from colloidal chitin by use of crude multi-chitinolytic enzyme complex obtained from *Paenibacillus illinoisensis* KJA-424. The production rate of GlcNAc increased continuously during incubation. The maximum production of GlcNAc was 1.71 mg/ml (yield of 62.2%) after 24 h of incubation. This enzymatic process was effective for the production of GlcNAc, facilitating its potential use in industrial applications (Jung, Souleimanov, Park and Smith, 2007).

To find out a cost effective, eco-friendly enzymatic method for the production of GlcNAc, fifteen fungal strains were evaluated for endochitinase and chitobiase production under solid-state fermentation using agro-industrial residues (Binod et al., 2007). The results showed that *Penicillium aculeatum* NRRL 2129 had maximum endochitinase activity whereas *Trichoderma harzianum* TUBF 927 had maximum chitobiase activity. When endochitinase and chitobiase were used in combination, the efficiency of degradation of colloidal chitin to GlcNAc was improved.

Some studies have been focused on the optimum production of extracellular chitinase enzyme system. The appropriate microorganisms can be isolated from soil and marine wastes, or obtained from specific collections. *Serratia marcescens* QMB 1466 is probably the most effective species for this purpose (Carroad and Tom, 1978, Cosio, Fisher and Carroad, 1982, Tom and Carroad, 1981). In Carroad and Tom

(1978)'s study, an extracellular chitinase enzyme system was obtained by a submerged culture of microorganisms with the chitin waste. When the enzymatic activity was 83 units/ml, after 40 h, 57.6% crude prepared chitin was hydrolyzed to GlcN. For colloidal chitin, the hydrolyzing time is longer. The hydrolysis of chitin by *Serratia marcescens* chitinase produced from *Serratia marcescens* QMB 1466 was studied by Tom and Carroad (1981). Hydrolysis increased with increasing enzyme activity and chitin slurry concentration, and with decreasing particle size. When the enzymatic activity was 193 units/ml, after 44 h, the GlcN yield was 78.0%. Pretreatment of shrimp processing waste including deproteination and demineralization was done before enzymatic hydrolysis. Enzymatic hydrolysis of pretreated chitin waste achieved 80% conversion in 24 hours with 73 units/ml enzymatic activity. Optimum temperature and pH were determined for maximum chitinase production in submerged culture, using pretreated chitin waste as substrate (Cosio, Fisher and Carroad, 1982). In the study of Wang et al. (2009), GlcNAc was produced by *Serratia* sp. TKU020 fermentation with squid pen wastes as the sole carbon/nitrogen source. After 4 days of fermentation, the maximum production of GlcNAc was 1.3 mg/ml.

Approximately 100 g/L of chitin from langostino crab shells was completely degraded by a combination of enzyme preparations from *Trichodermaatro viride* and *Serratia marcescens* to GlcNAc (78%), GlcN (2%), and chitobiose (10%) at 32°C in 12 days. The substrate did not have any pre-treatment including size reduction or swelling and during the incubation there was no removal of the inhibitory end-products from the reaction mixture (Donzelli, Ostroff and Harman, 2003).

From the enzymatic degradation of three European, virus-sensitive symbiotic *Chlorella* spp. strains cell walls by chitinase of *Streptomyses* sp., 7-17% GlcN was

obtained. The results showed that GlcN-containing cell walls of non-symbiotic *Chlorella* spp. belonging to *C. vulgaris*, *C. sorokiniana*, and *C. kessleri* (Kapaun, Loos and Reisser, 1992).

The effects of illumination with white linearly polarized light (WLPL) on the enzymatic degradation of chitin and chitosan were investigated (Konieczna-Molenda, Fiedorowicz, Zhong and Tomasik, 2008). Chitinase and chitosanase were illuminated at room temperature in separate vessels, and then mixed in reactors containing polysaccharides. Hydrolysis of chitosan to GlcN followed first order kinetics whereas hydrolysis of chitin to GlcNAc deviated from the first order kinetics. In both cases, an increase in the rate of hydrolysis depended on the illumination time. Efficient degradation required up to 60 min exposure of the enzyme to WLPL.

The enzymatic hydrolysis is conducted under much milder conditions with less toxic reagents and higher selectivity compared with acid hydrolysis process. However, the GlcN yield was very low and another step was required to convert GlcNAc to GlcN. Additionally, the enzymes are not available commercially. There are still raw starting material limitations.

2.2.2.3 Genetically modified microorganisms

Several studies have been reported on GlcN production from genetic engineered microorganism fermentation.

A recombinant *Escherichia coli* was modified by metabolic engineering means to develop an economically competitive fermentation process for GlcN and GlcNAc production (Deng et al., 2005, Deng, Wassink and Grund, 2006). In comparison with the shellfish acid hydrolysis approach, the production of GlcN and GlcNAc from the

metabolic engineered microorganism fermentation has the advantages such as the less environmental pollution, the stable raw material and the lack of allergy factors.

In the study of Deng et al. (2005), an *E. coli* K12 strain (ATCC 25947) was used as the host for metabolic engineering. The host was modified to minimize the GlcN transport and catabolism and over-express the biosynthesis gene (glmS). The amount of GlcN detected in the growth medium was increased from 4 mg/L of the host strain to 75 mg/L of the best mutant over-expressed strain at 26 h. To avoid the production inhibition of glmS by glucosmine-6-phosphate, mutant variants of the *E. coli* glmS were generated by error-prone PCR. It is effective in GlcN production that increased the GlcN level up to 6 g/L. However, rapid degradation of GlcN and the inhibitory effect of GlcN and its degradation products on the host cells were the limited factors for further GlcN improvement. By over-expressing a heterologous GlcN-6-P N-acetyltransferase gene (GNA1), a significant conceptual breakthrough for the pathway to GlcNAc, which is stable and inert to the *E. coli* host, was extended. Under the optimizing fermentation medium and other process conditions, GlcNAc level in the medium was increased to 110 g/L. In conclusion, using the modified *E. coli* strains that over-expressed the GlcN synthase (GlmS), inactivated the catabolic genes, and minimized the inhibitory effects of GlcN-6-P and GlcN on the host cell, a high-performance fermentation process for the production of GlcNAc was developed.

In the later research, it demonstrated that a kinetically unfavorable enzyme GlcN-6-Pdeaminase (NagB) could be used successfully in constructing a GlcNAc production metabolic pathway by coupling with efficient downstream reactions (Deng, Wassink and Grund, 2006). In the glmS deletion strains, overexpression of nagB produced a small amount of GlcN-6-P but sufficient to support the cell growth.

Overexpression of both nagB and GNA1 resulted in production of GlcNAc at levels comparable to strains overexpressing both GlmS and GNA1. It indicated that GNA1 acted as a strong driving force of the NagB-GNA1 pathway. When NagB was co-expressed with GNA1, it functioned essentially in the direction of GlcN-6-P synthesis. Compared with the GlmS-GNA1 pathway, the combination of NagB and GNA1 has unique advantages. GlmS uses glutamine as the amino donor; regeneration of glutamine consumes ATP. On the other hand, NagB uses a metabolically more economic amino donor, ammonia. Additionally, *E. coli* GlmS is a homodimer composed of subunits of 67 kDa. Most of the overexpressed GlmS protein is misfolded as inclusion bodies. Excessive aggregation of recombinant protein in the cytoplasm is often associated with cellular stress. Unlike *E. coli* GlmS, *E. coli* NagB is a homohexamer composed of much smaller subunits (about 30 kDa). Overexpressed NagB protein was mostly present in an active soluble form, and its overexpression is likely less stressful on the cell.

A step-wise dissolved oxygen (DO) control strategy was developed and may be useful for the industrial GlcN and GlcNAc production by recombinant *E. coli* (Chen, X. et al., 2012). In recombinant *E. coli*, the GlcN synthase encoding gene *glmS* and GlcN-6-P N-acetyltransferase encoding gene *gna1* were over-expressed. The influence of DO levels (10%, 20%, 30% and 40%) on the GlcN and GlcNAc production by recombinant *E. coli* was investigated. The results found that under different DO levels the highest specific GlcN and GlcNAc production rates were obtained at different culture stages. Therefore, a step-wise DO control strategy was developed, namely DO was controlled at 20% during 0-2 h, 30% during 2-8 h, 40% during 8-12 h and 30% during 12-18 h. From this DO step-wise regulated

fermentation, the total production of GlcN and GlcNAc reached 72.89 g/L, which was 1.37 times of that without DO control (53.31 g/L).

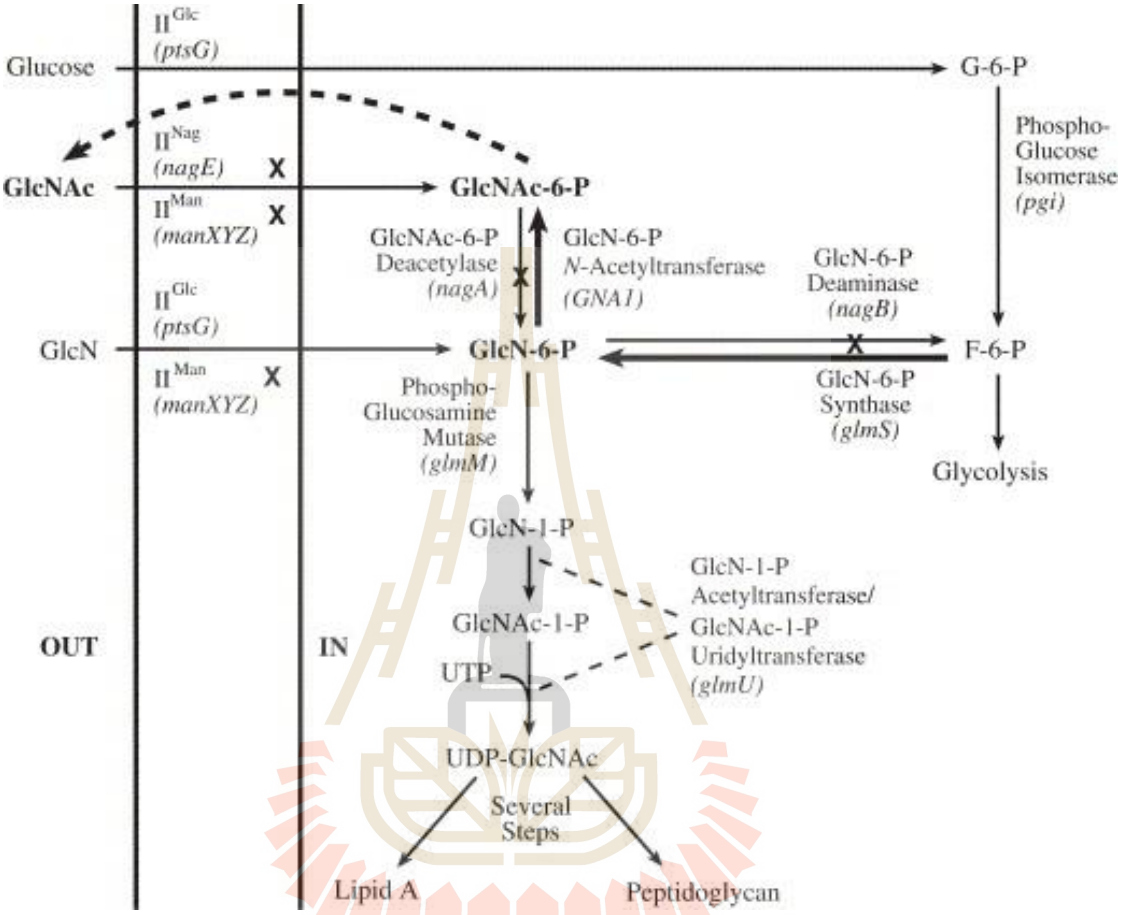


Figure 2.9 Pathway engineering for GlcNAc production in recombinant *E. coli*. Crosses indicate metabolic flux blocked by gene inactivation or deletion. Thicker lines indicate introduction and/or increase of the metabolic flux by gene over-expression. Dephosphorylation and secretion of GlcNAc-6-P are indicated as a dotted line. G-6-P: glucose-6-phosphate, F-6-P: fructose-6-phosphate, GlcN: glucosamine, GlcNAc: N-acetyl-glucosamine, II^{Glc}: glucose transporter, II^{NAG}: GlcNAc-specific transporter, II^{Man}: mannose transporter (Deng et al., 2005).

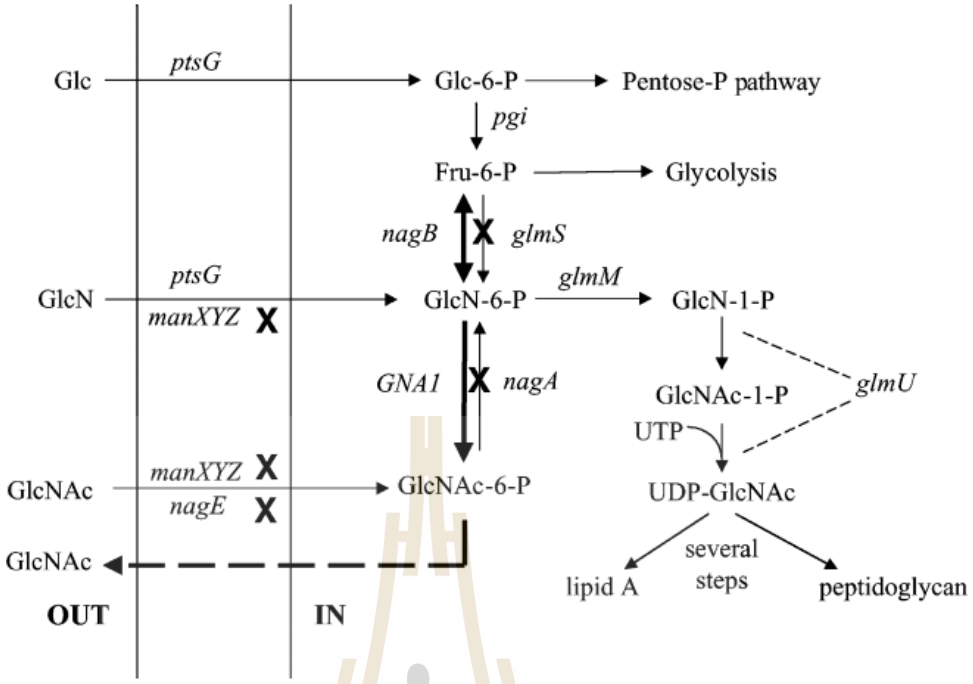


Figure 2.10 Pathway engineering for GlcNAc production in recombinant *E. coli*. Crosses indicate metabolic flux blocked by gene inactivation or deletion. Thicker lines indicate introduction and/or increase of the metabolic flux by gene overexpression. Dephosphorylation and secretion of GlcNAc-6-P are indicated as a dotted line. Glc: glucose, G-6-P: glucose-6-phosphate, F-6-P: fructose-6-phosphate, GlcN: glucosamine, GlcNAc: N-acetyl-glucosamine, *ptsG*: gene encoding glucose transporter, *manXYZ*: mannose transporter, *nagE*: GlcNAc transporter, *pgi*: phosphor-glucose isoerase, *glmS*: glucosamine synthase, *nagB*: GlcN-6-P deaminase, *nagA*: GlcN-6-P deacetylase, *GNAI*: GlcN-6-P N-acetyltransferase, *glmM*: phosphoglucosaminemutase, *glmU*: GlcN-1-P acetyltransferase/GlcNAc-1-P uridyltransferase, in: intracellular space, out: growth medium (Deng, Wassink and Grund, 2006).

2.2.2.4 Plant species

There are some patents described a method to generate GlcN from plants wherein fresh plant materials, or rehydrated dried plant materials or plant extracts through heat process. In the US patent 8378090, the process that produced GlcN from plant species was described (Petiard, Michaux and Courtois, 2013). A nitrogen-based fertilizer acting as GlcN precursor was used before harvest to obtain plant raw materials containing level of GlcN higher than 0.5% (5g per kg DW) of the dry matter. The plant was selected for its ability to generate free GlcN; in particular it may be selected from the group consisting of plant species containing sucrose, fructose or inulin such as *Cichorium*, *Daucus*, *Helianthus*, *Beta*. For example, root of chicory (*Cichoriumintybus*), carrot (*Daucuscarota*), tuber of Jerusalem artichoke (*Helianthus tuberosum*), and root of beet (*Beta vulgaris*). Fresh, dried or rehydrated raw plant materials were then heated using liquid maceration or drying process at a temperature between 70 and 100°C, for more than 10 h and less than one week, depending on the plant species and plant organ. The process was actual a chemical synthesis process that broadened the raw material range. The yield of GlcN was low and the plant cultivation needed a long time. However, the production was suitable for vegetarians.

2.3 ACE inhibitory peptides

The natural ACE inhibitory peptides were first obtained from snake venom (Ferreira, Bartelt and Greene, 1970, Kato and Suzuki, 1971, Ondetti et al., 1971). These ACE inhibitory peptides contained 5 to 13 amino acid residues per molecule, and proline at the carboxyl terminus of the molecule. Then, several studies found that

the synthetic nonapeptide SQ 20,881 (Teprotide, Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) lowered blood pressure in animal models of hypertension and human hypertension by blocking the pressor effect of RAS (Jaeger et al., 1978, Vinci et al., 1979). Since this discovery, peptide-based ACE inhibitory drugs were developed for clinical use in controlling hypertension (Wyvratt and Patchett, 1985).

To date, more and more studies have evidenced that food proteins have functions other than energetic and nutritional ones, and several peptides with potent ACE inhibitory activity have been isolated from them. Nowadays, more than 200 kinds of ACE-inhibitory peptides have been isolated and identified from proteins or their hydrolysates originating from natural and processed foods. Table 2.3 provides a partial summary of ACE inhibitory peptides derived from different food proteins. It can be seen that a variety of ACE inhibitory peptides with rather diverse molecular properties exist in different food proteins.

Table 2.3 Examples of ACE inhibitory peptides derived from different proteins.

Peptide sequence[a]	IC_{50}(μM)[b]	Origin	Reference
ER	667	Pork meat hydrolyzed by combination of pepsin and pancreatin	(Escudero, Sentandreu, Arihara and Toldrá, 2010)
KW	1.63	Sardine muscle hydrolyzed by *Bacillus licheniformis* alkaline protease	(Matsui et al., 1993)
IP	87.6	*Ulva rigida* C. Agardh protein hydrolyzed by combination of pepsin and bromelain	(Paiva, Lima, Neto and Baptista, 2016)
LY	110	Rapeseed protein isolate hydrolyzed by Alcalase	(He et al., 2013)
PK	4092	Fermented anchovy sauce	(Kim et al., 2016)
TF	810	Rapeseed protein isolate hydrolyzed by Alcalase	(He et al., 2013)
VW	12.9	Antarctic krill tail meat hydrolyzed by Thermoase PC10F	(Hatanaka, Miyahara, Suzuki and Sato, 2009)
AFL	65.9	*Ulva rigida* C. Agardh protein hydrolyzed by combination of pepsin and bromelain	(Paiva, Lima, Neto and Baptista, 2016)
AKK	3.13	Sardine muscle hydrolyzed by alkaline protease	(Matsufuji et al., 1994)

Table 2.3 Examples of ACE inhibitory peptides derived from different proteins (Continued).

Peptide sequence[a]	IC_{50}(μM)[b]	Origin	Reference
ASL	102.15	Silkworm pupa (*Bombyx mori*) protein hydrolyzed by combination of pepsin, trypsin and α-chymotrypsin	(Wu et al., 2015)
FVP	10.1	Soya milk hydrolyzed by PROTIN SD-NY10	(Tomatsu et al., 2013)
GCK	178	Fermented anchovy sauce	(Kim et al., 2016)
GPL	2.55	Bovine skin gelatin hydrolyzed by combination of Alcalase, pronase E and collagenase	(Kim, Byun, Park and Shahidi, 2001)
GPL	2.6	Alaska pollack skin gelatin hydrolyzed by combination of Alcalase, pronase E and collagenase	(Byun and Kim, 2001)
GPM	17.13		
GPV	4.67	Bovine skin gelatin hydrolyzed by combination of Alcalase, pronase E and collagenase	(Kim, Byun, Park and Shahidi, 2001)
IAV	27.0	Soya milk hydrolyzed by PROTIN SD-NY10	(Tomatsu et al., 2013)

Table 2.3 Examples of ACE inhibitory peptides derived from different proteins (Continued).

Peptide sequence[a]	$IC_{50}(\mu M)$[b]	Origin	Reference
IKW	0.21	Chicken muscle hydrolyzed by thermolysin	(Iroyukifujita, Eiichiyokoyama and Yoshikawa, 2000)
IPF	8.78	Fried whole egg hydrolyzed by combination of pepsin and pancreatin	(Majumder and Wu, 2009)
KLP	500	Pork meat hydrolyzed by combination of pepsin and pancreatin	(Escudero, Sentandreu, Arihara and Toldrá, 2010)
LKY	10.1	Antarctic krill tail meat hydrolyzed by Thermoase PC10F	(Hatanaka, Miyahara, Suzuki and Sato, 2009)
LIY	0.82	Human α_2-macroglobulin hydrolyzed by trypsin	(Nakagomi et al., 2000)
LPF	10.59	Fried whole egg hydrolyzed by combination of pepsin and pancreatin	(Majumder and Wu, 2009)
MAW	16.32	Cuttlefish muscle hydrolyzed by crude protease extract from cuttlefish hepatopacrease	(Balti et al., 2010)

Table 2.3 Examples of ACE inhibitory peptides derived from different proteins (Continued).

Peptide sequence[a]	IC_{50}(μM)[b]	Origin	Reference
MPF	17.98	Fried whole egg hydrolyzed by combination of pepsin and pancreatin	(Majumder and Wu, 2009)
NHP	1172	Fermented anchovy sauce	(Kim et al., 2016)
RPR	382	Pork meat hydrolyzed by combination of pepsin and pancreatin	(Escudero, Sentandreu, Arihara and Toldrá, 2010)
TTI	24.94	Fried whole egg hydrolyzed by combination of pepsin and pancreatin	(Majumder and Wu, 2009)
VAP	18.6	Grass carp protein hydrolyzed by Alcalase	(Chen, J. et al., 2012)
VDF	6.59	Fried whole egg hydrolyzed by combination of pepsin and pancreatin	(Majumder and Wu, 2009)
VNP	6.4	Rice protein hydrolyzed by combination of Alcalase and trypsin	(Chen et al., 2013a)
VNP	32.5	Soya milk hydrolyzed by PROTIN SD-NY10	(Tomatsu et al., 2013)
VWP	4.5	Rice protein hydrolyzed by combination of Alcalase and trypsin	(Chen et al., 2013a)

Table 2.3 Examples of ACE inhibitory peptides derived from different proteins (Continued).

Peptide sequence[a]	IC_{50}(μM)[b]	Origin	Reference
VYP	6.1	Cuttlefish muscle hydrolyzed by crude protease extract from cuttlefish hepatopacrease	(Balti et al., 2010)
WHP	4.8	Soya milk hydrolyzed by PROTIN SD-NY10	(Tomatsu et al., 2013)
DGGP	164	Fermented anchovy sauce	(Kim et al., 2016)
FFYY	1.9	Soya milk hydrolyzed by PROTIN SD-NY10	(Tomatsu et al., 2013)
FLEK	79.5	Cupuassu seed protein isolate hydrolyzed by Alcalase	(da Cruz, Pimenta, de Melo and Nascimento, 2016)
KPLL	11.98	Chicken breast meat hydrolyzed by simulated *in vitro* GI digestion	(Sangsawad, Roytrakul and Yongsawatdigul, 2017)
LEPP	100.1	Soya milk hydrolyzed by PROTIN SD-NY10	(Tomatsu et al., 2013)
LRFF	116.9	Milk fermented with yeast *Kluyveromyces marxianus*	(Li et al., 2015)

Table 2.3 Examples of ACE inhibitory peptides derived from different proteins (Continued).

Peptide sequence[a]	IC_{50}(μM)[b]	Origin	Reference
PAFG	35.9	*Enteromorpha clathrata* protein hydrolyzed by Alcalase	(Pan, Wang, Jing and Yao, 2016)
RALP	650	Rapeseed protein isolate hydrolyzed by Alcalase	(He et al., 2013)
VEGY	128.4	*Chlorella ellipsoidea* hydrolyzed by Alcalase	(Ko et al., 2012)
VIIF	8.7	Cuttlefish muscle hydrolyzed by crude protease extract from cuttlefish hepatopacrease	(Balti et al., 2010)
VRYL	24.1	Manchego cheese	(Ruiz, Ramos and Recio, 2004)
WNPR	880.0	Soya milk hydrolyzed by PROTIN SD-NY10	(Tomatsu et al., 2013)
ACKEP	126	Pistachio kernel hydrolyzed by combination of pepsin and trypsin	(Li et al., 2014)
ELFTT	6.35	Chicken breast meat hydrolyzed by simulated *in vitro* GI digestion	(Sangsawad, Roytrakul and Yongsawatdigul, 2017)

Table 2.3 Examples of ACE inhibitory peptides derived from different proteins (Continued).

Peptide sequence[a]	IC_{50}(μM)[b]	Origin	Reference
ELFTT	6.35	Chicken breast meat hydrolyzed by simulated *in vitro* GI digestion	(Sangsawad, Roytrakul and Yongsawatdigul, 2017)
ERYPI	8.76	Fried whole egg hydrolyzed by combination of pepsin and pancreatin	(Majumder and Wu, 2009)
ITTNP	549.0	Porcine myosin hydrolyzed by thermolysin	(Arihara et al., 2001)
MEKHS	63	Cupuassu seed protein isolate hydrolyzed by Alcalase	(da Cruz, Pimenta, de Melo and Nascimento, 2016)
MNPPK	945.5	Porcine myosin hydrolyzed by thermolysin	(Arihara et al., 2001)
RYLGY	0.71	Casein hydrolyze by pepsin	(Contreras et al., 2009)
VAMPF	0.44	Small-spotted catshark discards hydrolyzed by combination of subtilisin and trypsin	(García-Moreno, Espejo-Carpio, Guadix and Guadix, 2015)
VELYP	5.22	Cuttlefish (*Sepia officinalis*) muscle protein hydrolyzed by cuttlefish hepatopancreas	(Balti et al., 2015)
YTAGV	23.38	Fried whole egg hydrolyzed by combination of pepsin and pancreatin	(Majumder and Wu, 2009)

Table 2.3 Examples of ACE inhibitory peptides derived from different proteins (Continued).

Peptide sequence[a]	IC_{50}(μM)[b]	Origin	Reference
KNGDGY	51.63	Cuttlefish (*Sepia officinalis*) muscle protein hydrolyzed by crude proteases from *Bacillus mojavensis* A21	(Balti et al., 2015)
KPLLCS	0.37	Chicken breast meat hydrolyzed by simulated *in vitro* GI digestion	(Sangsawad, Roytrakul and Yongsawatdigul, 2017)
MLVFAV	3.07	Defatted skipjack roe hydrolyzed by Alcalase	(Intarasirisawat, Benjakul, Wu and Visessanguan, 2013)
RSIKGF	32.74	Cuttlefish (*Sepia officinalis*) muscle protein hydrolyzed by crude proteases from *Bacillus mojavensis* A21	(Balti et al., 2015)
STHGVW	19.30		
VLSRYP	36.7	Milk fermented with yeast *Kluyveromyces marxianus*	(Li et al., 2015)
AYFYPEL	6.58	Casein hydrolyzed by pepsin	(Contreras et al., 2009)
EKSYELP	14.41	Cuttlefish (*Sepia officinalis*) muscle protein hydrolyzed by cuttlefish hepatopancreas	(Balti et al., 2015)

Table 2.3 Examples of ACE inhibitory peptides derived from different proteins (Continued).

Peptide sequence[a]	IC_{50}(μM)[b]	Origin	Reference
EVSQGRP	50	Sea cucumber (*Stichopus horrens*) hydrolyzed by Alcalase	(Forghani et al., 2016)
FNVPLYE	7.72	Salmon byproduct protein hydrolyzed by Alcalase	(Ahn, Jeon, Kim and Je, 2012)
GIHETTY	25.66	Cuttlefish (*Sepia officinalis*) muscle protein hydrolyzed by cuttlefish hepatopancreas	(Balti et al., 2015)
GLPLNLP	18.7	Chum salmon (*Oncorhynchus keta*) skin hydrolyzed by trypsin	(Lee, Jeon and Byun, 2014)
MILLLFR	0.12	Tilapia mince hydrolyzed by *Virgibacillus halodenitrificans* SK1-3-7 proteinases	(Toopcham, Roytrakul and Yongsawatdigul, 2015)
MVVDKLF	59.34	Cupuassu seed protein isolate hydrolyzed by Alcalase	(da Cruz, Pimenta, de Melo and Nascimento, 2016)
YQKFPQL	20.08	Casein hydrolyzed by pepsin	(Contreras et al., 2009)
AFVGYVLP	18.02	Cuttlefish (*Sepia officinalis*) muscle protein hydrolyzed by crude proteases from *Bacillus mojavensis* A21	(Balti et al., 2015)

Table 2.3 Examples of ACE inhibitory peptides derived from different proteins (Continued).

Peptide sequence[a]	**IC_{50}(μM)**[b]	**Origin**	**Reference**
FGASTRGA	14.7	Alaska Pollack frame protein hydrolyzed by pepsin	(Je, Park, Kwon and Kim, 2004)
GSGKHVSP	3.11	Cupuassu seed protein isolate hydrolyzed by Alcalase	(da Cruz, Pimenta, de Melo and Nascimento, 2016)
KKYNVPQL	77.1	Manchego cheese	(Ruiz, Ramos and Recio, 2004)
LHPGDAQR	10.3	Soya milk hydrolyzed by PROTIN SD-NY10	(Tomatsu et al., 2013)
PVNNPQIH	206.7	Small red bean (*Phaseolus vulgaris*) hydrolyzed by combination of Alcalase, papain and *in vitro* GI simulation	(Rui, Boye, Simpson and Prasher, 2013)
SFHPYFSY	82.71	Cuttlefish (*Sepia officinalis*) muscle protein hydrolyzed by crude proteases from *Bacillus mojavensis* A21	(Balti et al., 2015)
VWDPPKFD	9.10	Salmon byproduct protein hydrolyzed by Alcalase	(Ahn, Jeon, Kim and Je, 2012)

Table 2.3 Examples of ACE inhibitory peptides derived from different proteins (Continued).

Peptide sequence[a]	IC_{50}(μM)[b]	Origin	Reference
ACPGPNPGRP	2.03	Box jellyfish (*Chiropsalmus quadrigatus* Haeckel) venom hydrolyzed by combination of pepsin and papain	(So et al., 2016)
LLMLDNDLPP	35.7	Pacific cod skin gelatin hydrolyzed by combination of pepsin, trypsin and α-chymotrypsin	(Himaya, Ngo, Ryu and Kim, 2012)
FEDYVPLSCF	10.77	Salmon by product protein hydrolyzed by Alcalase	(Ahn, Jeon, Kim and Je, 2012)
VSRHFASYAN	210	Sea cucumber (*Stichopus horrens*) hydrolyzed by Alcalase	(Forghani et al., 2016)
AWLHPGAPKVF	135	*Phascolosoma esculenta* water-soluble protein hydrolyzed by pepsin	(Du et al., 2013)
VECYGPNRPQF	29.6	Algae protein waste hydrolyzed by pepsin	(Sheih, Fang and Wu, 2009)
CRQNTLGHNTQTSIAO	80	Sea cucumber (*Stichopus horrens*) hydrolyzed by Alcalase	(Forghani et al., 2016)
GDLGKTTTVSNWSPPKYKDTP	11.28	Tuna frame protein hydrolyzed by pepsin	(Lee, Qian and Kim, 2010)

[a] One-letter amino acid codes were used.

[b] Peptide concentration needed to inhibit 50% ACE activity.

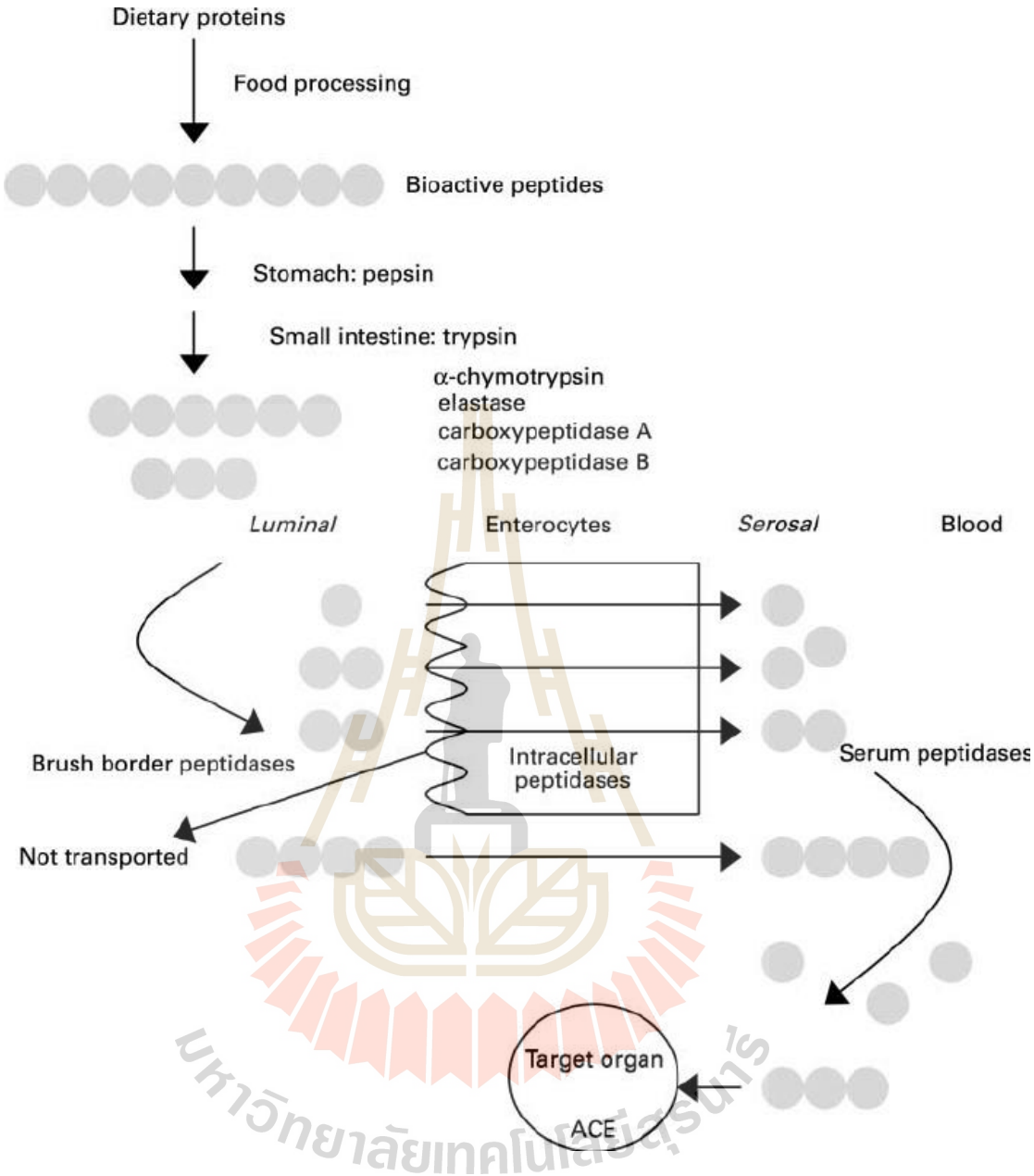


Figure 2.11 The potential activation and inactivation of angiotensin-I converting enzyme (ACE) inhibitory peptides in the human body during GI digestion and absorption, and in the blood (Vermeirssen, Van Camp and Verstraete, 2004).

These ACE inhibitory peptides are inactive within the protein sequence but may be released by hydrolysis, and, once released, they show biological activity

(Aluko, 2015, Miguel, Contreras, Recio and Aleixandre, 2009). They can be generated *in vivo* by the action of GI enzymes, and can also be obtained *in vitro* using specific enzymes, or can be produced during the manufacture of certain foods (Hatanaka, Miyahara, Suzuki and Sato, 2009, Himaya, Ngo, Ryu and Kim, 2012). Following proteolysis, the protein hydrolysate can be separated into various fractions on basis of peptide size, charge, or hydrophobicity (Banerjee and Shanthi, 2012, Chen, J. et al., 2012, Pérez-Vega, Olivera-Castillo, Gómez-Ruiz and Hernández-Ledesma, 2013, Segura Campos, Chel Guerrero and Betancur Ancona, 2010). Spectrophotometric, fluorimetric, chromatographic and capillary electrophoresis techniques have been used to isolate the active peptides and to measure their ability to inhibit ACE i*n vitro* (Chen et al., 2013b, Hatanaka, Miyahara, Suzuki and Sato, 2009, Lahogue et al., 2010, Li, Liu, Shi and Le, 2005, Wu, Aluko and Muir, 2002). Protein hydrolysate, peptide fractions, and isolated homogeneous peptides can be analyzed to determine amino acid composition or amino acid sequence (Arihara et al., 2001, Li et al., 2014, Nakahara et al., 2009), which provides information on the structural composition of the products.

In recent years, the research on mushrooms and their production have received increased attention because of the recognition that mushrooms are nutritious food with health-stimulating properties and medicinal effects (Kalač, 2013, Lavanya and Subhashini, 2013). Some edible mushrooms have been reported to significantly reduce blood pressure after oral administration. Examples are *Grifola frondosa*, *Lyophyllum decastes* Sing., *Pleurotus cornucopiae* and *P. nebrodensis* (Hagiwara et al., 2005, Kokean, Nishii, Sakakura and Furuichi, 2005, Miyazawa, Okazaki and Ohga, 2008, Talpur et al., 2002). The protein content in mushrooms is ranked below most animal

meats but above most other foods, such as milk, vegetables and fruits (Miles and Chang, 2004). Thus, this makes them a good starting material for the identification of peptides with biological activities including ACE inhibitory activity. ACE inhibitory peptides and proteins have also been successfully purified from mushrooms (Table 2.4), such as *Grifola frondosa* (Choi et al., 2001*) Hypsizygus marmoreus (*Kang et al., 2013*) Lactarius camphorates* (Yan and Gao, 2012*) Pholiota* spp. (Koo et al., 2006, Lee et al., 2003) *Pleurotus* spp. *(*Ibadallah, Abdullah and Shuib, 2015, Jang et al., 2011, Lau, Abdullah and Shuib, 2013*) Tricholoma* spp. *(*Geng et al., 2016, Lee et al., 2004*)*. However, there is a paucity of study on ACE inhibitory peptides from mushrooms by hydrolysis.

2.3.1 Production of ACE inhibitory peptides

ACE inhibitory peptides can be produced from food proteins in the following ways: (a) enzymatic hydrolysis by digestive enzymes during GI digestion (Balti et al., 2010, Escudero, Sentandreu, Arihara and Toldrá, 2010), (b) proteolysis by food-grade enzymes derived from different origins (animal, microorganisms or plants) (Hatanaka, Miyahara, Suzuki and Sato, 2009, Intarasirisawat, Benjakul, Wu and Visessanguan, 2013), (c) fermentation or maturation during food processing (Ashar and Chand, 2004, Rojas-Ronquillo et al., 2012). In many studies, combinations of (a), (b) and (c), have proven effective in the generation of ACE inhibitory peptides (Gibbs, Zougman, Masse and Mulligan, 2004). If the peptidic sequence is known, it is also possible to synthesize the peptide by chemical or enzymatic synthesis or by recombinant DNA technology (Gill, López-Fandiño, Jorba and Vulfson, 1996, Gobbetti, Minervini and Rizzello, 2004).

Table 2.4 ACE inhibitory peptides from mushrooms of different genera.

Mushroom species	Preparation method	Peptides	Inhibitory pattern on ACE	IC_{50}[a]	Reference
Agaricus bisporus	Water extract	PSSNK	Non-competitive	129 μM	(Lau, Abdullah, Shuib and Aminudin, 2014)
		RIGLF	Competitive	116 μM	
		AHEPVK	Competitive	63 μM	
Grifola frondosa	Water extract	VIEKYP	Competitive	0.097 mg	(Choi et al., 2001)
Hypsizygus marmoreus	Water extract	LSMGSASLSP	Non-competitive	0.19 mg/ml	(Kang et al., 2013)
Lactarius camphorates	Water extract	N. D	N. D	1.646 ± 0.061 mg/mL	(Yan and Gao, 2012)
Leucopaxillus tricolor	Water extract	DGPTMHRQAVADFKQ	Competitive	1.64 mg/ml	(Geng et al., 2015)
Pholiota adiposa	Water extract	GEGGP	N. D.	0.044 mg	(Koo et al., 2006)

Table 2.4 ACE inhibitory peptides from mushrooms of different genera (Continued).

Mushroom species	Preparation method	Peptides	Inhibitory pattern on ACE	IC_{50}[a]	Reference
Pholiota spp.	Water extract	N. D	N. D	0.2 mg	(Lee et al., 2003)
Pleurotus cornucopiae	Water extract	RLPSEFDLSAFLRA	Competitive	0.46 mg/ml	(Jang et al., 2011)
		RLSGQTIEVTSEYLFRH	Non-competitive	1.14 mg/ml	
Pleurotus cystidiosus	Water extract	AHEPVK	Competitive	62.8 μM	(Lau, Abdullah and Shuib, 2013)
		GPSMR	N. D.	277.5 μM	
Pleurotus pulmonarius	Water extract	N.D.	N. D.	12 μg/ml	(Ibadallah, Abdullah and Shuib, 2015)
Tricholoma giganteum	Water extract	GEP	Competitive	0.04 mg	(Lee et al., 2004)
Tricholoma matsutake	Water extract	WALKGYK	Non-competitive	0.04 μM	(Geng et al., 2016)

N. D.: not detected.

[a] Peptide concentration needed to inhibit 50% ACE activity.

2.3.1.1 Gastrointestinal digestion

It has been recognized that dietary proteins and peptides are susceptible to hydrolysis during the different stages of GI digestion, namely ingestion, digestion and absorption (Vermeirssen, Van Camp and Verstraete, 2004). Once ingested, these proteins and peptides are subjected to hydrolysis by different enzymes present in the GI tract such as pepsin, trypsin, chymotrypsin and peptidases at the surface of epithelial cells to release peptides of various lengths. Some of these peptides may exert a direct function at the GI tract. However, other peptides can be absorbed to reach target organs and tissues through systemic circulation (Shimizu, 2004).

In order to examine the effect of GI proteases on the release and breakdown of ACE inhibitory peptides from food proteins, simulated GI digestion processes have been carried out on various protein sources, such as milk proteins (Hernández-Ledesma, Amigo, Ramos and Recio, 2004a, 2004b, Hernandez-Ledesma, Quiros, Amigo and Recio, 2007, Lignitto et al., 2010, Ruiz, Ramos and Recio, 2004), egg proteins (Miguel, Aleixandre, Ramos and López-Fandiño, 2006), meat proteins (Escudero, Sentandreu, Arihara and Toldrá, 2010, Jang, Jo and Lee, 2007), fish proteins (Cinq-Mars, Hu, Kitts and Li-Chan, 2007, Samaranayaka, Kitts and Li-Chan, 2010), as well as vegetable proteins (Akıllıoğlu and Karakaya, 2009, Jiménez-Escrig, Alaiz, Vioque and Rupérez, 2010, Tovar-Pérez, Guerrero-Legarreta, Farrés-González and Soriano-Santos, 2009, Vermeirssen et al., 2003). As an example, Hernandez-Ledesma, Quiros, Amigo and Recio (2007) identified peptides with ACE inhibitory and antioxidant activity in hydrolyzates of several samples of human milk

and infant formulas after digestion with pepsin and pancreatin simulating infant GI conditions.

Food processing, such as thermal treatment (Bertrand-Harb et al., 2002) and high hydrostatic pressures (Bonomi et al., 2003), may enhance protein digestibility and peptide release. For example, hydrolysis of ovalbumin by pepsin under high pressure (200-400 MPa) promoted the proteolysis and the quick production of the ACE inhibitory peptides YAEERYPIL, FRADHPFL and RADHPFL (Quirós, Chichón, Recio and López-Fandiño, 2007). The method employed for cooking can also affect digestibility. In this way, Majumder and Wu (2009) have studied the effect of simulated GI digestion of cooked eggs on the release of ACE inhibitory peptides. These authors found that fried egg digests showed more potent ACE inhibitory activity than boiled egg digests, and postulated that the lower protein denaturation in boiled eggs may results in a lower protein digestibility.

2.3.1.2 Enzymatic hydrolysis

So far, ACE inhibitory peptides are most commonly produced by enzymatic hydrolysis. During the process of enzymatic hydrolysis, active enzymes cleaves proteins to produce peptides of different sizes. As different proteolytic enzymes cleave proteins based on their specificity, peptides with different sizes, sequences, and functional properties are produced. Food proteins have been shown to encrypt active peptides in their sequences. These sequences may not exert activity while they remain in the parent proteins, but once correctly released by enzymatic hydrolysis they are likely to show various physiological activities. A large number of studies have demonstrated the release of ACE inhibitory peptides from food proteins, by hydrolysis with commercially available microbial-derived food-grade proteinases

(Ahn, Jeon, Kim and Je, 2012, Hatanaka, Miyahara, Suzuki and Sato, 2009). It is advantageous as these enzymes are low-cost and safe, and the product yields are very high (Mao et al., 2007). Ueno, Mizuno and Yamamoto (2004) purified and characterized an endopeptidase from *L. helveticus* CM4 and demonstrated that this peptidase can generate ACE inhibitory peptides using synthetic pro-peptides as substrates. Mizuno et al. (2004) measured the ACE inhibitory activity of casein hydrolyzates upon treatment with nine different commercially available proteolytic enzymes. Among these enzymes, a protease extracted from *Aspergillus oryzae* acted specifically on casein to release VPP and IPP, and the obtained casein hydrolysate demonstrated a significant dose-dependent antihypertensive effect in SHR.

Recently, the interest of food technologists has turned to the use of different techniques, such as high pressure and heat-denaturing and power ultrasound to modify protein structure and increase enzymatic hydrolysis (Garcia-Mora et al., 2016). As compared to the proteolysis at atmospheric pressure, qualitative and quantitative differences were detected in the hydrolysis pattern when proteolysis with trypsin was carried out under high pressure treatments (Chicón, Belloque, Recio and López-Fandiño, 2006, Chicón, López-Fandiño, Quirós and Belloque, 2006). Hernández-Ledesma, Ramos, Recio and Amigo (2006) reported that heating of β-Lg during enzyme treatments with thermolysin enhances the formation of peptides with ACE inhibitory activity, and one of the peptides released under these heat-denaturing conditions was LQKW that had previously been described as a potent ACE inhibitor (Hernández-Ledesma, Recio, Ramos and Amigo, 2002). Prolonged exposure to high-intensity ultrasound has been shown to inhibit the catalytic activity of a number of food enzymes (Kadkhodaee and Povey, 2008). However, in some cases, enzymes

have been found to increase activity following short exposures to ultrasound (Lee, Nguyen, Koo and Ha, 2008). Jia et al. (2010) found that the use of ultrasonic treatment during proteolysis could facilitate the enzymatic hydrolysis of peptide DWGP, whereas ultrasonic pre-treatment could promote the release of ACE-inhibitory peptides from this region.

2.3.1.3 Fermentation and maturation process

During fermentation process, intracellular peptidases of lactic acid bacteria (LAB) hydrolyze milk proteins, mainly caseins, into peptides and amino acids which are used as nitrogen sources necessary for their growth (Juillard, Guillot, Le Bars and Gripon, 1998). Hence, bioactive peptides can be generated by starter and non-starter bacteria used in the manufacture of fermented dairy products (Ashar and Chand, 2004, Fitzgerald and Murray, 2006, Korhonen and Pihlanto, 2006, Nakamura et al., 1995, Nakamura, Yamamoto, Sakai and Takano, 1995). Proteolytic system of *Lactobacillus helveticus, Lactobacillus delbrueckii* ssp. *bulgaricus, Lactococcus lactis* ssp. *diacetylactis, Lactococcus lactis* ssp. *cremoris,* and *Streptococcus salivarius* ssp. *thermophylus* strains have demonstrated to hydrolyze milk proteins and release ACE inhibitory peptides (Fitzgerald and Murray, 2006, Korhonen and Pihlanto, 2006, Lopez-Fandino, Otte and Van Camp, 2006). Some of the peptides identified (and/or the fermented milk containing them) also have shown to lower blood pressure in hypertensive rats and humans (Fuglsang, Nilsson and Nyborg, 2002, Fuglsang, Rattray, Nilsson and Nyborg, 2003, Seppo, Jauhiainen, Poussa and Korpela, 2003, Sipola et al., 2002, Tuomilehto et al., 2004, Yamamoto, Maeno and Takano, 1999). Moreover, ACE inhibitory activity in milk has been found to increase during fermentation with Calpis sour milk starter containing *Lactobacillus helveticus* and

Saccharomyces cerevisiae (Nakamura et al., 1995). Two ACE inhibitory peptides, Val-Pro-Pro and Ile-Pro-Pro, have in fact been isolated and identified from Calpis sour milk. At present they are commercialized in Japan (Ameal S®/Calpis®, Calpis Co. Ltd., Tokyo, Japan) and Finland (Valio Evolus® Double Effect, Valio Ltd., Finland). These fermented milks have shown beneficial effects on blood pressure in several rat models and human studies without any adverse effects (Aihara et al., 2005, Hata et al., 1996, Hirota et al., 2007, Jauhiainen et al., 2005, Masuda, Nakamura and Takano, 1996, Mizushima et al., 2004, Seppo, Jauhiainen, Poussa and Korpela, 2003, Sipola et al., 2002, Tuomilehto et al., 2004).

Ashar and Chand (2004) identified an ACE inhibitory peptide from milk fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus*, and Pihlanto, Virtanen and Korhonen (2010) reported two peptides responsible for the ACE inhibitory activity of milk fermented with *Lactobacillus jensenii*. In combination with *S. salivarius* ssp. *thermophylus* and *L. lactis* biovar. *diacetylactis*, a hypotensive structure with a sequence of SKVYP was obtained from β-casein. Quirós and co-workers (2007) identified two peptides in fermented milk with *Enterococcus faecalis* that corresponded to β-CN fragments LHLPLP and LVYPFPGPIPNSLPQNIPP, with potent ACE inhibitory activity and proven antihypertensive effect when orally administered to spontaneously hypertensive rats (SHR) after acute and long-term administration (Miguel et al., 2005, Miguel et al., 2006, Quirós et al., 2007).

During the maturation of cheese, the major milk proteins are degraded into a large number of peptides due to the action of endogenous milk enzymes, added coagulants and microbial enzymes. A number of studies have shown that ACE inhibitory peptides can be produced during cheese making, in particular during the

ripening process. These peptides have been characterized in different commercial cheeses, such as Edam, Gouda, Camembert, Havarti and Blue cheeses, and Italian, Spanish and Finnish cheeses (Gómez-Ruiz et al., 2006, Lignitto et al., 2010, Ruiz, Ramos and Recio, 2004, Saito et al., 2000, Smacchi and Gobbetti, 1998). Some of these ACE inhibitory peptides have shown *in vivo* activities (Miguel, Gómez - Ruiz, Recio and Aleixandre, 2010, Saito et al., 2000). ACE inhibitory peptides VPP and IPP have also been identified and quantified in different cheese varieties by Butikofer and co-workers who found high amounts of VPP and IPP in several commercially available hard and semi-hard cheeses (Bütikofer et al., 2008, Bütikofer, Meyer, Sieber and Wechsler, 2007). However, significant differences exists between samples of the same cheese variety, as well as between different varieties. Thus, it is necessary to develop a reproducible cheese-making process with selected cultures to produce higher concentrations of these peptides that could be used for clinical trials (Meyer et al., 2009).

Fermented soy products, traditionally consumed in Eastern countries, have been also found to be an important source of ACE inhibitory peptides. A potent antihypertensive peptide has been identified and characterized in a Korean soy product denominated “chunggugjang” and obtained by soy fermentation with *Bacillus subtilis* CH-1023 (Korhonen and Pihlanto, 2003). Other ACE inhibitory peptides have been identified in soy paste (Shin et al., 2001), soy sauce (Nakahara et al., 2009, Okamoto et al., 1995), natto and tempeh (Gibbs, Zougman, Masse and Mulligan, 2004), and other fermented soy products (Ibe et al., 2009, Li et al., 2009, Rho et al., 2009).

2.3.1.4 Genetic recombination in bacteria

During the last years, several techniques based on genetic engineering were being developed. One of the challenges of these approaches is the susceptibility of short ACE inhibitory peptides to degradation by proteases or peptidases. Moreover, the expression products may be harmful to the host, impacting the high-level expression of the gene. This shortcoming has been conquered by expression of ACE inhibitory peptides in the forms of a fusion protein or a tandem gene. ACE inhibitory peptides with sequences HHL, HVLPVP, FFVAPFPEVFGK, and GHIATFQER have been expressed successfully in *Escherichia coli* (Jeong et al., 2007, Liu et al., 2007, Lv, Huo and Fu, 2003, Park et al., 1998), although special proteases are needed to release the target active protein, thus increasing the cost of separation and purification after enzymatic hydrolysis. Recently, Rao et al. (2009) expressed an ACE inhibitory peptide multimer, a common precursor of 11 kinds of ACE inhibitory peptides, and the release was confirmed by simulated GI digestion. Although promising results are being obtained, to date, the use of genetic modified microorganisms in food products is controversial.

2.3.2 Fractionation and purification of ACE inhibitory peptides

Generally, after the treatments mentioned in Section 2.3.1 (GI digestion, enzymatic hydrolysis, fermentation and maturation process, genetic recombination in bacteria), a mixture that contains peptides with different chain lengths, amino acid compositions, and ACE inhibitory efficacies was produced. The peptide mixture has been fractionated and enriched by means of various methods based on chain length, hydrophobicity, or net charge. Depending on the selection criteria, fractionation and purification always increase the ACE inhibitory potency of peptides compared to the

original mixture (Girgih et al., 2011, Li et al., 2014, Zhang et al., 2009). Further amino acid sequence determination can offer a better understanding of structure-activity relationship of ACE inhibitory peptides.

2.3.2.1 Membrane ultrafiltration

Membrane ultrafiltration is a variety of membrane filtration in which hydrostatic pressure forces liquid against a semi-permeable membrane. The suspended solids and solutes of high MW are retained, while water and low MW solutes pass through the membrane. This separation process is used in industry and research for purifying and concentrating macromolecular (10^3-10^6 Da) solutions, especially protein solutions. Membrane ultrafiltration is not fundamentally different from reverse osmosis, microfiltration, or nanofiltration, except in terms of the size of the molecules it retains (Zhang et al., 2009). The importance of peptide size is related to absorption potential into the blood from the gastrointestinal tract (GIT) as well as ability to interact with target enzymes involved in blood pressure regulation. *In vitro* studies have shown that smaller-size peptides may be more active than the bigger peptides (Ko et al., 2012, Pérez-Vega, Olivera-Castillo, Gómez-Ruiz and Hernández-Ledesma, 2013, Segura Campos, Chel Guerrero and Betancur Ancona, 2010, Zhang et al., 2009). Membrane ultrafiltration offers the possibility of fractionating peptides into a wide range of peptide sizes using the most common membranes with the standard MWCO sizes 1, 3, 5, and 10 kDa. However, other membrane types with 2- or 6-kDa MWCO sizes have also been used for peptide separation and isolation (Lin, Lv and Li, 2012, Liu, Zhang, Zhang and Liu, 2012). Membrane fractionation can be performed to obtain a single or two products by using a single membrane with a desired MWCO size (del Castillo et al., 2007, Fujita,

Yamagami and Ohshima, 2001, García-Tejedor et al., 2014, Hayes et al., 2007, Li et al., 2011, Liu, Zhang, Zhang and Liu, 2012, Memarpoor-Yazdi, Asoodeh and Chamani, 2012) or different fractions with various membranes (Girgih et al., 2011, Ruiz-Ruiz, Dávila-Ortíz, Chel-Guerrero and Betancur-Ancona, 2013, Wang, Tian and Wang, 2011). To obtain fractions that have distinct differences in peptide size, the hydrolysate is first passed through a membrane with the smallest MWCO size, such as the 1 kDa membrane; the flow-through solution (permeate) is then collected as the < 1 kDa peptide fraction. The retained solution (retentate) can be passed through the 3 kDa membrane, and permeate will contain peptides with sizes bigger than 1 kDa but less than 3 kDa (1-3 kDa fraction). The retentate from the 3 kDa membrane can be passed through a 5 kDa membrane to obtain a 3-5 kDa permeate fraction. Finally, the 5 kDa membrane retentate is then passed through the 10 kDa membrane to obtain a permeate with 5-10 kDa peptides. The fractionation process can be reversed by starting with the highest membrane MWCO size and finishing with the lowest (Jung, W.-K. et al., 2006, Zhang et al., 2009). For example, the protein hydrolysate can be first passed through a 10 kDa membrane and the permeate collected and passed through a 5 kDa (or 6 kDa) membrane; the retentate will be collected as the 5-10 kDa (or 6-10 kDa) fraction (Lin, Lv and Li, 2012, Puchalska, García and Marina, 2014). The permeate from the 5 kDa (or 6 kDa) membrane is then passed through a 3 kDa (or 2 kDa) membrane whose permeate is passed through a 1 kDa membrane to obtain retentates that are 3-5 and 1-3 kDa peptide fractions. The final permeate obtained from the 1 kDa membrane will be taken as the <1 kDa peptide fraction. During ultrafiltration, it is common to add distilled water at intervals to reduce solution viscosity and improve the rate of peptide permeation of the membrane; this process is

called diafiltration and can be used to enhance the yield and speed of the membrane ultrafiltration process. However, the membranes do not have 100% efficiency, and it is possible that a small amount of peptides with sizes higher than the MWCO size could pass through into the permeate. To reduce fouling caused by peptides that stick to the membrane during ultrafiltration, it is better to use membranes that have been designed specifically for protein separation. This is because membrane fouling can reduce efficiency of the filtration process and lead to low peptide yields in addition to extended filtration periods. A membrane ultrafiltration reactor has been applied for the continuous extraction of permeates enriched with bioactive fragments, in order to produce antithrombotic peptide (Bouhallab and Touzé, 1995). Thus, the membrane ultrafiltration provides new possibilities for enriching peptides with a low molecular mass and that it is easily up-scaled to gram or even kilogram quantities.

2.3.2.2 Fast protein liquid chromatography

The medium-pressure fast protein liquid chromatography (FPLC) is usually used for peptide purification. Two main column chromatography methods (gel filtration or ion exchange columns) are most used (Banerjee and Shanthi, 2012, Intarasirisawat, Benjakul, Wu and Visessanguan, 2013, Lee, Qian and Kim, 2010, Sheih, Fang and Wu, 2009, Tomatsu et al., 2013, Wang, Tian and Wang, 2011, You and Wu, 2011). After the separation through FPLC column, fractions within each peak are pooled, freeze-dried, and assayed for potential ACE inhibitory activity. The peak that shows the highest *in vitro* ACE inhibition activity is then subjected to further separation during which fractions are also collected and assayed for activity. Pure peptides have also been isolated through initial ion-exchange chromatography followed by fractionation and refractionation of the most active peaks by gel filtration

chromatography (Banerjee and Shanthi, 2012, Majumder and Wu, 2009). Gel filtration chromatography can also be used to separate the peptide mixture into peptide fractions of known molecular sizes followed by assay of each fraction for potential *in vitro* or *in vivo* antihypertensive properties (Escudero et al., 2012, Ruiz-Ruiz, Dávila-Ortíz, Chel-Guerrero and Betancur-Ancona, 2013). Similar to ultrafiltration fractionation, the results from the gel filtration chromatography separation can be used to estimate the relationship between peptide size and *in vitro* or *in vivo* antihypertensive property.

2.3.3 *In vitro* ACE inhibitory activity assays

Several methods have been devised for estimating potential ACE inhibitory activity of peptides include spectrophotometric, fluorometry, radiochemical, capillary electrophoresis and HPLC methods. For the above mentioned methods, spectrophotometric and HPLC are commonly used. The spectrophotometric method was initially developed by Cushman and Cheung (1971). In this method, hippuryl-histidyl-leucine (HHL) was used as a substrate. The cleavage of the hippuryl-histidine bond releases free hippuric acid, which can then be extracted into ethyl acetate. The solvent is evaporated and the hippuric acid residue dissolved in distilled water followed by spectrophotometric measurement at 228 nm. Alternatively, the reaction mixture can be separated on a reverse-phase HPLC column to quantify the hippuric acid peak (Wang, Tian and Wang, 2011, Wu, Aluko and Muir, 2002). In the presence of peptide inhibitors, ACE-mediated production of hippuric acid is reduced, and the percentage ratio to the value in the absence of peptide can be calculated as the percent inhibition. A major drawback of the spectrophotometric assay is that if excessive heat is used for the ethyl acetate evaporation, some of the

hippuric acid may not be dissolved in water, which causes underestimation of enzyme activity and overestimation of peptide inhibitory capacity. The ethyl acetate may be evaporated with nitrogen gas but if solvent residues remain, there is overestimation of enzyme activity and underestimation of peptide inhibitory activity because the ethyl acetate also absorbs UV radiation at 228 nm, where hippuric acid is normally measured. Moreover, the ethyl acetate extract can sometimes be contaminated with the substrate (HHL), which also absorbs at 228 nm and can lead to overestimation of ACE activity and underestimation of peptide inhibitory activity (Chen et al., 2013b, Wu, Aluko and Muir, 2002). A recent report has confirmed the higher sensitivity and precision of the HPLC method when compared to the spectrophotometric method (Chen et al., 2013b). In contrast, the spectrophotometric method can simultaneously measure several samples with simple operations, suitable for analysis of ACE inhibition activity of food protein enzymatic hydrolysates.

The assay developed by (Chen et al., 2013b) was later modified by other researchers using spectrophotometry in the visible light wavelength range. For this modified method, the ethyl acetate extraction step was replaced by specific binding reaction between HL and 2, 4, 6-trinitrobenzene sulphonate or a colorimetric reaction between HA with benzene sulfonyl chloride in the presence of quinoline (Li, Liu, Shi and Le, 2005, Serra et al., 2005).

Another commonly used ACE assay involves N-3-(2-furyl acryloyl)-phenylalanylglycylglycine (FAPGG) as the substrate (Holmquist, Bünning and Riordan, 1979). ACE hydrolyzes FAPGG into furylacryloyl-L-phenylalanine (FAP) and glycylglycine (GG); the reaction can then be followed by continuous UV absorption measurement to determine the decrease in absorbance at 345 nm as a result

of the Phe-Gly peptide bond cleavage (Hatanaka, Miyahara, Suzuki and Sato, 2009, Udenigwe, Lin, Hou and Aluko, 2009). The rate of absorption decrease in the presence of peptide inhibitors will be less and can be subtracted from the reaction rate in the absence of the inhibitor to obtain percentage inhibition. Alternatively, the released FAP can be quantified by injecting the reaction mixture directly onto a reverse-phase HPLC column with detection at 305 nm (Lahogue et al., 2010). In general, the FAPGG method processed the simplicity, high sensitivity and cost effectiveness because of the lack of the procedure for separating HA from the reaction mixture (Shalaby, Zakora and Otte, 2006). Therefore, it is better suited to routine analysis of several samples when compared to the HHL assay.

2.3.4 Structure-activity relationship for ACE inhibitory peptides

Many ACE inhibitory peptides were identified from different food sources (Table 2.3). The amino acid sequencing studies indicated that the majority of them are relatively small molecules containing of 2 to 12 amino acid residues, the length of the sequence seems to be determinant in the final activity (Ruiz, Ramos and Recio, 2004, Saiga et al., 2006). This can be explained by the study of Natesh, Schwager, Sturrock and Acharya (2003), that the active site of ACE (Figure 2.12) cannot accommodate large peptide molecules.

Although the structure-activity relationship of food derived ACE-inhibitory peptides has not yet been fully established, correlation among different peptide inhibitors of ACE indicate that C-terminal tripeptide plays a predominant role in binding to the active site of ACE (Hernández-Ledesma, del Mar Contreras and Recio, 2011, Li, Le, Shi and Shrestha, 2004). The C-terminal tripeptide can interact with the subsites S1, S1′, and S2′ at the active site of ACE. Many studies have shown that the

most effective ACE inhibitory peptides identified have hydrophobic, aromatic or branched chain amino acids at their C-terminal and branched chain amino acids at their N-terminal (Murray and FitzGerald, 2007).

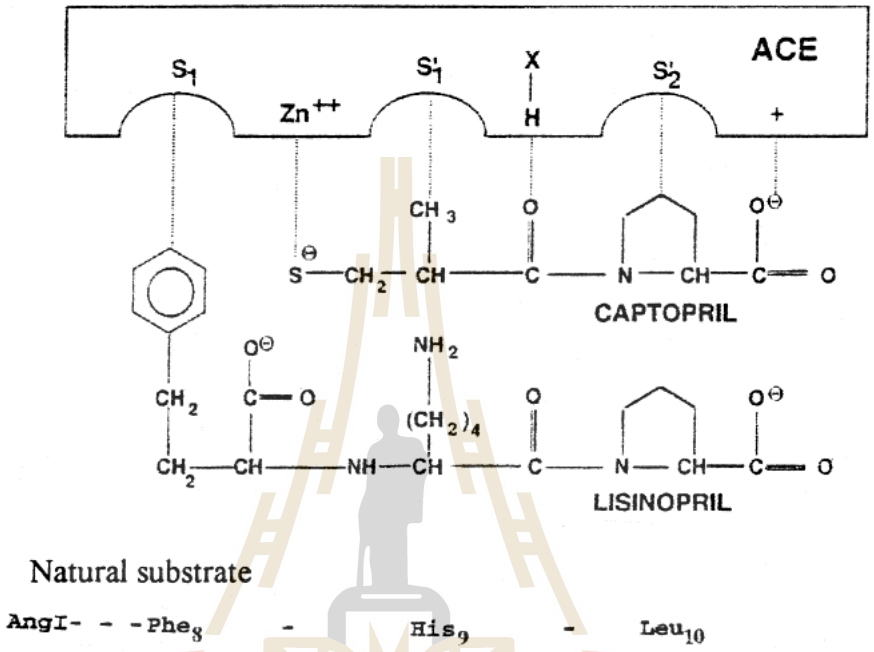


Figure 2.12 Proposed binding model for interaction between substrates or competitive inhibitors and the active site of angiotensin-I converting enzyme (ACE). Circular clefts (labeled S_1, S_1', and S_2') represent subsites that can potentially interact with the corresponding amino acid residues of peptide substrates or inhibitors. The zinc ion of ACE is appropriately located between subsites S_1 and S_1' to participate in hydrolytic cleavage of the peptide bond between antepenultimate and penultimate amino acid residues of the substrate, resulting in release of the dipeptide product. A hydrogen bond donating group (X-H), which could bind the terminal, nonscissile peptide bond of a peptide substrate, is located between subsites S_1' and S_2', a positively charged group on the active site of ACE

forms ionic bond with the C-terminal negatively charged amino acid carboxyl group of the substrate. Studies with substrates and with various classes of competitive inhibitors have indicated that subsites S_1, S_1' and S_2' are all more or less hydrophobic pockets (Li, Le, Shi and Shrestha, 2004).

The presence of proline at the antepenultimate residue, appears to enhance binding to ACE (Vermeirssen, Van Camp and Verstraete, 2004), although aromatic amino acids in this position also seem to positively affect the inhibitory capacity of peptides. In order to compare aromatic amino acids with proline at the antepenultimate position, the peptides LHLYLP and LHLWLP were synthesized where Pro is substituted Tyr and Trp, respectively. These sequences showed IC_{50} values of 18.9 and 9.0 μM, respectively, which is five-times and twice lower ACE-inhibitory capacity than LHLPLP (Quirós et al., 2009). Among the aromatic residues, the larger amino acid tryptophan at the C-terminal showed higher ACE inhibitory activity. Replacing tyrosine with a bulkier tryptophan, the results showed that IKW was two times more potent than IKY (Jimsheena and Gowda, 2010). Kobayashi et al. (2008) also reported that IKW was four times more potent than IKY against rabbit lung ACE. Furthermore, branched chain aliphatic amino acids such as isoleucine, leucine, and valine at C-terminal may contribute significantly to increase ACE-inhibitory potential (Ruiz, Ramos and Recio, 2004). In 2001, Kim, Byun, Park and Fereidoon reported that the presence of leucine as the C-terminal amino acid (GPL) instead of valine (GPV) increased the inhibitory activity of the peptide two folds. Also, in the study of Ruiz, Ramos and Recio (2004), peptide KKYNVPQL exhibited a 10 fold greater inhibitory

activity than KKYNVPQ (IC_{50} values 77.1 and 716.9 μM, respectively) which might be also caused by the presence of leucine as the C-terminal residue.

In addition, the presence of the positive charge of lysine (ε-amino group) and arginine (guanidino group) at the C-terminal residue may contribute substantially to the inhibitory potency (FitzGerald and Meisel, 2000). Given these considerations, when the middle residue glutamic acid (negatively charged) was substituted by lysine (positively charged), the peptide IKY showed six times more potent than the peptide IEY (Jimsheena and Gowda, 2010). The activity of the peptide LHLPLR with an IC_{50} value of 1.8 μM was twice more potent than the activity of LHLPLP (Quirós et al., 2009).

The catalytic sites of ACE have different conformational requirements, thus, in order to inhibit ACE activity more completely, there is a need for developing a complex mixture of peptides with slightly different conformational features (Gobbetti, Minervini and Rizzello, 2004). Peptides can adopt different configurations depending on the environmental conditions, which determine their bioactivity. e.g., bradykinin, as an extended or random coil structure, is open and sensitive for cleavage by ACE. A β-turn at the C-terminal end of bradykinin in water gives only a weak interaction with ACE (Desai, Coutinho and Srivastava, 2002). ACE also shows high stereo-specificity for an amino acid residue at position three from the C-terminal. It has been demonstrated that ACE has an absolute requirement for the L-configuration of the residue in this position, but it shows little stereo-specificity at position four (Li, Le, Shi and Shrestha, 2004), which is also supported by the report of Maruyama et al. (1987) that D-VAP shows little inhibitory activity with IC_{50} value of 550 μmol/L but peptides VAP and D-FVAP with IC_{50} value of 2.0 μmol/L and 17 μmol/L, respectively.

Furthermore, the change of a *trans*- to a *cis*- form of proline at the C-terminal position of an ACE inhibitory peptide can cause significant changes in its interaction with the enzyme. Gómez-Ruiz, Recio and Belloque (2004) studied two different preparations for DKIHP (β-casein f47-51), an ACE-inhibitory peptide obtained from Manchego cheese. One preparation, with a unique conformer containing *trans*-proline, gave a significant ACE-inhibitory activity (IC_{50}=113.18 μM). The second one contained three different conformers, two with *trans*-proline and one with *cis*-proline, and showed a lower ACE-inhibitory activity (IC_{50}=577.92 μM) compared which the unique conformer. The study by Viitanen, Vapaatalo and Valjakka (2014) further supported that the configuration of proline-containing peptides plays a significant role in enzyme inhibition.

In recent years, quantitative structure-activity relationship (QSAR) of ACE inhibitory peptides was conducted to establish statistical computer models to further elucidate the structural elements in these peptides that are critical for their interaction with the active sites of ACE, as well as to facilitate identification or design of more new potent ACE inhibitory peptides (He et al., 2011, Pripp, Isaksson, Stepaniak and Sørhaug, 2004, Wu, Aluko and Nakai, 2006). Pripp, Isaksson, Stepaniak and Sørhaug (2004) thoroughly examined ACE inhibitory peptides derived from milk proteins by QSAR methodology using physicochemical descriptors. For peptides up to six amino acids, increased side chain hydrophobicity at the C-terminal and decreased side chain size of the penultimate amino acid enhanced ACE inhibitory potency. No relationship was found between the N-terminal structure and the ACE inhibitory activity. Further, Wu, Aluko and Nakai (2006) modeled food based ACE inhibitory peptides consisting of 168 dipeptides and 140 tripeptides from published literature and proposed ACE

inhibitor peptides with higher potency than reported earlier. For tripeptides, the most favorable residues for the C-terminal were aromatic or hydrophobic amino acids, while positively charged amino acids were preferred for the intermediate domain, and hydrophobic amino acids such as alanine, proline, tyrosine, and valine were preferred for the N-terminal. For long-chain peptides, the C-terminal tetrapeptide residues were more important to their ACE inhibitory activity than the C-terminal tripeptide residues. The most likely preferred amino acid residues starting from C-terminal are tyrosine and cysteine for the first position, histidine, tryptophan and methionine for the second position with isoleucine, leucine, valine and methionine for the third position, and tryptophan for the fourth position (Wu, Aluko and Nakai, 2006). A QSAR model was built with an artificial neural network (ANN) approach based on structural or activity data of 58 dipeptides (including peptide activity, hydrophilic amino acids content, three-dimensional shape, size, and electrical parameters). Then, the model was applied in ACE inhibitory peptides preparation from defatted wheat germ protein (DWGP). According to the QSAR model, the C-terminal of the peptide was found to have principal importance on ACE inhibitory activity, that is, if the C-terminal is hydrophobic amino acid, the peptide's ACE inhibitory activity will be high, and proteins which contain abundant hydrophobic amino acids are suitable to produce ACE inhibitory peptides. DWGP is a good protein material to produce ACE inhibitory peptides because it contains 42.84% of hydrophobic amino acids, and structural information analysis from the QSAR model showed that proteases of Alcalase and Neutrase were suitable candidates for ACE inhibitory peptides preparation from DWGP (He et al., 2011).

CHAPTER III

MATERIALS AND METHODS

3.1 Raw materials and sample preparation

Five species of edible mushrooms that are widely used for human consumption were selected to produce GlcN and ACE inhibitory peptides in this study. These include enoki mushroom (*Flammulina velutipes*) (fresh), oyster mushroom (*Pleurotus ostreatus*) (fresh), straw mushroom (*Volvariella volvacea*) (fresh), shiitake (*Lentinus edodes*) (dried), and wood ear mushroom (*Auricularia auricular*) (dried). The mushrooms were purchased from a local market, while the shrimp shell waste sample that serves as the control was collected from a restaurant in Nakhon Ratchasima province, Thailand.

Figure 3.1 Photos of five mushrooms. a: enoki mushroom; b: oyster mushroom; c: straw mushroom; d: shiitake; and e: wood ear mushroom.

The three fresh mushrooms, namely enoki mushroom, oyster mushroom, straw mushroom, were washed, cut into small pieces and dried in a Gamma 2-16 LSC freeze-drier (Martin Christ GmbH, Osterode am Harz, Germany). In the freeze-drying process, mushroom pieces were frozen at -85°C for 1 h in the freeze-drier chamber, dried at -65°C for 72 h, and then finally dried at 30°C for 24 h. The entire process was carried out at -85°C cold collector temperature and 0.001 mbar chamber pressure. The shrimp shell waste sample was dried using the same method described above. After freeze-drying, five dried mushrooms and shrimp shell waste were ground using an IKA-WERKE M20 universal laboratory miller with a cooling jacket and 4-edged blade and then sieved to obtain a fraction below 40-mesh. The ground mushroom and shrimp shell waste samples were well-sealed in the plastic bags and kept at 4°C throughout the study.

3.2 Chemicals

All chemicals of reagent grade were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), Carlo Erba Reagenti (Rodano, Milano, Italy) and Acros Organics (New Jersey, USA). All solvents were of analytical grade except HPLC grade solvent for HPLC analysis. D-(+)-Glucosamine hydrochloride (minimum 99% purity), N-(9H-Fluoren-2-ylmethoxycarbonyloxy) succinimide (FMOC-Su) (98% purity), triethylamine (TEA) and trifluoroacetic acid (TFA) were specifically used for the determination of GlcN. ACE from rabbit lung and FAPGG were specifically used for the ACE inhibitory analysis. Captopril tablets (specification: 25 mg per tablet) were bought from the local drugstore.

3.3 Proximate composition analysis

Five ground mushroom samples were analyzed for the proximate composition according to AOAC official method 934.01 (AOAC, 2000). These include moisture content, crude fat content, crude protein content and ash content. All chemical components were analyzed in triplicate and reported as mean on the percentage of dry matter (DM).

3.3.1 Moisture content

Moisture content of ground mushroom and shrimp shell waste samples was determined by thermo-gravimetric method using a convective hot-air oven (Gallenkamp Plus II, Gallenkamp Ltd., Leicester, UK). An amount of 1.5 g sample was accurately weighed in a clean, dried and pre-weighed can. The can and its contents were placed in an oven at 105°C for 24 h until a constant weight was obtained. Then the can and its contents were placed in desiccator for 30 min to cool and weighed again. The moisture content was calculated on a wet basis (w. b.) by the following formula:

$$\text{Moisture content}\ (\%, \text{w.b.}) = \frac{Sample\ weight\ (g) - Dried\ sample\ weight\ (g)}{Sample\ weight\ (g)} \times 100 \quad (3.1)$$

3.3.2 Crude fat content

Crude fat content of ground mushroom samples was determined by soxhlet extraction using petroleum ether through a Soxtec^{TM} 2050 auto fat extraction system. Approximately 1 g of sample was wrapped in filter paper, placed in fat free thimble and then introduced in the extraction tube. The receiving aluminum beaker was cleaned, dried, weighed and filled with petroleum ether and fitted into the apparatus. Next, aluminum beaker with the extract was transferred into an oven at

105°C for 2 h and cooled in a desiccator. The crude fat content was determined by using the following formula:

$$\text{Crude fat content (\%, DM)} = \frac{Weight\ of\ petroleum\ ether\ extract\ (g)}{Dry\ matter\ of\ sample\ (g)} \times 100 \quad (3.2)$$

3.3.3 Crude protein content

Total nitrogen in the ground mushroom samples was determined by Kjeldahl method. Dried samples (1.0 g) were placed in digestion flasks. Fifteen milliliters of concentrated sulfuric acid was added together with 5 g of digestion mixture of potassium sulphate and copper sulphate i.e. K_2SO : $CuSO_4$ (10:1). The flask was swirled to mix the contents thoroughly then heated to start digestion at 400°C until the mixture become clear (blue green in color). Distillation of the cooled digest was performed in a Foss Kjeltec™ 8100 distillation unit (FOSS Analytical AB, Höganäs, Sweden). Seventy milliliters of distilled water was introduced in the distillation tube and 50 mL of 40% NaOH was gradually added through the similar approach. Distillation was continued for at least 4.5 min and NH_3 produced was collected as NH_4OH in a conical flask containing 25 mL of 4% boric acid solution with a few drops of modified methyl red indicator. During distillation yellowish color appears due to NH_4OH. The distillate was then titrated against standard 0.1 M HCl solution till the appearance of pink color. A blank was also run through all steps as above.

$$\text{Crude protein content (\%, DM)} = \frac{(S-B)\times N\times 0.014}{Dry\ matter\ of\ sample\ (g)} \times F \times 100 \quad (3.3)$$

Where S = Sample titration reading (mL); B = Blank titration reading (mL); N = Normality of HCl (M); *0.014* = Milliequivalent weight of Nitrogen; F = 4.38

Usually, crude protein content was determined by the Kjeldahl method and a conversion factor of 6.25 was used for calculation. However, for mushrooms, such values were overestimated due to a high proportion of non-protein nitrogen,

particularly in chitin. A factor of 4.38 has been recommended and mostly used in recent publications.

3.3.4 Ash content

For the determination of ash, clean empty porcelain crucible was placed in a temperature controlled muffle furnace at 600°C for 4 h, cooled in desiccator and then weight of empty crucible was noted. Two grams of ground mushroom sample was weighed in the crucible and placed in the muffle furnace at 600°C for 6 h. The crucible was then cooled and weighed and the content of ash was calculated by the following formula:

$$\text{Ash content (\%, DM)} = \frac{Ash\ weight\ (g)}{Dry\ matter\ of\ sample} \times 100 \qquad (3.4)$$

3.4 Production of glucosamine

3.4.1 Chitin content

Chitin in ground mushroom and shrimp shell waste samples were extracted after alkali and acid treatments, and determined by the gravimetric method. Crude chitin was extracted after treatments with alkali and acid by a procedure adapted from Ifuku, Nomura, Morimoto and Saimoto (2011). Firstly, ground sample (25 g) was stirred with 250 mL distilled water for 1 hour at room temperature to remove the water soluble glucans and minerals. Then, the slurry was centrifuged (12000 × g, 20 min, 22°C). After centrifugation, the residue was treated with 2% (w/v) NaOH (250 mL) for 24 hours at 80°C to remove the proteins and alkali soluble glucans. After centrifugation and washing with distilled water, the residue was treated with 2 M HCl (500 mL) for 48 hours at room temperature to remove residual mineral salts and then washed with distilled water. The residue (extracted wet chitin) was dried

by vacuum oven at 70°C for 24 hours. The obtained dry chitin was weighted to calculate the chitin content of ground samples.

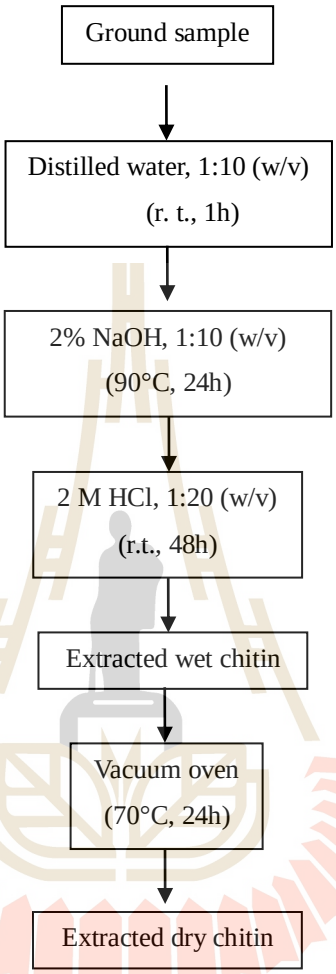


Figure 3.2 Extraction procedure of chitin from ground samples.

3.4.2 Determination of glucosamine

3.4.2.1 Derivatization and chromatographic conditions

The procedure for GlcN determination was based on the AOAC official method 2005.01 (2005) with minor modification. The method was based on precolumn derivatization with FMOC-Su. The GlcN free base was released by adding TEA to 1 ml GlcN standards and samples. The amount of TEA (0, 0.15, 0.3,

0.5, 0.75 and 1 μL) and the released time (0, 0.5, 1, 2, 4, 6, 8, 10, 12, 14 and 16 h) were investigated to improve the accuracy of GlcN determination. Then, 100 μL released solution was derivatized with 0.5 ml 15 mM FMOC-Su in the sonicator water bath at 50°C for 30 min. After cooled to room temperature, 4 mL of mixture of mobile phase A (water containing 0.05% TFA, pH 2.4)/B (acetonitrile) (1/1, v/v) was added. After that, the solution was filtered through 0.45 μm filter into a HPLC vial for injection. A 10 μL sample was injected onto the column, and separation was performed on a 4.6×250 mm, 5 μm, ZORBAZ ODS C18 analytical column (Agilent), employing a C18 precolumn guard cartridge. The temperature of column was maintained at 40°C. Samples were eluted with the following gradient mode (Table 3.1) of mobile phase A and mobile phase B at a flow rate of 0.8 mL/min. Detection was performed at a wavelength (λ) of 265 nm with an analytical time of 17 min.

Table 3.1 The gradient mode of mobile phase A and mobile phase B for GlcN determination by HPLC.

Time, min	Ratio: A : B
0.0-6.0	70:30 isocartuc
6.0-11.0	Change to 0:100
11.0-15.0	Change to 70:30
15.0-17.0	70:30 isocartuc

3.4.2.2 Construction of calibration curve

In order to prepare stock solution of GlcN chloride, 280 mg GlcN chloride was dissolved in 100 mL deionized water and stored at 4°C. GlcN

standards were prepared in concentrations ranging from 0.02 to 1.2 mg/mL from the stock solution. The calibration curve was constructed between HPLC peak area and the GlcN concentration using weighted least regression. The linear regression equation was calculated as $y=ax + b$, where y is the sum of HPLC peak area of two peaks under the curve, x is the concentration of GlcN in mg/mL. A coefficient of determination is required more than 0.99.

3.4.2.3 Precision and accuracy

To determine the inter-day precision, three replicates of six samples (0.02-1.00 mg/mL) were analyzed on the same day. The intra-day precision was assessed using the same samples on three different days. To determine the accuracy, six samples were analyzed by comparing the tested concentration with the theoretical concentration.

To validate the method, the standard addition technique was used. GlcN standard (0.05, 0.10, or 0.15 mg) was added to three samples, the GlcN concentration of original hydrolysates and obtained mixtures were measured by the HPLC method, and the recovery was calculated.

3.4.2.4 Stability after derivatization

To determine the stability of GlcN derivative, samples containing 0.08, 0.50, and 1.00 mg/mL GlcN were processed and derivatized as described in Section 3.4.1.1. The derivatized samples were stored at 4°C and analyzed on the 0^{th}, 7^{th}, 14^{th} and 30^{th} day. Peak areas were compared to determine the stability of GlcN derivative.

3.4.3 Characterization of glucosamine

Characterization of GlcN was performed on 20 cm×10 cm high-performance silica gel 60F254 GLP plates (Merck, Darmstadt, Germany) according to published work (Mojarrad et al., 2007). The plates were precleaned using dichloromethane-methanol (1:1) and dried in a fume hood before use. The solutions of standard and obtained samples were applied to the plates. 1-Butanol-glacial acetic acid-deionized water (3:1:1) was used as the mobile phase. The development time was about 2.5 h. After development, the mobile phase was evaporated from the plate by drying the plate in a fume hood for 10 min. The plate was then sprayed heavily and evenly with ninhydrin reagent (0.3 g of ninhydrin in 100 mL of 1-butanol plus 3 mL of glacial acetic acid) and dried in the fume hood for ca. 10 min. The plate was then heated on a plate heater at 115°C for several minutes to produce red zones of glucosamine on a white background.

3.4.4 Acid hydrolysis of ground samples

Acid hydrolysis of mushrooms and shrimp waste was performed with a Gerhardt Kjeldahltherm digestion unit equipped with digital temperature controller (Gerhardt GmbH & Co. KG, Königswinter, Germany). The acid hydrolysis reaction was stopped by cooling at 4°C, and then the resulting hydrolysate was neutralized with calcium carbonate. The neutralized hydrolysate was used for GlcN determination and characterization.

The conversion of glucosamine form chitin was defined as:

$$\text{Conversion } (\%) = \frac{M_g}{M_c} \times 100\% \qquad (3.5)$$

in which M_g represents the mass of glucosamine in the mushroom hydrolysate, and M_c represents the mass of chitin/chitosan in the mushroom sample.

3.4.4.1 Comparison of the efficiency of hydrochloric acid and sulfuric acid for glucosamine production

To investigate the efficiency of hydrochloric acid and sulfuric acid for GlcN production, five mushrooms were used as the raw materials, while shrimp waste was used as the control. The parameters selected for the catalyst efficiency comparison were: dry sample, ratio of raw material to acid volume (1:10, w/v), acid concentration (6 M), hydrolysis temperature (80°C), and hydrolysis time (8h). After hydrolysis, the reaction was stopped by cooling the mixture to room temperature and then neutralized with calcium carbonate. The neutralized hydrolysate was used for glucosamine quantitative determination.

3.4.4.2 Selection of important parameters in sulfuric acid hydrolysis

One Variable At a Time (OVAT) was used to select the important parameters for further optimization. A certain amount of mushroom sample, different type and concentration acid were well-mixed by a vortex mixer, then stirred and heated under different temperature for a period of time. For preliminary study we have investigated several parameters of acid hydrolysis of mushroom sample such as acid type and concentration, ratio of raw material and acid volume, reaction temperature and time. The levels for each parameters were set as follows: acid type and concentration: hydrochloride acid (1, 2, 4, 6, 9 and 12 M) and sulfuric acid (1, 2, 4, 6, 9, 12, 15 and 18 M); raw material status (fresh and dried); ratio of raw material to acid volume (w/v, 1:5, 1:10; 1:15, 1:20, 1:25, 1:30 and 1:35), reaction temperature (40, 60, 80 and 100°C) and time (0.5, 1, 2, 3, 4, 6, 10, 12, 15, 18, 24, 36 and 48 h).

3.4.5 Optimization of acid hydrolysis conditions

3.4.5.1 Experimental design

In the present study, the software Design Expert (Trial Version 10, Stat-Ease Inc. Minneapolis, Minn., U.S.A.) was employed for experimental design, data analysis, and model building. Response surface methodology (RSM) based on a statistical experimental design (BBD) was used to investigate the effects of the independent variables on the response (*Y*, GlcN yield), determine the optimum conditions and maximize the GlcN yield from oyster mushroom. From preliminary results, independent variables and their proper ranges were selected. Factors considered important were hydrolysis temperature (70-110°C), time (2-6 h), and acid concentration (3-9 M). The symbols and levels are shown in Table 3.2. In this scheme the variable levels (-1 for the lower level, 1 for the upper level, and o for the mean value) are codified based on the following equation (Eq. 3.6) (Suxia et al., 2012):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad i = 1, 2, 3 \tag{3.6}$$

Where x_i was the coded value of the independent variable, X_i was the real value of the independent variable, X_0 was the real value of the independent variable on the center point, and ΔX_i was the step-change value. Table 3.3 showed the experimental design. In all experimental assays, dry sample was used, and the ratio of raw material to acid volume (1:5, w/v) was kept constant. The experiment design contains 17 trials with 5 center points which allows to calculate the pure error sum of squares and the response function at intermediate level and to estimate the system performance at any experimental point within the selected range. The value of the responses was the mean of triplications. Experiments were randomized to maximize the effects of unexplained variability in the observed responses because of extraneous factors.

Table 3.2 Variables and their levels used in the experiments.

Independent variables	**Levels**		
	-1	**0**	**1**
Hydrolysis temperature (°C) (X_1)	70	90	110
Hydrolysis time (h) (X_2)	2	4	6
Acid concentration (M) (X_3)	3	6	9

In addition, a suitable approximation for the true functional relationship between the response function and the set of independent variables was constructed from the experimental results. If the response function is well modeled by the linear function, the optimization strategy will be continued by extrapolating along the path of steepest ascent, and locating a stationary point using canonical analysis. On the other hand, if the response function is not well modeled by the linear function, then a polynomial of higher degree must be sought, such as 2nd order model, which requires fitting response surfaces (Serrato-Joya et al., 2006). In this case, our experimental results were evaluated and the latter case was the one observed.

The experimental data was fitted to the second order polynomial (Eq. 3.7) by a non-linear regression method.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (3.7)$$

where Y is the predicted response variable; β_0, β_i, β_{ii}, and β_{ij} are constant regression coefficients for intercept, linearity, square, and interaction terms of the model, respectively; and X_i and X_j ($i = 1, 2, 3$; $j = 1, 2, 3$, $I \neq j$) represent the coded independent variables.

Table 3.3 Box-Behnken design matrix of three variables.

Run no.	Hydrolysis temperature (X_1, °C)	Time (X_2, h)	Acid concentration (X_3, M)
1	-1 (70)	-1 (2)	0 (6)
2	1 (110)	-1 (2)	0 (6)
3	-1 (70)	1 (6)	0 (6)
4	1 (110)	1 (6)	0 (6)
5	-1 (70)	0 (4)	-1 (3)
6	1 (110)	0 (4)	-1 (3)
7	-1 (70)	0 (4)	1 (9)
8	1 (110)	0 (4)	1 (9)
9	0 (90)	-1 (2)	-1 (3)
10	0 (90)	1 (6)	-1 (3)
11	0 (90)	-1 (2)	1 (9)
12	0 (90)	1 (6)	1 (9)
13	0 (90)	0 (4)	0 (6)
14	0 (90)	0 (4)	0 (6)
15	0 (90)	0 (4)	0 (6)
16	0 (90)	0 (4)	0 (6)
17	0 (90)	0 (4)	0 (6)

3.4.5.2 Percentage contributions of process variables

Based on the sum of squares obtained from the analysis of variance (ANOVA), the percentage contributions (PC) for each individual process variables were calculated by the following equations described by Yetilmezsoy, Demirel, and Vanderbei (2009).

$$TPC_i = \frac{\sum_{i=1}^{n} SS_i}{\sum_{i=1}^{n}\sum_{j=1}^{n} SS_i + SS_{ii} + SS_{ij}} \times 100 \quad (3.8)$$

$$TPC_{ii} = \frac{\sum_{i=1}^{n} SS_{ii}}{\sum_{i=1}^{n}\sum_{j=1}^{n} SS_i + SS_{ii} + SS_{ij}} \times 100 \quad (3.9)$$

$$TPC_{ij} = \frac{\sum_{i=1}^{n}\sum_{j=1}^{n} SS_{ij}}{\sum_{i=1}^{n}\sum_{j=1}^{n} SS_i + SS_{ii} + SS_{ij}} \times 100 \quad (3.10)$$

Where TPC_i, TPC_{ij} and TPC_{ii} are total percentage contributions (TPC) of linear, interactive and quadratic terms; SS_i, SS_{ij} and SS_{ii} are the computed sum of squares (*SS*) for linear, interactive and quadratic terms respectively.

3.4.5.3 Optimization and verification of the predicted optimized conditions

After the results were obtained, Derringer's desired function methodology (Derringer and Suich, 1980) was performed to evaluate the optimum conditions to yield maximum amount of GlcN from oyster mushroom by sulfuric acid hydrolysis.

The general approach of desirability function is to first transform the response into a dimensionless individual desirability function (d_i) that varies from 0 to 1 (lowest to highest desirability). From the geometric means of different individual d_i values, overall desirability function (G) was obtained by combining the individual desirability values.

$$G = (d_1 \times d_2 \times d_3 \times \cdots \times d_n)^{1/n} \quad (3.11)$$

Where d_i indicates the desirability of the response and n is the number of responses in the measure. If any of the responses is beyond the desirability, then overall function will be turned into zero. It can be extended to

$$G = \left(d_1^{\alpha_1} \times d_2^{\alpha_2} \times d_3^{\alpha_3} \times \cdots \times d_n^{1/n}\right)^{1/n},$$

$$0 \leq \alpha_i \leq 1(i = 1,2,3,\cdots,n),\ \alpha_1 + \alpha_2 + \cdots + \alpha_n = 1 \qquad (3.12)$$

Where d_i indicates the desirability of the different responses Y_i (i = 1, 2, 3, . . ., n) and i represents the importance of responses. So, maximum overall desirability function G depends on the α_i (importance) value. For optimization of any response, the response is to be maximized, minimized or assigned a target value. In this study, the response was assigned to be maximized. The following desirability function criterion was used to obtain maximum yield of GlcN from oyster mushroom.

d_i = 0 if response < low value.

$0 \leq d_i \leq 1$ as response varies from low to high.

d_i = 1 if response > high value.

To determine the validity of the developed mathematical model equation, triplicate experiments were performed under the optimal conditions as predicted by the model. The average value of the experiments was compared with the predicted values of the developed model and find out the accuracy, appropriateness and suitability of the developed model.

3.4.6 Kinetics of glucosamine production from different mushrooms

For kinetics study, 10 g ground mushroom sample was hydrolyzed following the addition of 100 mL 6 M sulfuric acid and heat (90, 100 or 110°C). During the 6 h hydrolysis, the subsamples were taken at 30 min intervals. Then the digestion mixture was cooled to room temperature and neutralized to pH = 7. The final solution volume was recorded. GlcN concentration was determined by HPLC method.

Due to the difficulty in finding a strict mechanism for hydrolysis reactions, it is usual to use simplified models for the determination of kinetics. The mechanism of chitosan/chitin hydrolysis was studied by several researchers (Einbu, Grasdalen and

Vårum, 2007, Einbu and Vårum, 2008, Shabrukova, Shestakova, Zainetdinova and Gamayurova, 2002, Vårum, Ottøy and Smidsrød, 2001, Yan and Evenocheck, 2012). In weak acid, oligosaccharides are dominant in the hydrolysate products at low temperature. When a strong acid is used, chitosan/chitin could be fully hydrolyzed to glucosamine.

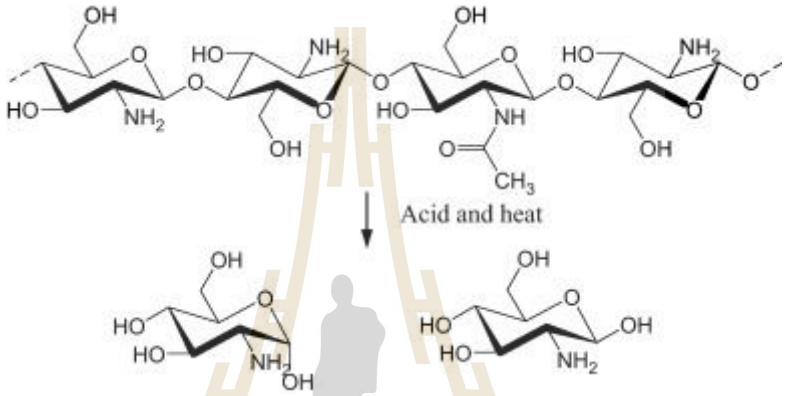


Figure 3.3 Schematic view of GlcN production from chitin (Yan and Evenocheck, 2012).

In the literature, hydrolysis of chitin/chitosan is known to follow pseudo-homogeneous irreversible first-order kinetics (Yan and Evenocheck, 2012). The first-order model was widely applied to the hydrolysis of cellulose and hemicellulosic fraction using sulfuric acid hydrolysis (Aguilar, Ramırez, Garrote and Vázquez, 2002, Tellez-Luis, Ramırez and Vázquez, 2002). This model used successfully was proposed by Saeman (1945):

$$\text{Polymers} \xrightarrow{k} Monomers \tag{3.13}$$

In our study, it can be specified in Eq. (3.14):

$$\text{Chitin/chitosan} \xrightarrow{k} GlcN \tag{3.14}$$

Where *k* is the rate constant of the hydrolysis reaction. The rate law for a reaction that is first-order with respect to a reactant A is:

$$\mathrm{r} = \frac{-d[A]}{dt} = k[A] \tag{3.15}$$

The integrated first-order rate law is:

$$\ln[A] = -\mathrm{kt} + \ln[A]_0 \tag{3.16}$$

Or

$$kt = \ln[A]_0 - \ln[A] \tag{3.17}$$

A plot of $\ln[A]_0 - \ln[A]$ *vs.* time *t* gives a straight line with a slope of *k*.

Arrhenius' equation gives the dependence of the rate constant of a chemical reaction on the absolute temperature, a pre-exponential factor and other constants of the reaction.

$$k = \mathrm{A}e^{-Ea/(RT)} \tag{3.18}$$

Where *k* is the rate constant; *T* is the absolute temperature (in Kelvin); *A* is the pre-exponential factor, a constant for each chemical reaction that defines the rate due to frequency of collisions in the correct orientation; *Ea* is the activation energy for the reaction (in J/mol or kJ/mol); *R* is the universal gas constant, 8.3144598 J/mol/K.

3.5 Purification and identification of glucosamine and ACE inhibitory peptides

3.5.1 ACE inhibitory activity assay

The ACE inhibitory activity was measured using a spectrophotometric method following the protocol described by Hatanaka, Miyahara, Suzuki and Sato (2009) with minor modification. The synthetic tripeptide substrate, FAPGG, was used in a 96-well microplate assay. For the assay, 10 µL of sample solution added with 5 µL

ACE solution (100 mU/mL) was pre-incubated at 37ºC for 10 min. The reaction was initiated by adding 50 µL substrate FAPGG solution (0.39 mM FAPGG in 0.1 M sodium borate buffer containing 0.3 M NaCl at pH = 8.3) and incubated for 2 h at 37ºC. The substrate FAPGG was hydrolyzed to FAP and GG by ACE, resulting in a decrease in absorbance at 340 nm as measured using a Biotek Epoch microplate spectrophotometer (Winooski, VT, USA). The ACE inhibitory activity was calculated using the following equation:

$$\text{ACE inhibitory activity (\%)} = \frac{C-S}{C-B} \times 100 \qquad (3.19)$$

where *C* is the absorbance of blank (hydrochloric acid was added before ACE), *S* is the absorbance in the presence of both ACE and sample, *B* is absorbance without sample (buffer instead of the sample).

The IC_{50} value was defined as the concentration of peptide required to inhibit 50% of the ACE activity under the above assay conditions. The peptide concentration was determined by Bradford method.

3.5.2 Purification procedure

3.5.2.1 Decoloring process

Decoloring experiments were carried out in a rotary flask of 250 mL which containing 100 mL neutralized mushroom hydrolysate and 6 g decoloring agent. The mixture was stirred at 240 rpm for 1 h at room temperature. At the end of decoloring test, the slurry was transferred to a filtering equipment and filtered by vacuum suction. GlcN content of filtrate was analyzed by HPLC described before and GlcN yield was calculated. The most suitable decoloring agent was selected based on its decoloring efficiency and GlcN yield.

3.5.2.2 Ultrafiltration

Decolorized mushroom hydrolysate was separated into two fractions (retentate and permeate) by ultrafiltration using a 1 kDa MWCO membrane. Both fractions were assayed for GlcN yield and ACE inhibitory activity.

3.5.2.3 Ethanol precipitation

The permeate fraction obtained from ultrafiltration was further concentrated in a vacuum rotary evaporator at 45ºC and precipitated by the slow addition of ethanol. Ethanol was added to concentrated mushroom hydrolysate until the ethanol-to-hydrolysate volume ratio was 5:1. The mixture was stirred for 30 min and kept at 4ºC for 24 h. After centrifugation, the ethanol-soluble fraction with higher GlcN yield (purified GlcN fraction) was dried by vacuum evaporation at 45ºC. The precipitate fraction possessing higher ACE inhibitory activity was dried in a vacuum oven at 60ºC for 24 h and collected for subsequent chromatographic purification of ACE inhibitory peptides.

3.5.2.4 Chromatographic purification

An AKTA explorer 100 system (GE Healthcare Bioscience, Piscataway, NJ, USA) equipped with an automatic fraction collector was employed. The dried precipitate was dissolved in 50 mM Tris-HCl buffer (pH 8.0) and loaded onto a HiTrap DEAE Sepharose FF ion exchange column (GE Healthcare, Chalfont St Giles, UK), which was pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0). A segmented gradient of 0-1 M NaCl in 50 mM Tris-HCl buffer (pH 8.0) was carried out at a flow rate of 0.5 mL/min. Elution was monitored by absorbance at 220 nm and collected in 2 mL volume fractions. Ion exchange chromatography was repeated 20 times. Fractions were collected according to elution peaks, freeze-dried, and assayed

for ACE inhibitory activity. Then the fraction with the highest ACE inhibitory activity was redissolved in deionized water and applied to a Superdex Peptide 10/300 GL gel filtration column (10 mm i.d.×300 mm). The column was equilibrated and eluted with deionized water at a flow rate of 0.5 mL/min. Elution was monitored by absorbance at 220 nm and collected in 2 mL volume fractions. Gel filtration chromatography was repeated 20 times. The fraction with the highest activity was collected and freeze-dried for further analyses of residual activity, inhibition pattern and peptide analysis.

3.5.3 Residual activity assay of the purified ACE inhibitory peptides

For the study of the temperature effect on the ACE inhibitory activity, the solutions of purified peptides were incubated at different temperatures (4, 20, 40, 60, 80, and 100ºC) for 2 h and the residual ACE inhibitory activity was assayed as described in section 3.5.1.

For the study of the pH effect on the ACE inhibitory activity, the solutions of purified For the study of the temperature effect on the ACE inhibitory activity, the solutions of purified peptides were incubated at 37ºC for 2 h under different pH values varying from 2 to 12. After the pH values were adjusted to 7.0, the residual ACE inhibitory activity was determined as described in section 3.5.1.

For the study of the gastrointestinal proteases effect on the ACE inhibitory activity, the residual activity of purified peptides after gastrointestinal proteases digestion was assessed as reported by Chen et al. (2012). The purified peptides were dissolved at 0.8 mg/mL in 1% pepsin (0.1 mM KCl-HCl buffer at pH=2.0) or chymotrypsin solution (0.1 mM KCl-NaOH buffer at pH=7.0) and reacted at 37ºC for 4 h. The reactions were terminated by boiling for 15 min. After centrifugation, the supernatant was neutralized and assayed for ACE inhibitory activity as mentioned in

Section 3.5.1. Control treatments were prepared by mixing inactivated pepsin or chymotrypsin (by boiling for 15 min) with the purified peptides and reacted at 37°C for 4 h. Reactions were terminated by boiling for 15 min. After centrifugation at 10,000×g for 25 min, the supernatant was transferred, neutralized for acidity, and assayed for ACE inhibitory activity as mentioned in Section 3.5.1.

3.5.4 Inhibition pattern of the purified ACE inhibitory peptides

To clarify the ACE inhibition patterns on ACE of the purified peptides, different concentrations of purified ACE inhibitory peptides were incubated with different concentrations of substrate (FAPGG), and each reaction mixture was analyzed as described in Section 3.5.1. Basically, various concentrations (0.1, 0.2, 0.4, and 0.8 mM) of FAPGG were incubated with ACE solution in the absence and presence of purified ACE inhibitory peptides (0.75 and 0.15 mg/ml) at 37 °C. The ACE inhibition pattern was estimated by constructing Lineweaver-Burk plots. The Lineweaver-Burk plot was plotted where the reciprocal of FAPGG concentration was used as the independent variable (*X*-axis) and the reciprocal of absorption decrement at 220 nm as the dependent variable (*Y*-axis).The kinetic parameters K_i (inhibitor constant), V_{max} (maximum velocity) and K_m (Michaelis-constant) for the binding of inhibitor to ACE were also calculated according to the formulation as

$$\frac{1}{v} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) \quad (3.20)$$

Where v is the velocity of generating product;

K_m is Michaelis constant;

$[S]$ is substrate concentration;

V_{max} is maximum velocity;

$[I]$ is inhibitor concentration;

K_i is inhibitor constant.

3.5.5 Peptide analysis of the purified ACE inhibitory peptides

Peptide analysis was performed by a NanoAcquity system (Waters Corp. Milford, MA) and SYNAPT HDMS mass spectrometer (Waters Corp. Manchester, UK). Peptides were separated on a nanocolumn (Acclaim PepMap 100 C18, 3 mm, 100A, 75 mm i. d. × 150 mm). Eluent A was 0.1% formic acid in water. Eluent B was 80% acetonitrile in water containing 0.1% formic acid. Elution was performed using a linear gradient from 0 to 70% of eluent B at a flow rate of 300 nL/min for 13 min. Raw data obtained from the mass spectrometer were generated in a Micromass file (PKL) supported for online MASCOT MS/MS Ions Search (http://www.matrixscience.com/) against the NCBI database. Search parameters were: enzyme (no cleave), taxonomy (All entries), peptide mass tolerance (1.2 Da), fragment mass tolerance (0.6 Da), peptide charge state (1+, 2+ and 3+), instrument type (ESI-QUAD-TOF), and max missed cleavages (1). The potential peptide candidates were searched against the BIOPEP database (Minkiewicz et al., 2008) and SATPdb database (Singh et al., 2016) to determine their novelty and subsequently filtered according to the score of PeptideRanker (Mooney, Haslam, Pollastri and Shields, 2012). Three selected peptide sequences (ASPYAFGL, MLCSTTF, and LASLFGNDP) were chemically synthesized using a solid phase peptide synthesis method (GL Biochem Ltd., Shanghai, China) at the highest available purity (98%), which was verified by HPLC method. The sequences of synthesized peptides were confirmed by their molecular mass and spectra acquired from LC-MS/ESI. The ACE inhibitory activity of synthesized peptide was determined as previously described in Section 3.5.1. Captopril, a well-known ACE inhibitor, was used as the comparison.

3.6 Storage stability of neutralized mushroom hydrolysates

3.6.1 Liquid samples

Mushroom hydrolysate after decolorization and ultrafiltration was concentrated under vacuum (resulting in approximately 20 mg/mL GlcN). Then, 1 mL concentrated mushroom hydrolysate was transferred into 2 mL sealed transparent glass vials (in the presence of air) and stored at three different temperatures: -20°C and 4°C, representing the refrigeration temperature, and 26°C, representing room temperature. The samples were collected after the following storage times: 0, 15, 30, 45, and 60 days, and GlcN concentration and ACE inhibitory activity were analyzed as described above. The analyses were carried out in triplicate and totally six vials were used for each storage time analyzed. The results of all analyses were compared to the results immediately after vacuum concentration and expressed as a percentage of these results (i.e., the results immediately after vacuum concentration were taken as 100%).

3.6.2 Powdered samples

Mushroom hydrolysate after decolorization and ultrafiltration was dried in a Gamma 2-16 LSC freeze-drier (Martin Christ GmbH, Osterode am Harz, Germany). In the freeze-drying process, mushroom hydrolysate was frozen at -85°C for 1 h in the freeze-drier chamber and dried at -65°C for 72 h, and then finally dried at 30°C for 24 h. The entire process was carried out at -85°C cold collector temperature and 0.001 mbar chamber pressure. Then, the freeze-dried sample was transferred into sealed plastic bags and stored at -20, 4, and 26°C for 60 days. At each sampling day (0, 15, 30, 45, and 60), 1 g sample was collected and dissolved in 10 mL DI water , the GlcN concentration and ACE inhibitory activity were analyzed as described above. The results of all analyses were compared to the results immediately

after freeze-drying and expressed as a percentage of these results (i.e., the results immediately after freeze-drying were taken as 100%). Before carrying out any tests, the residual insoluble particles of solutions were removed by cellulose acetate (CA) membrane filtration (0.45 µm pore size, National Scientific Company, Rockwood, TN).

3.7 Statistical analysis

All experiments were performed in triplicates and all data were presented as the mean±standard deviation (SD). A p-value < 0.05 was considered statistically significant. Data obtained from response surface methodology were analyzed by linear multiple regression using the Design Expert software (Trial Version 10.0, Stat-Ease Inc., Minneapolis, MN, U.S.A.). One-way ANOVA and paired sample t test were used for testing any significant differences among the treatments. Tukey's test was used to assess differences in means. Statistical analyses were performed by SPSS software (version 16.0; SPSS Inc., Chicago, Ill, U.S.A.). The software, GraphPad Prism for Windows (version 7.02, GraphPad Software, La Jolla, CA, U.S.A.), was used to calculated IC_{50} values of synthesized peptides and Captoril. All the calculations of descriptive analysis were carried out in the Microsoft Excel® 2013 running under windows.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Proximate composition analysis

The proximate composition of five ground mushroom samples were determined and the results were tabulated in Table 4.1. Moisture was determined on the basis of fresh weight. Other chemical compositions including crude fat, crude protein, and ash were presented on the basis of DW.

The DW of fresh mushrooms, mainly composed of carbohydrates, proteins, fiber, and minerals, is relatively low, commonly ranging between 8% and 14%. Therefore, when considering the chemical composition of mushrooms, it is worthwhile to keep in mind that water content is the parameter that is to some degree, varied for fresh mushroom. The moisture content of fresh mushrooms can be influenced by the change of weather conditions during cultivation and the moisture evaporation after harvest. The high moisture content of fresh mushrooms indicates that they are highly perishable. Sunday, Israel and Odey (2016) suggested that removal of moisture during processing may extend shelf life of mushrooms, thereby increasing the concentration of nutrients. Even after drying process, the moisture content maybe different from each other because of its inherent nature or drying method and conditions. There is a consensus that the moisture content of fresh fruiting bodies is 90%, and data published on the chemical composition of mushrooms needs to be normalized to dry matter contents.

Table 4.1 Proximate composition (%, DM) of ground mushroom samples.

Mushroom	Moisture	Ash	Crude fat	Crude protein
Enoki mushroom	5.99 ± 0.13	9.90 ± 0.11	2.84 ± 0.28	27.06 ± 0.26
Oyster mushroom	10.05 ± 0.64	9.80 ± 0.19	3.67 ± 0.28	33.08 ± 1.56
Straw mushroom	11.45 ± 0.22	8.41 ± 0.15	0.77 ± 0.19	44.10 ± 0.17
Shiitake	12.27 ± 0.09	6.58 ± 0.11	3.00 ± 1.19	24.64 ± 0.08
Wood ear mushroom	16.30 ± 0.41	4.36 ± 0.11	6.15 ± 0.43	15.97 ± 0.16

Results are expressed as Mean ± SD (n = 3).

Among these five kinds of mushroom, it was observed that enoki mushroom had the lowest moisture content (5.99%); while wood ear mushroom had the highest moisture content (16.30%). As regards fat, it is well known that mushrooms are characterized by low fat content (Barros et al., 2007, Dıez and Alvarez, 2001). Fat contents of mushrooms investigated in the present work express this characteristic since values were in range of 0.77 to 6.15%. This also implied that mushrooms can function effectively in low-fat diet such as those required by patients with cardiovascular diseases, obesity, etc. (Gropper and Smith, 2012). The ash content of five mushrooms were ranged from 4.36 to 9.90%. These results are consistent with those reported by other authors (Agrahar-Murugkar and Subbulakshmi, 2005, Manzi et al., 1999, Sanmee et al., 2003). In these studies, the reported ash contents ranged from 5.4 to 27.6% of dry weight. The protein contents of mushrooms are influenced by the compost composition, flush number, harvest time and strain. In literature, the reported total protein content of mushrooms range from 14.0 to 44.3% on a DW basis (Agrahar-Murugkar and Subbulakshmi, 2005, Appolinaire, Hubert, Parfait and Patrice,

2015, KIRBAĞ and AKYÜZ, 2010). Some papers reported crude protein contents determined by the Kjeldahl method using the usual conversion factor of 6.25. However, such values were overestimated due to a high proportion on non-protein nitrogen, particularly in chitin. A factor of 4.38 has been mostly used in recent publications (Kalač, 2013). In this study, the total nitrogen contents of mushrooms were determined by the Kjeldahl method and the corresponding protein content was calculated using the conversion factor of 4.38 (Table 4.1). The values were ranged from 15 to 33% and were within the reported range.

The carbohydrate content of mushroom could be calculated by subtracting the total percentage of all fractions (ash, crude fat, and crude protein) from 100 (Adejumo and Awosanya, 2005). The results showed that about one-half of mushroom DM (46.72 to 73.52%) were carbohydrates. Kalač (2013) reported that the main carbohydrates are chitin, glycogen, β-glucans, trehalose, and mannitol.

4.2 Production of glucosamine

4.2.1 Determination of chitin

The chitin was extracted from ground mushroom samples by a series of chemical treatments described in Figure 3.2 to remove associated components: proteins, pigments, glucans, and minerals. The procedure and treatments were as follows. At the first stage, distilled water was used to remove water soluble glucans and minerals. At the second stage, sodium hydroxide was used to dissolve, hydrolyze, and remove proteins and alkali-soluble glucans. Then, hydrochloric acid was added to remove minerals. At the last two stages, it is known that partial neutral saccharides and acid-soluble protein compounds were also separated. The crude chitin content of ground mushroom samples varied between 1.30 and 5.78% (Table 4.2). The values

were in agreement with the result of previous research (Manzi, Aguzzi and Pizzoferrato, 2001, Mario, Rapanà, Tomati and Galli, 2008, Nitschke, Altenbach, Malolepszy and Mölleken, 2011, Tshinyangu and Hennebert, 1996, Vetter, 2007). The slight different results may be caused by mushroom strains, cultivation conditions, pretreatment and so on (Jiang et al., 2010, Kalač, 2009, Zivanovic and Buescher, 2004). The higher chitin content indicates that straw mushroom from the local market could be considered as a good source of chitin for the production of GlcN.

Table 4.2 Chitin content (%, DM) of ground mushroom samples.

Mushroom	**Chitin content**
Enoki mushroom	2.64 ± 0.12
Oyster mushroom	2.70 ± 0.00
Straw mushroom	5.78 ± 0.23
Shiitake	3.85 ± 0.17
Wood ear mushroom	1.30 ± 0.10

Results are expressed as Mean ± SD (n = 3).

4.2.2 Determination of glucosamine

The poor control of GlcN content in commercial products has been highlighted as an important cause of inconsistent outcomes in clinical trials (Magaña, Wrobel, Escobosa and Wrobel, 2014). In this regard, analytical control of GlcN in the commercial products becomes an issue. A variety of different methods are available for this purpose, including simple spectrophotometric assays (Wu, Hussain and Fassihi, 2005), thin layer chromatography (TLC) (Esters et al., 2006), liquid

chromatography (LC) or capillary electrophoresis (CE) separations with spectrophotometric, contactless conductivity, amperometric or fluorimetric detection (Akamatsu and Mitsuhashi, 2012, Campo et al., 2001, EI-Saharty and Bary, 2002, Jáč et al., 2008, Skelley and Mathies, 2006, Volpi, 2009, Zhang et al., 2006, Zhou, Waszkuc and Mohammed, 2004, 2005, Zhu, Cai, Yang and Su, 2005). Among various methods reported previously, the most common approach relies of suitable derivatization followed by LC separation with spectrophotometric or spectrofluorimetric detection (Aghazadeh-Habashi, Sattari, Pasutto and Jamali, 2002, Jamali and Ibrahim, 2010, Shao et al., 2004, Sitanggang, Wu and Wang, 2009, Zhang et al., 2006, Zhou, Waszkuc and Mohammed, 2004, 2005, Zhu, Cai, Yang and Su, 2005). Because GlcN lacks any UV-absorbing chromophore, HPLC, coupled with pre-column derivatization, is necessary for GlcN determination. The derivatization agents used so far have been: *o*-phtaldialdehyde (OPA) (Eikenes, Fongen, Roed and Stenstrøm, 2005), 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) (Zhu, Cai, Yang and Su, 2005), 9-fluorenylmethyl-chloroformate (Huang et al., 2006), FMOC-Su (Zhou, Waszkuc and Mohammed, 2004, 2005), dansyl chloride (Qi, Zhang, Zuo and Chen, 2006), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Wang et al., 2008), 2-aminobenzoic acid (Volpi, 2009), and phenylisothiocyanate (Tekko, Bonner and Williams, 2006).

In this study, we utilized the AOAC official method for the analysis of GlcN (Zhou, Waszkuc and Mohammed, 2005). The GlcN free base is released by adding TEA to the solution and derivatized with FMOC-Su. The formation of FMOC- GlcN is shown in Figure 4.1. The derivative is separated by HPLC and measured with UV detection.

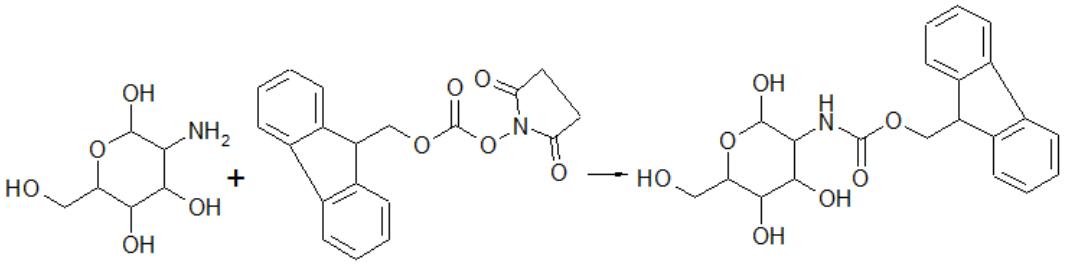


Figure 4.1 Formation of 9-fluorenylmethoxycarbonyl-gluccosamine derivative (Jamali and Ibrahim, 2010).

4.2.2.1 Optimum conditions of release

As mentioned before, the GlcN free base was released by TEA and then derivatized with FMOC-Su. Therefore, the release conditions, namely TEA amount, released time and pH value of reaction medium, were very important for the derivatization and the following GlcN quantitative determination.

1) The TEA amount and released time

The effects of TEA amount and released time on the release of GlcN are shown in Figure 4.2 and 4.3. In Figure 4.2, the HPLC peak area of GlcN derivative increased with the TEA amount from 0 to 0.75 μL, then kept constant with TEA amount from 0.75 to 1.00 μL. When 0.75 μL TEA was added, 10 h was enough to complete the release of GlcN. With extended released time (16 h), there is no significant change of the final HPLC peak area of GlcN derivative (Figure 4.3). Therefore, the optimum TEA amount and released time were 0.75 μL and 10 h, respectively.

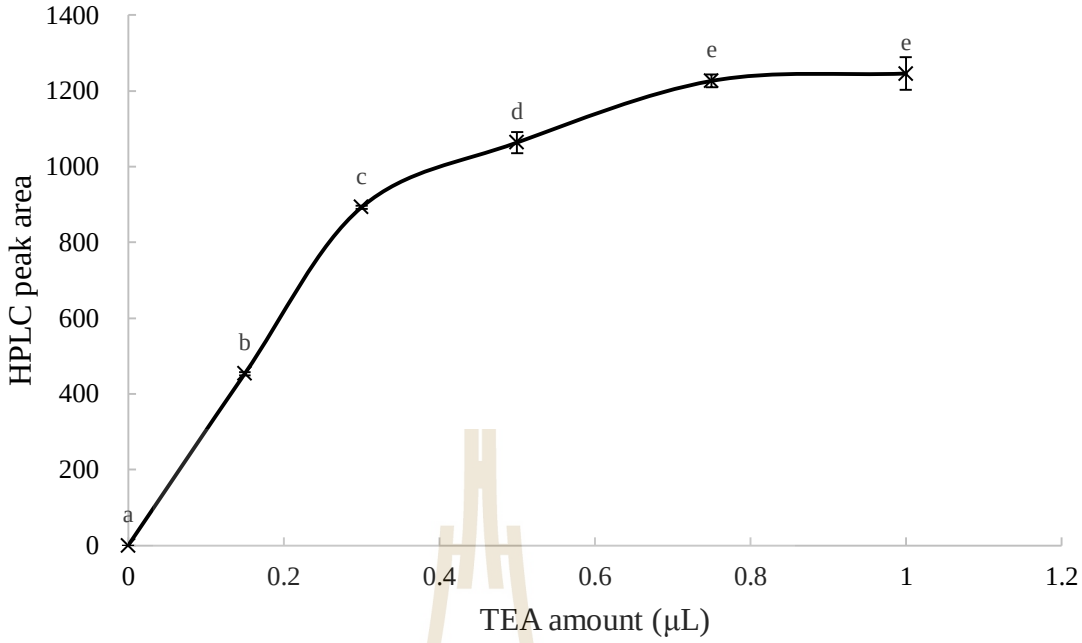


Figure 4.2 The effect of TEA amount on the release of GlcN. Values followed by different letters indicate significant differences ($p < 0.05$) between samples when analyzed by one-way ANOVA and post-hoc Tukey's HSD test.

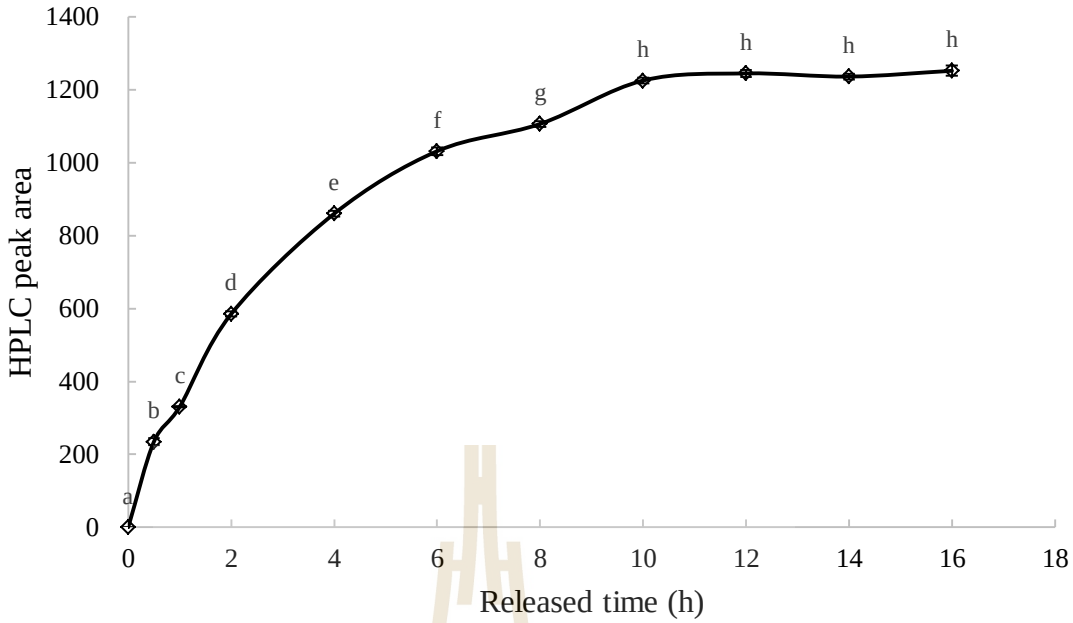


Figure 4.3 The effect of released time on the release of GlcN. Values followed by different letters indicate significant differences ($p < 0.05$) between samples when analyzed by one-way ANOVA and post-hoc Tukey's HSD test.

2) pH of the medium for release

The pH of the medium was important in controlling both the release and the derivatization of GlcN. The rate of release increased with increasing pH, but decreased sharply with pH from 7 to 12. In Figure 4.4, the result suggested that the optimum pH value was in the range of 5 to 7. Under these conditions, the HPLC peak areas of GlcN derivative were the highest.

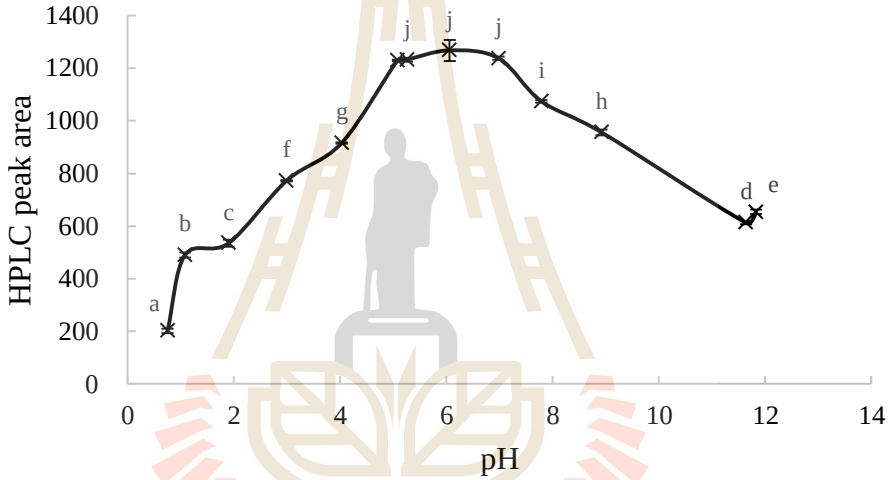


Figure 4.4 The effect of pH on the derivatization of GlcN. Values followed by different letters indicate significant differences ($p < 0.05$) between samples when analyzed by one-way ANOVA and post-hoc Tukey's HSD test.

4.2.2.2 Precision and accuracy of the HPLC method

The standard curve for GlcN showed linearity over the selected concentration range from 0.02 to 1.20 mg/mL, and excellent correlation coefficient ($R \geqq 0.99$). A typical regression equation for the quantitative determination of GlcN was found to be $y = 1020.70x + 23.807$ ($R^2 = 0.9993$, $n = 7$).

Different blind standard samples, prepared from the stock solution, were determined using the suggested HPLC procedures. The intra-day RSD were in the range of 0.26 to 2.09 % and the inter-day RSD were in the range of 0.09 to 3.66 %. Results are found to be reproducible, as shown in Table 4.3, with mean percentage accuracy of 100.88 ± 1.26. The HPLC analysis of GlcN in mushroom hydrolysates using the calculated linear regression equation, showed GlcN concentration of 0.0831 ± 0.0075, 0.5186 ± 0.0093 and 1.0298 ± 0.0428, respectively. The validity of the method was further checked using the standard addition technique where the percentage recovery was found to be 101.25 ± 0.99, 100.36 ± 1.82 and 99.94 ± 1.84, respectively (Table 4.4).

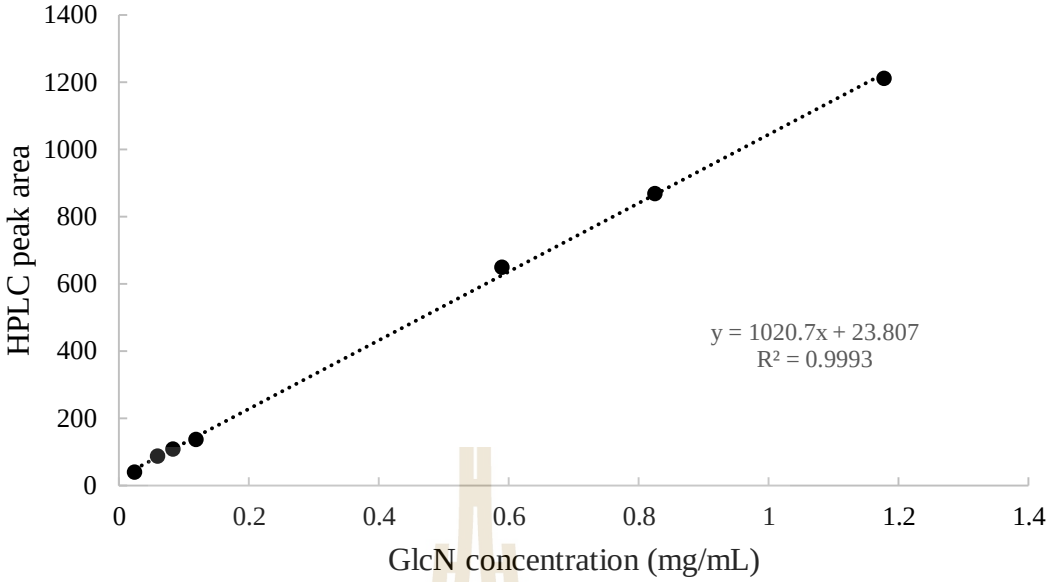


Figure 4.5 Calibration curve of GlcN.

GlcN has 2 natural stereoisomers (α and β), and the inter-conversion of these 2 in aqueous solution is not preventable (Zhou, Waszkuc and Mohammed, 2004, 2005), resulting in 2 peaks in the typical chromatogram of GlcN (Figure 4.6). The retention time (R_r) is 11.310 and 11.587 min. The sum of the areas of these 2 peaks is used to quantify the GlcN.

Table 4.3 HPLC determination of GlcN in standard solutions.

Experiment no.	Taken (mg/mL)	Found (mg/mL)[a]	Accuracy (%)	RSD (%)	
				Intra-day	Inter-day
1	0.02	0.0199	99.38	2.09	1.11
2	0.04	0.0404	100.91	1.76	0.61
3	0.08	0.0822	102.72	0.63	0.45
4	0.20	0.2004	100.21	0.38	2.85
5	0.60	0.6119	101.98	0.26	0.09
6	1.00	1.0009	100.09	0.37	3.66
Mean ± SD			100.88 ± 1.26		

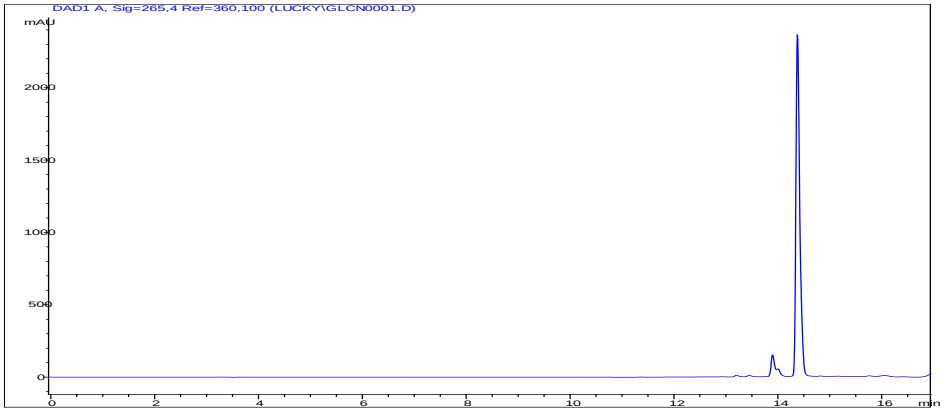
[a] Average of three determinations.

Table 4.4 HPLC determination of GlcN in mushroom hydrolysates.

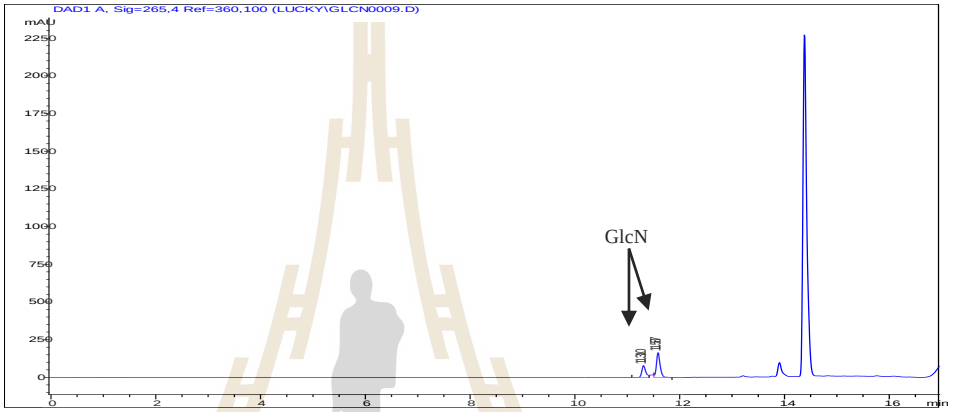
Sample no.	Found (mg/mL)[a]	Added (mg/mL)	Found (mg/mL)[a]	Recovery (%)
1	0.0831 ± 0.0075	0.05	0.0503	100.55
		0.10	0.1024	102.38
		0.15	0.1512	100.83
Mean ± SD				101.25 ± 0.99
2	0.5186 ± 0.0093	0.05	0.0503	100.55
		0.10	0.1021	102.09
		0.15	0.1477	98.45
Mean ± SD				100.36 ± 1.82
3	1.0298 ± 0.0428	0.05	0.0504	100.86
		0.10	0.1011	101.14
		0.15	0.1467	97.82
Mean ± SD				99.94 ± 1.84

[a] Average of three determinations.

A



B



C

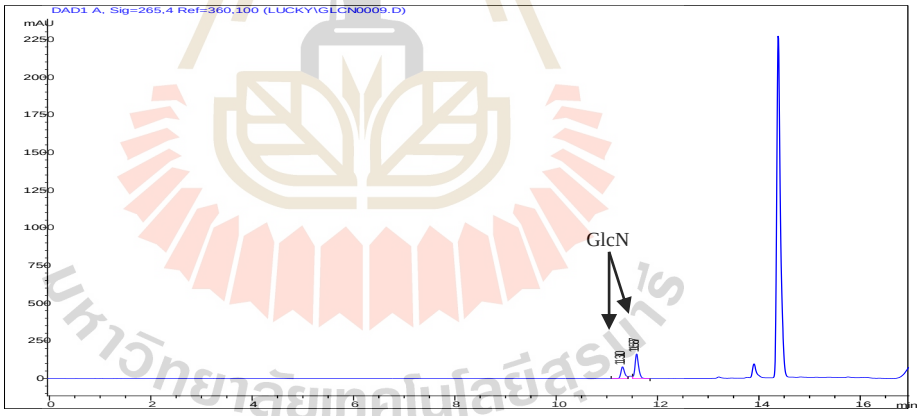


Figure 4.6 Typical chromatogram of GlcN. A: blank, B: GlcN standard, and C: GlcN sample from mushroom.

4.2.2.3 Stability after derivatization

The results (Figure 4.7) demonstrated that there were no significant differences ($\alpha = 0.05$) among the peak areas at zero time and after 7, 14, and 30 day stored at 4°C, indicating GlcN derivative was stable for at least 30 days when stored at 4°C.

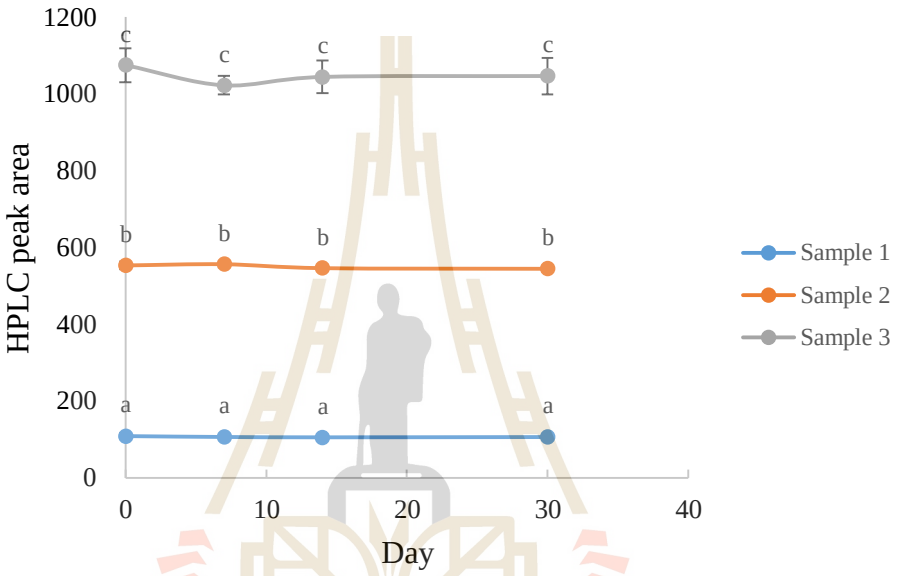


Figure 4.7 HPLC peak areas of GlcN derivatives during 30-day storage at 4°C. Sample 1, 2, and 3, which were prepared from GlcN standard, represented samples with low, middle, and high GlcN concentration. Values followed by different letters indicate significant differences ($p < 0.05$) between samples when analyzed by one-way ANOVA and post-hoc Tukey's HSD test.

4.2.3 Characterization of glucosamine

TLC was used to characterize GlcN in standard solution and samples from this study. GlcN formed a narrow, flat, red, band-shaped zone with an R_f value of 0.4 on the silica gel developed with 1-butanol-glacial acetic acid-DI water (3:1:1) mobile phase (Figure 4.8). The calculated R_f value is same as the previous study (Mojarrad et al., 2007).

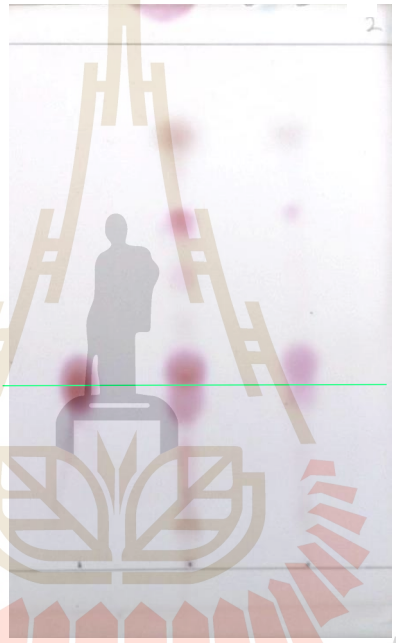


Figure 4.8 TLC chromatogram of GlcN. Line 1: GlcN standard; line 2 and line 3: GlcN samples from oyster mushroom.

4.2.4 Important parameters screening of acid hydrolysis

4.2.4.1 Comparison of the efficiency of hydrochloric acid and sulfuric acid for glucosamine production

Most of the glucosamine was commercially prepared from the exoskeletons of shellfish or other marine resources. Concentrated hydrochloric acid was usually employed to hydrolyze chitin extracted from crab and shrimp shells (Sitanggang, Wu, Wang and Ho, 2010). The hydrolysis is usually performed by 2.5-10 M hydrochloric acid at 80-140°C for 1-23 h (Wu, Zivanovic, Draughon and Sams, 2004, Zhu, Cai, Yang and Su, 2005). However, this method becomes limited by its low glucosamine yield (below 65%) (Sashiwa et al., 2002). On the other hand, sulfuric acid is widely used for the hydrolysis of cellulosic materials and analysis of total sugars in impure samples (Bondeson, Mathew and Oksman, 2006, Herrera et al., 2004, Lavarack, Griffin and Rodman, 2002, Meinita, Hong and Jeong, 2012, Ruiz and Date, 1996).

In order to investigate the efficiency of sulfuric acid hydrolysis for GlcN production, the GlcN yields of five mushrooms by hydrochloric acid and sulfuric acid hydrolysis were compared. The acid hydrolysis conditions were as follows: ratio of raw material to acid volume 1:10, hydrolysis temperature 80°C, time 8 h, and 6M HCl/H_2SO_4. Five ground mushroom samples (enoki mushroom, oyster mushroom, straw mushroom, shiitake, and wood ear mushroom) were used as the raw materials. Shrimp waste sample was used as the control to confirm the efficiency of sulfuric acid hydrolysis.

Table 4.5 The GlcN yields of five mushrooms and shrimp shell waste sample.

Raw material	GlcN yield (mg/g DM)[a]	
	HCl hydrolysis	**H_2SO_4 hydrolysis**
Enoki mushroom	6.55 ± 0.36	13.69 ± 2.09
Oyster mushroom	5.93 ± 0.84	14.94 ± 4.72
Straw mushroom	7.25 ± 1.87	29.36 ± 3.81
Shiitake	11.95 ± 1.19	25.63 ± 1.00
Wood ear mushroom	4.09 ± 0.46	8.74 ± 0.81
Shrimp shell waste	4.65 ± 1.38	75.62 ± 3.16

[a] Average of three determinations.

Acid hydrolysis conditions: ratio of raw material to acid volume 1:10, hydrolysis temperature 80°C, time 8 h, and 6M HCl/H_2SO_4.

The GlcN yield obtained from sulfuric acid hydrolysis (75.6188±3.1626 mg/g DM) was numerically higher than the value from hydrochloric acid (4.6493±1.3754 mg/g DM). Similar effect was observed using the mushrooms as the GlcN yields of five mushrooms by sulfuric acid hydrolysis were numerically higher than that of hydrochloric acid (2.09-4.05 times) (Table 4.5). To test the hypothesis that GlcN yield from sulfuric acid hydrolysis (n = 6, M = 27.9988, SD = 24.5809) were statistically higher than that from hydrochloric acid hydrolysis (n = 6, M = 6.7367, SD = 2.8095), an independent *t* test was conducted. The assumption of homogeneity of variances was test and satisfied via Levene's *F* test, F (10) = 4.335, p = 0.064. The GlcN yield from sulfuric acid hydrolysis were statistically significant higher than that from hydrochloric acid hydrolysis, t (10) = 2.105, p = 0.031 (one-tailed). This indicates that sulfuric acid has stronger ability than hydrochloric acid to convert chitin into GlcN

from five mushrooms. These findings are similar to the previous studies that sulfuric acid is more efficient for polymer degradation (Lavarack, Griffin and Rodman, 2002, Meinita, Hong and Jeong, 2012, Yoshioka, Motoki and Okuwaki, 2001). Ravens (1960) explained that the hydrolysis of poly (ethylene terephthalate) (PET) is principally determined by the solubility of PET in hydrochloric acid. In this study, the higher GlcN yield from sulfuric acid hydrolysis could be due to the quicker dissolution rate and higher solubility of chitin in sulfuric acid. In the study of Lavarack, Griffin and Rodman (2002), sulfuric acid was found to be more active for the formation of xylose from sugarcane bagasse hemicellulose compared to hydrochloric acid. Similarly, Meinita, Hong and Jeong (2012) found that sulfuric acid was more active at hydrolyzing *Kappaphycus alvarezii*. Conversely, Herrera et al. (2004) reported that hydrochloric acid was more powerful as the catalyst in the hydrolysis of sorghum straw than sulfuric acid. Therefore, the raw material type played an important role in the efficiency of acid hydrolysis. In this study, sulfuric acid was found to be more efficient than hydrochloric acid as the catalyst for the production of glucosamine from five mushrooms.

4.2.4.2 Selection of important parameters in sulfuric acid hydrolysis

There are several parameters which affect the GlcN yield by sulfuric acid hydrolysis, i.e., raw material form, acid concentration, ratio of raw material to acid volume, hydrolysis temperature and time. The important parameters of sulfuric acid hydrolysis were screened by OVAT using oyster mushroom as the raw material.

From the analysis, the raw material form affected the GlcN yield significantly. Moisture content was found to be a critical factor since the high moisture content of

fresh oyster mushroom led to extreme low GlcN yield. This result suggested that dried sample is more suitable for GlcN production. Then, the effect of sulfuric acid concentration, ratio of raw material to acid volume, hydrolysis temperature, and time were investigated (Figure 4.9). A one-way ANOVA was used to determine if there is a significant difference in GlcN yield from different level of the dependent variables (sulfuric acid concentration, ratio of raw material to acid volume, hydrolysis temperature, and time), while post-hoc Tukey's HSD test was used to evaluate where differences exist.

As shown in Figure 4.9 (1), the GlcN yield increased when the sulfuric acid concentration was increased from 1 to 6 M. A further increase in sulfuric acid concentration (6M to 9M) did not enhance the yield of GlcN. On the other hand, there is no significant difference between different ratios of raw material to acid volume (Figure 4.9 (2)). Hydrolysis temperature had an important impact on GlcN yield. The GlcN yield was increased when the hydrolysis temperature increased. When the temperature was increased from 90°C to 110°C, there is no significant improvement of GlcN yield (Figure 4.9 (3)). To investigate the effect of hydrolysis time on GlcN yield, the GlcN yield was monitored in 48h. It was identified that the acid hydrolysis was finished in 6h, after, no significant change of the GlcN yield was found (Figure 4.9 (4)).

From the results of OVAT, dried oyster mushroom had higher GlcN yield than the fresh one. The other three important parameters: hydrolysis temperature, time and acid concentration were selected for further optimization of the GlcN production by sulfuric acid hydrolysis.

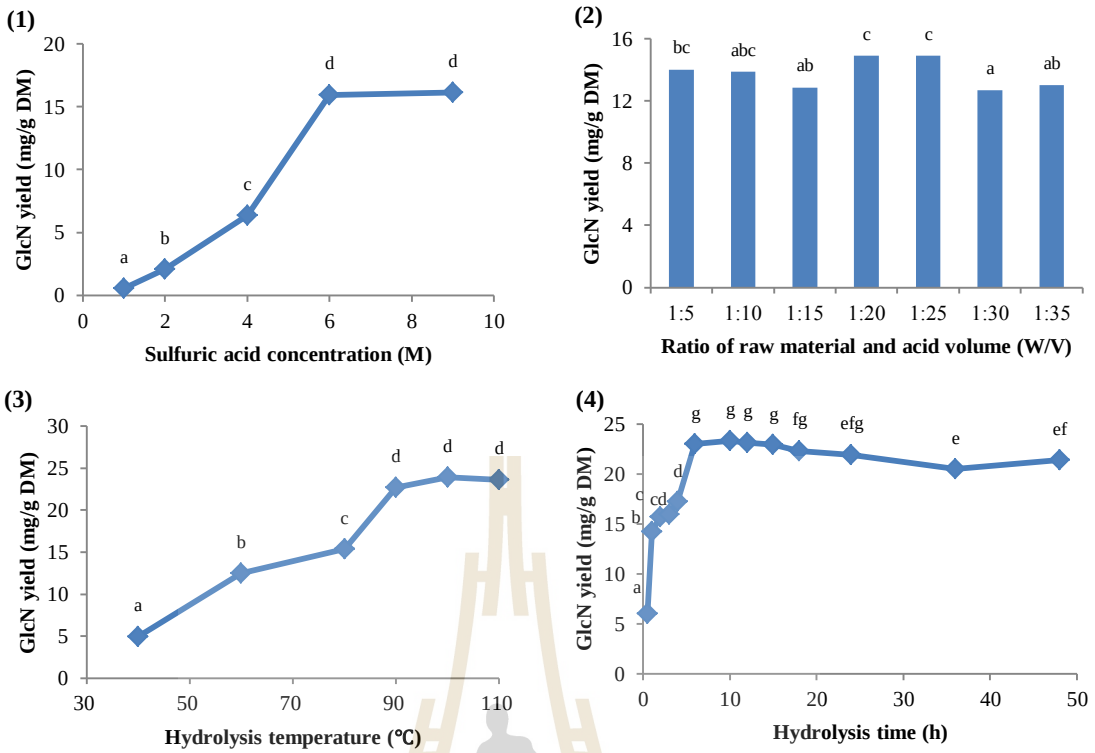


Figure 4.9 The effect of sulfuric acid concentration, ratio of raw material to acid volume, hydrolysis temperature, and time on GlcN yield. (1): sulfuric acid concentration; (2): ratio of raw material to acid volume; (3): hydrolysis temperature; (4): hydrolysis time. Shared letters indicate means that are not significant different ($p < 0.05$) by one-way ANOVA with post-hoc Tukey's HSD.

4.2.5 Optimization of acid hydrolysis conditions

4.2.5.1 BBD analysis

In order to study the combined effect of independent variables (hydrolysis temperature, time and acid concentration) on the GlcN yield, statistically designed experiments with different combinations of variables were performed. The results are shown in Table 4.6 that includes the experimental design, the observed and predicted values.

Model adequacy was checked based on the experimental data to determine whether the approximating model would give poor or misleading results. Four high degree polynomial models (linear, interactive, quadratic and cubic) were fitted to the experimental data. Three different tests (the sequential model sum of squares, lack of fit tests and model summary statistics) were carried out to conclude about the adequacy of models among various models to represent the maximum GlcN yield and the results are exhibited in Table 4.7. “Sequential model sum of squares” was used to select the highest order polynomial where the additional terms are significant and the model is not aliased. “Lack of fit tests” wanted the selected model to have insignificant lack-of-fit. “Model summary statistics” focused on the model with the maximum R_a^2 and R_p^2. From Table 4.7, Quadratic model was found to have maximum R^2, R_a^2, $R_p^2$and also exhibited low p-values. The quadratic model is found to be the most suitable model for the GlcN production from oyster mushroom. The adequacy of model was further justified through ANOVA.

Table 4.6 Box-Behnken design matrix of three variables with the observed and predicted response.

Run no.	Hydrolysis temperature (X_1, °C)	Time (X_2, h)	Acid concentration (X_3, M)	GlcN yield (Y, mg/g DM)		Residual error	%Error	Absolute error
				Observed	Predicted			
1	-1 (70)	-1 (2)	0 (6)	15.0535 ± 0.3266	16.1150	-1.1615	-7.0515	1.1615
2	1 (110)	-1 (2)	0 (6)	23.1161 ± 0.6573	23.2950	-0.1789	-0.7739	0.1789
3	-1 (70)	1 (6)	0 (6)	15.8825 ± 0.1335	15.7050	0.1775	1.1176	0.1775
4	1 (110)	1 (6)	0 (6)	24.6634 ± 1.2014	23.6050	1.0584	4.2914	1.0584
5	-1 (70)	0 (4)	-1 (3)	8.4325 ± 0.0515	8.0300	0.4025	4.7732	0.4025
6	1 (110)	0 (4)	-1 (3)	19.9385 ± 0.3303	20.4100	-0.4715	-2.3648	0.4715
7	-1 (70)	0 (4)	1 (9)	11.9814 ± 0.0822	11.5100	0.4714	3.9344	0.4714
8	1 (110)	0 (4)	1 (9)	13.8207 ± 0.3589	14.2100	-0.3893	-2.8168	0.3893
9	0 (90)	-1 (2)	-1 (3)	16.5233 ± 1.0361	15.8640	0.6593	3.9901	0.6593
10	0 (90)	1 (6)	-1 (3)	15.3767 ± 1.5783	15.9560	-0.5793	-3.7674	0.5793
11	0 (90)	-1 (2)	1 (9)	15.2313 ± 0.0330	14.6460	0.5853	3.8427	0.5853
12	0 (90)	1 (6)	1 (9)	13.7995 ± 0.3174	14.4540	-0.6545	-4.7429	0.6545
13	0 (90)	0 (4)	0 (6)	25.9933 ± 0.8473	26.150	-0.1567	-0.6028	0.1567
14	0 (90)	0 (4)	0 (6)	26.1036 ± 0.9625	26.1500	-0.0464	-0.1778	0.0464
15	0 (90)	0 (4)	0 (6)	25.3929 ± 0.8576	26.1500	-0.7571	-2.9815	0.7571
16	0 (90)	0 (4)	0 (6)	26.9242 ± 0.7754	26.1500	0.7742	2.8755	0.7742
17	0 (90)	0 (4)	0 (6)	26.3427 ± 0.9025	26.1500	0.1927	0.7315	0.1927

Table 4.7 Sequential model fitting for the yield of GlcN.

Source	Sum of squares	DF	Mean square	*F* value	Prob > *F*	Remarks
Sequential model sum of squares						
Mean	6197.04	1	6197.04			
Linear	117.62	3	39.21	1.10	0.3836	
Interactive	23.51	3	7.84	0.18	0.9086	
Quadratic	433.28	3	144.43	172.94	< 0.0001	Suggested
Cubic	4.61	3	1.54	4.97	0.0777	Aliased
Residual	1.24	4	0.31	-	-	
Total	6777.30	17	398.66	-	-	
Lack of fit tests						
Linear	461.40	9	51.27	165.86	< 0.0001	
Interactive	437.89	6	72.98	236.11	< 0.0001	
Quadratic	4.61	3	1.54	4.97	0.0777	Suggested
Cubic	0.000	0	-	-	-	Aliased
Pure error	1.24	4	0.31	-	-	
Model summary statistics						
Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	Press	Remarks
Linear	5.97	0.2027	0.0187	-0.2529	726.98	
Interactive	6.63	0.2432	-0.2108	-1.1585	1252.48	
Quadratic	0.91	0.9899	0.9770	0.8696	75.69	Suggested
Cubic	0.56	0.9979	0.9915	-	-	Aliased

4.2.5.2 Fitting of second order polynomial equation

The mathematical model representing the GlcN yield as a function of the independent variables within the region under investigation was expressed by a second-order polynomial equation. The final equation obtained in terms of coded factors is given below (Eq. (4.1)):

$$Y = 26.15 + 3.77X_1 - 0.025X_2 - 0.68X_3 + 0.18X_1X_2 - 2.42X_1X_3 - 0.071X_2X_3 - 4.08X_1^2 - 2.39X_2^2 - 8.53X_3^2 \quad (4.1)$$

4.2.5.3 BBD statistical analysis

ANOVA is a statistical technique that subdivides the total variation in a set of data into component parts associated with specific sources of variation for the purpose of testing hypotheses on the parameters of the model. The significance and fitness of the model was checked and the significance of the regression coefficients were evaluated by their corresponding *p*-values is presented in Table 4.8.

In general, exploration and optimization of a fitted response surface may produce poor or misleading results unless the model exhibits a good fit, so it is essential to check the model's adequacy. The Model *F*-value of 76.42 implies that the model is significant at $p < 0.0001$ and adequate to represent the actual relationship between the response (GlcN yield) and the significant variables. The "Lack of Fit *F*-value" of 4.97 and the associated *p*-value of 0.0777 imply the Lack of Fit is insignificant due to relative pure error. In this study, the *p*-value is less than 0.05 indicating the significance of model terms. From the *p*-values of each model terms, it could be concluded that one linear coefficient (X_1), three quadratic coefficients (X_1^2, X_2^2 and X_3^2) and one interactive coefficient (X_1X_3) were significant with small *p*-values and indicate the pattern of the interactions between the variables.

Table 4.8 ANOVA of the regression model for the prediction of GlcN yield.

Source	Coefficient estimate	SS[a)]	DF[b)]	Standard error	MS[c)]	*F*-value	*p*-value
Model	26.15	574.41	9	0.41	63.82	76.42	< 0.0001
X_1	3.77	113.92	1	0.32	113.92	136.41	< 0.0001
X_2	-0.025	5.106E-003	1	0.32	5.106E-003	6.113E-003	0.9399
X_3	-0.68	3.70	1	0.32	3.70	4.43	0.0734
X_1X_2	0.18	0.13	1	0.46	0.13	0.15	0.7060
X_1X_3	-2.42	23.36	1	0.46	23.36	27.97	0.0011
X_2X_3	-0.071	0.020	1	0.46	0.020	0.024	0.8804
X_1^2	-4.08	70.12	1	0.45	70.12	83.96	< 0.0001
X_2^2	-2.39	24.08	1	0.45	24.08	28.84	0.0010
X_3^2	-8.53	306.15	1	0.45	306.15	366.59	< 0.0001
Residual (error)		5.85	7		0.84		
Lack of fit		4.61	3		1.54	4.97	0.0777
Pure error		1.24	4		0.31		
Cor Total		580.26	16				
Adeq. Prec.	25.850						

[a)] SS, sum of squares.

[b)] DF, degrees of freedom.

[c)] MS, mean square.

The coefficient of determination (R^2) is defined as the ratio of the explained variation to the total variation and is a measurement of the degree of fitness. The small value of R^2 indicates the poor relevance of the dependent variable in the model. The model can fit the actual data well and analyze the trends in the responses when R^2 approaches unity. The R^2 (0.9899) value of the quadratic regression model indicates that only 0.0101% of the total variations was not explained by the model and the regression model well defined the true behavior of the system. The goodness of fit of the model was further evaluated by R, R_a^2, R_p^2 and CV. The value of R_a^2 (0.9770) is very high and close to the R^2 confirming that the model was highly significant. The R_a^2 and R_p^2 should be within approximately 0.20 of each other to be in reasonable agreement. If they are not, there may be a problem with either the data or the model. In our case, the R_p^2 of 0.8696 is in reasonable agreement with the R_a^2 of 0.9770. Moreover, a very high value of the correlation coefficient (R = 0.9949) exhibited an excellent correlation between the experimental and predicted response values. The low CV (4.79%) clearly indicated that the deviations between experimental and predicted values are low and showed a good precision and reliability of the experiments carried out.

Adequate precision is a measure of the range in predicted response relative to its associated error, in other words a signal-to-noise ratio (Mourabet et al., 2012). Its desired value is 4 or more. The ratio was found to be > 25, which indicates an adequate signal and confirms that, this model is significant for this GlcN production process.

Mallow's C_p statistic (Eq. (4.2)) can be used to determine how many terms can be omitted from the response surface model (Yetilmezsoy, Demirel and Vanderbei, 2009). For a response surface model including all terms, $C_p = p$, where p is the number of parameters or variables in the regression model including the intercept term. For

response surface models with omitted terms, $C_p \sim p$ indicates a good model with little bias, and $C_p \leq p$ indicates a very good prediction model. The goal is to remove terms from the response surface model until a minimum C_p value near p is obtained. If $C_p > p$, this indicates that too many terms have been removed or some remaining terms are not necessary. In our case, Mallow's C_p statistic (C_p = 10) indicated the third condition ($C_p \leq p$ and p = 10 including β_0, β_1, $\cdots$, β_3^2), showing a very good prediction model.

$$C_p = \frac{SS_E}{MSS_E} + 2p - n \tag{4.2}$$

The autocorrelation between errors in the model and linear association between adjacent residuals was evaluated by Durbin-Watson (DW) statistic (Eq. (4.3)) method. The DW statistic range varies between 0 and 4. The DW value below 2 indicates positive autocorrelation and above 2 indicates negative autocorrelation (Khajeh, 2011). Our study shows that, the DW value of 2.3059 is close to 2, indicating a good of fit of the model.

$$\mathrm{W} = \frac{\sum_{i=2}^{n}(e_i - e_{i-1})^2}{\sum_{i=1}^{n} e_i^2} \tag{4.3}$$

The chi-square (χ^2) test (Eq. (4.4)) was used to check the significant difference between the experimental and predicted values. In our study, the calculated χ^2 value (0.3325) was found to be less than the tabulated value (26.296), exhibiting that there was no significant difference between predicted and experimental data.

$$\chi_{cal}^2 = \frac{\sum_{i=1}^{n}(O_i - E_i)^2}{E_i} \tag{4.4}$$

Bias (Eq. (4.5)) is an estimator used to find out the normal distribution of errors between experimental and predicted value. In our study, bias value of 1.0007 indicted that, the errors are normally distributed and shows the good fit of the model.

$$\text{Bias} = \exp\left(\frac{1}{n}\sum_{i=1}^{n} ln\frac{Y_O}{Y_p}\right) \quad (4.5)$$

4.2.5.4 Diagnostics of model adequacy

Model adequacy checking was performed to determine whether the approximating model would give poor or misleading results. Figure 4.10 shows the residual and the influence plots for the experimental data obtained from this study. Raw residuals, which cannot be explained by the model, represent the deviations between experimental and predicted values. For these, Shapiro-Wilk (*W*) normality test was carried out and normality test gave an insignificant value of W statistics ($W = 0.977$, $p = 0.9$), indicating model predict very well for GlcN production from oyster mushroom.

The predicted values obtained were quite close to the experimental values, and the points of all predicted and experimental response values fall very close to the 45^o line (Figure 4.10a), indicating that the model developed was successful in capturing the correlation between the process variables on the response. Figure 4.10b shows the normal% probability plot of residuals for response was normally distributed, as they lie reasonably close on a straight line and shows no deviation of the variance. The good fit of the model was analyzed by constructing the internally studentized residuals versus experimental runs and shows that all the data points lay within the limits (Figure 4.10c). Since all leverage values were less than 1 (Figure 4.10d), there are no outliers or unexpected errors in the model. However, difference in beta values plot (Figure 4.10e) showed no undue influence of any observation on any of the regression coefficients. Since the Cook's distance values are in the determined range (Figure 4.10f), there is no strong evidence of influential observations in experimental data.

Hence, no obvious patterns were found in the analysis of model and indicated the accuracy of the developed model.

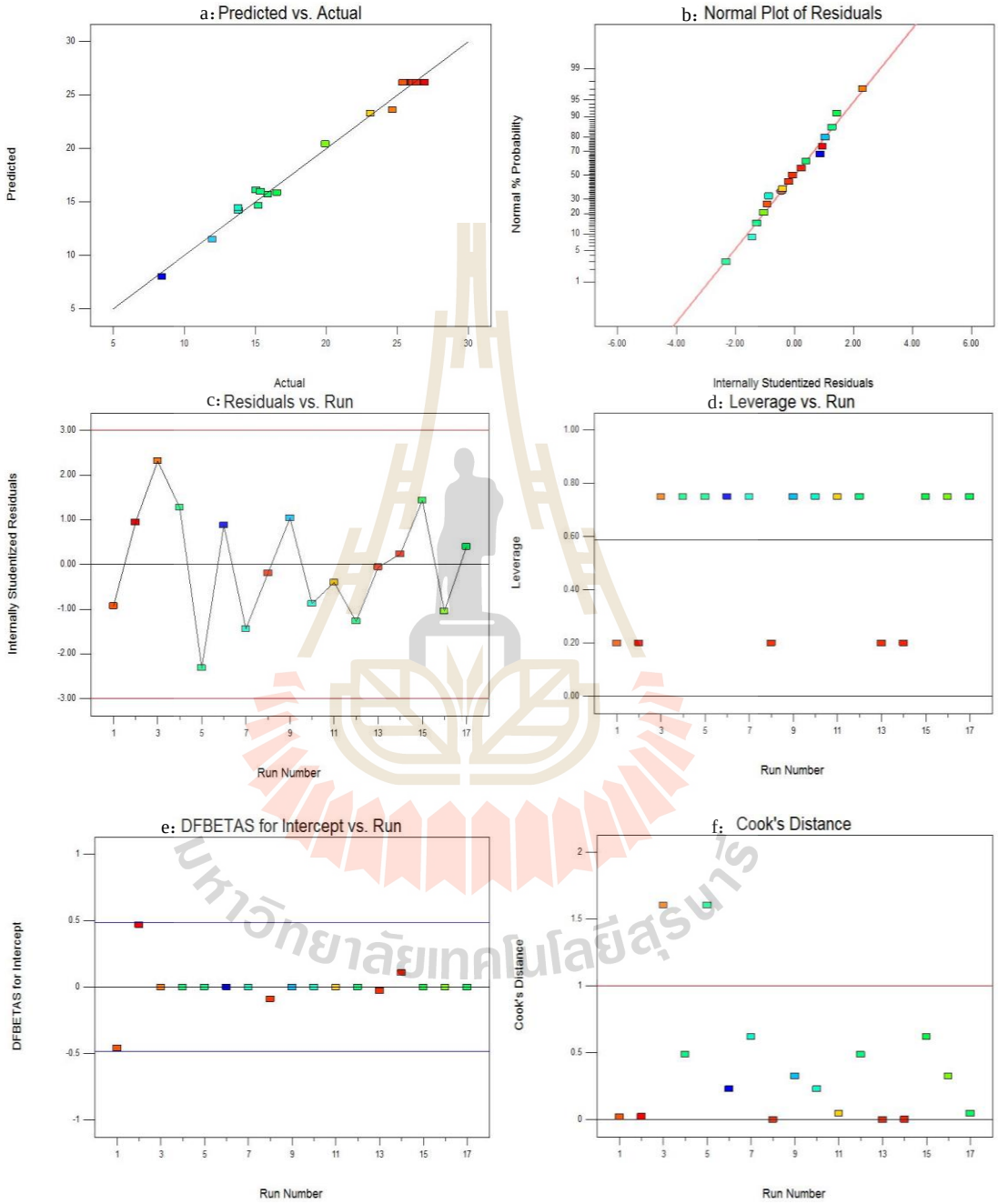


Figure 4.10 Diagnostic plots for the model adequacy.

4.2.5.5 Percentage contribution of process variables

Based on the sum of squares obtained from the ANOVA, the percentage contributions (PC) for each individual process variables were calculated and indicated the percentage contributions of process variables on the response. The quadratic terms showed the highest level of contribution (73.94%) on the TPC compared with the other terms and this was followed by the linear terms (21.72%). Among the all terms, the interactive terms exhibited the lowest level of significance (4.34%) and did not showed a large effect in prediction of the GlcN yield. Hence, TPC values proved that, the quadratic independent variables have a direct relationship on the dependent variable.

4.2.5.6 Effect of process variables on glucosamine yield

The second-order polynomial regression model resulted in three response surface graphs for production of GlcN. Interpretation of response surface model and contour plots are the graphical representations of regression equation. They provide visual interpretation of the relationship between responses and experimental levels of each variable and the type of interactions between two test variables. The maximum predicted values were indicated by the surface confined in the smallest ellipse in the contour diagram. The shapes of the contour plots, circular or elliptical, indicated whether the reciprocal interactions between the variables are significant or not. The circular contour plots indicated the interactions between corresponding variables are negligible, while the elliptical contour plots indicating the interactions between corresponding variables are significant (Ahmad, Alkharfy, Wani and Raish, 2015).

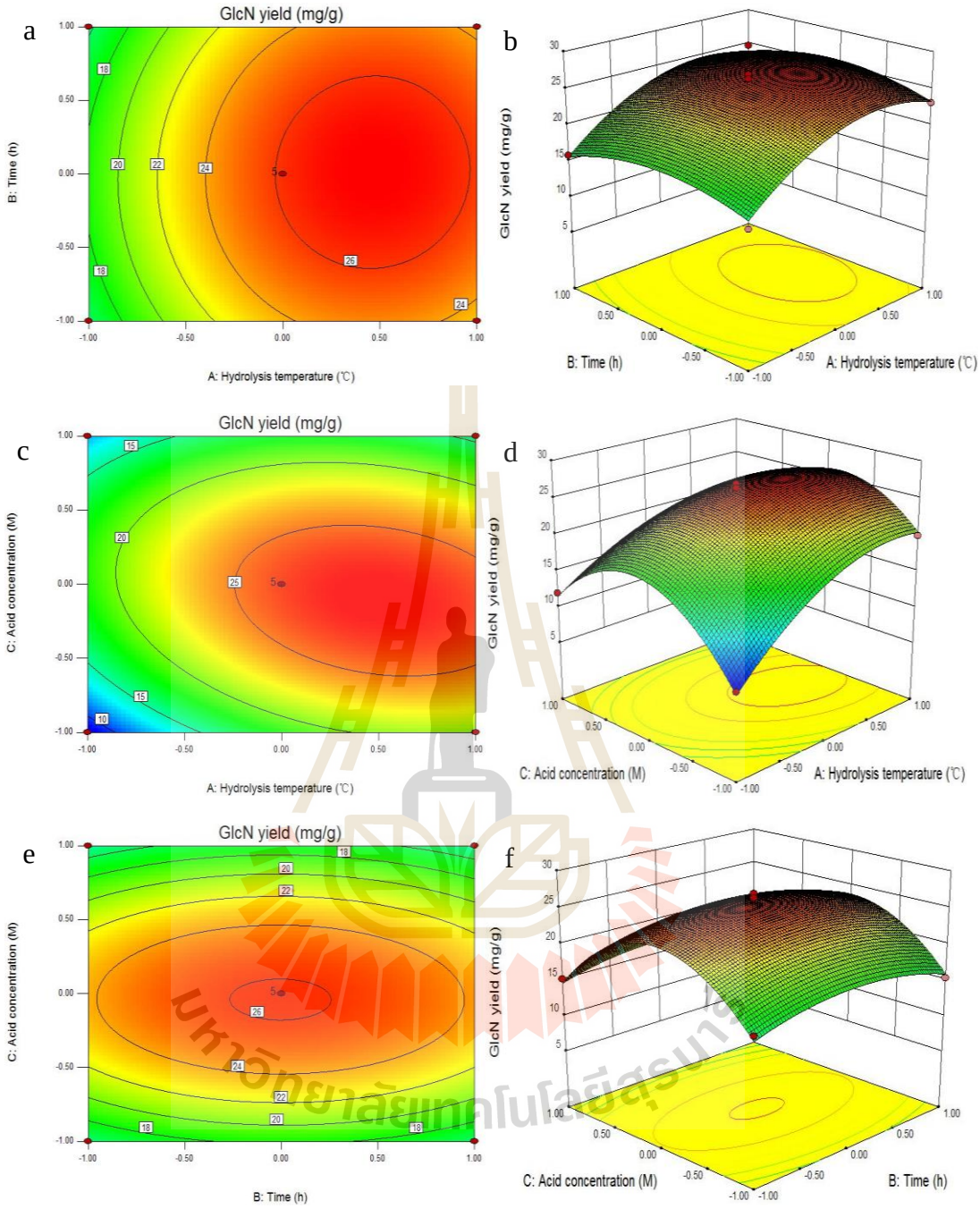


Figure 4.11 The effect of parameters on GlcN yield using contour plot and response surface plot. a and b: hydrolysis temperature *vs*. time; c and d: hydrolysis temperature *vs*. acid concentration; e and f: time *vs*. acid concentration.

These graphs were drawn by imposing two other variables at their zero level, which were shown in Figure 4.11. It was observed that there was a relative significant interaction between every two variables, and there was a maximum predicted yield as indicated by the surface confined in the smallest ellipse in the contour diagrams.

Figure 4.11 a and b represent the effects of hydrolysis temperature and time on the yield of GlcN. Hydrolysis temperature had positive effects on the response. It can be seen that by increasing the hydrolysis temperature GlcN yield increased as well, reached a maximum value, and the further increase had slight effect. Figure 4.11 c and d depict the interaction effect of hydrolysis temperature and acid concentration on the yield. When the hydrolysis temperature was increased from 70°C to 100°C, the yield ascended greatly with the enhanced acid concentration. The effects of hydrolysis time and acid concentration on the yield of GlcN could be seen in Figure 4.11 e and f. As the hydrolysis time reached to a high level, the yield increased with a rising of acid concentration. Therefore, a slightly higher temperature was required to achieve maximum increase.

4.2.5.7 Optimization and verification of the model

Derringer's desired function methodology was employed to optimize the sulfuric acid hydrolysis conditions on the maximum production of GlcN from oyster mushroom as follows: hydrolysis temperature of 100°C, time of 4.03 h and sulfuric acid concentration of 5.67 M. Under these conditions, the predicted GlcN yield was 27.1231 mg/g with a desirability value of 0.980. A desirability ramp was developed from optimal points via numerical optimization technique (Figure 4.12). For their validation of the optimum conditions, triplicate confirmatory experiments were carried out under the optimized conditions and the average GlcN yield was

26.9934 ± 0.4159 mg/g. The results are closely related with the data obtained from optimization analysis using desirability functions, indicating Box-Behnken design incorporate with desirability function could be effectively used to optimize the production parameters on GlcN.

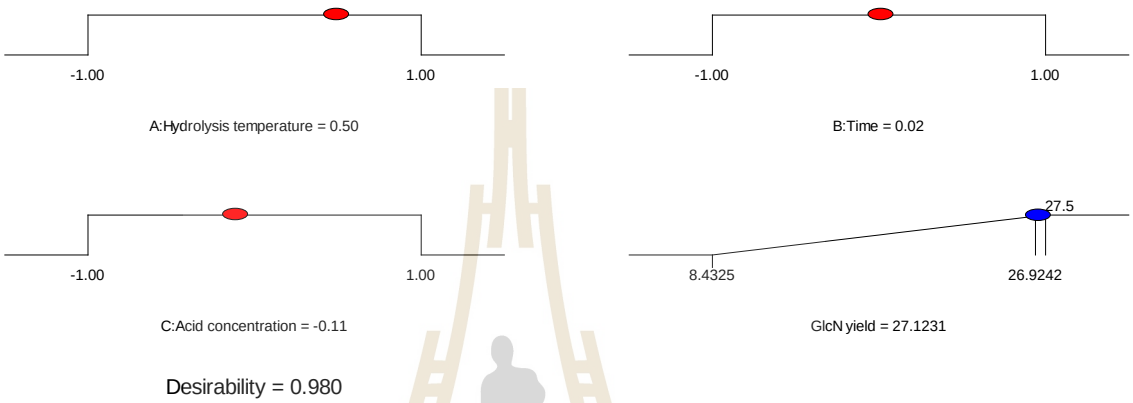


Figure 4.12 Desirability ramp for optimization.

4.2.6 Production of glucosamine from five mushrooms by sulfuric acid hydrolysis

Under the optimum conditions obtained from Section 4.2.5.7, other four mushrooms were used to produce GlcN (Table 4.9). From the comparison between the GlcN yield by sulfuric acid hydrolysis before and after BBD optimization, the latter GlcN yield was numerically higher than the former (1.41-1.93 times). A paired sample *t* test was conducted to evaluate whether a statistically significant difference existed between the mean GlcN yield before and after BBD optimization. The results were significant, t (4) = 3.43, p = 0.027, indicating that there is a significant increase in GlcN yield by sulfuric acid hydrolysis before and after BBD optimization. The mean increase was 13.30 with the 95% confidence interval for the difference between the means of 2.53 to 24.07. This indicates that the efficiency of sulfuric acid for GlcN

production improved greatly through BBD optimization. On the other hand, the different GlcN yield from different mushroom may be caused by mushroom strains, cultivation conditions, pretreatment among others (Jiang et al., 2010, Kalač, 2009, Zivanovic and Buescher, 2004). Among the five mushroom used in this study, the results from one-way ANOVA with post-hoc Tukey's HSD test revealed that straw mushroom had the highest GlcN yield (56.8132 ± 3.1671 mg/g DM). It indicated that straw mushroom had the highest chitin content among these five mushrooms and it could be considered as a better source of chitin for the production of GlcN. Moreover, the relatively high conversion (greater than 95%) implied sulfuric acid hydrolysis was an efficiency method for GlcN production.

The choice of suitable acid and control of hydrolysis conditions are crucial for the production of GlcN. Our results indicating that the chitin content of mushroom was a crucial factor for GlcN yield. As compared to crustacean shells, the traditional and current commercial source of GlcN, mushrooms exhibited lower GlcN yields which are attributed to the difference in chitin contents of raw materials. However, the supply of crustacean shells can be limited. On the other hand, mushrooms can be cultivated throughout the year in a short period without geographical and seasonal restrictions. Additionally, mushrooms are relatively consistent in composition and are not associated with inorganic materials; thus, no demineralization treatment is required and the heavy metal hazard can be avoided (Mario, Rapanà, Tomati and Galli, 2008). Moreover, GlcN from mushrooms is suitable for vegetarians and shellfish-allergic consumers. Therefore, mushrooms' potential as an economical and low-cost source of GlcN should be encouraged.

Table 4.9 The chitin content, GlcN yield and conversion of five mushrooms.

Mushroom	Chitin content (mg/g DM)	H_2SO_4 hydrolysis [1]		H_2SO_4 hydrolysis [2]	
		GlcN yield (mg/g DM)	Conversion (%)	GlcN yield (mg/g DM)	Conversion (%)
Enoki mushroom	26.43 ± 1.2061	13.6947 ± 2.0872	51.81	25.2615 ± 1.2479 [b]	95.58
Oyster mushroom	27.03 ± 0.0070	14.9436 ± 4.7200	55.29	26.9934 ± 0.4778 [b]	99.86
Straw mushroom	57.83 ± 2.3227	29.3638 ± 3.8112	50.78	56.8132 ± 3.5748 [d]	98.24
Shiitake	38.52 ± 1.7264	25.632 ± 1.0002	66.54	37.4460 ± 1.1173 [c]	97.21
Wood ear mushroom	12.96 ± 1.0074	8.7396 ± 0.8128	67.44	12.3593 ± 1.1899 [a]	95.36

[1] Hydrolysis conditions: dry mushroom sample; ratio of raw material to acid volume, 1:10; sulfuric acid concentration, 6 M; hydrolysis temperature, 80°C; and time, 8h.

[2] Hydrolysis conditions: dry mushroom sample; ratio of raw material to acid volume, 1:10; sulfuric acid concentration, 5.67 M; hydrolysis temperature, 100°C; and time, 4.03 h.

Values in 5th column followed by different superscript letters indicate significant differences ($p < 0.05$) between samples when analyzed by one-way ANOVA and post-hoc Tukey's HSD test.

4.2.7 Kinetics of glucosamine production from different mushrooms

4.2.7.1 Effect of temperature on glucosamine yield

Ground mushroom sample was first dispersed in 5.67 M sulfuric acid solution and then hydrolyzed at three different temperatures. The GlcN concentration in the hydrolysate solution was analyzed. The GlcN yield was calculated and plotted against hydrolysis time. Figure 4.13 shows the GlcN yield during the hydrolysis process with respect to hydrolysis time at various temperatures.

As shown in Figure 4.13 a and b, using 5.67 M sulfuric acid, after 6 h of hydrolysis, chitosan/chitin in enoki mushroom and oyster mushroom were fully hydrolyzed into GlcN. For straw mushroom, maximum GlcN yield was observed in 7.5 h at 90°C, 7 h at 100°C, and 6.5 h at 110°C, respectively. These results indicated when increased the hydrolysis temperature, the reaction time should be shortened. The maximum GlcN yield of shiitake was obtained using 6 M sulfuric acid at 110°C in 6.5 h. Hydrolysis conditions of 6 M sulfuric acid, 110°C and 5 h were the optimum conditions for wood ear mushroom. Based on these results, the hydrolysis conditions selected in our study were suitable for GlcN production by sulfuric acid hydrolysis and the GlcN decomposition was avoided under these conditions.

4.2.7.2 Kinetics of glucosamine production

The initial stage of the hydrolysis was fitted with a first-order reaction model. $[A]_0$ was calculated based on the determined chitin content of each mushroom, and $[A]_t$ = the difference between original chitin concentration and liberated glucosamine at reaction time *t*. The plots of $\ln[A]_0 - \ln[A]$ *vs.* *t* at different temperatures were presented in Figure 4.14. The correlation coefficients are all above 0.9.

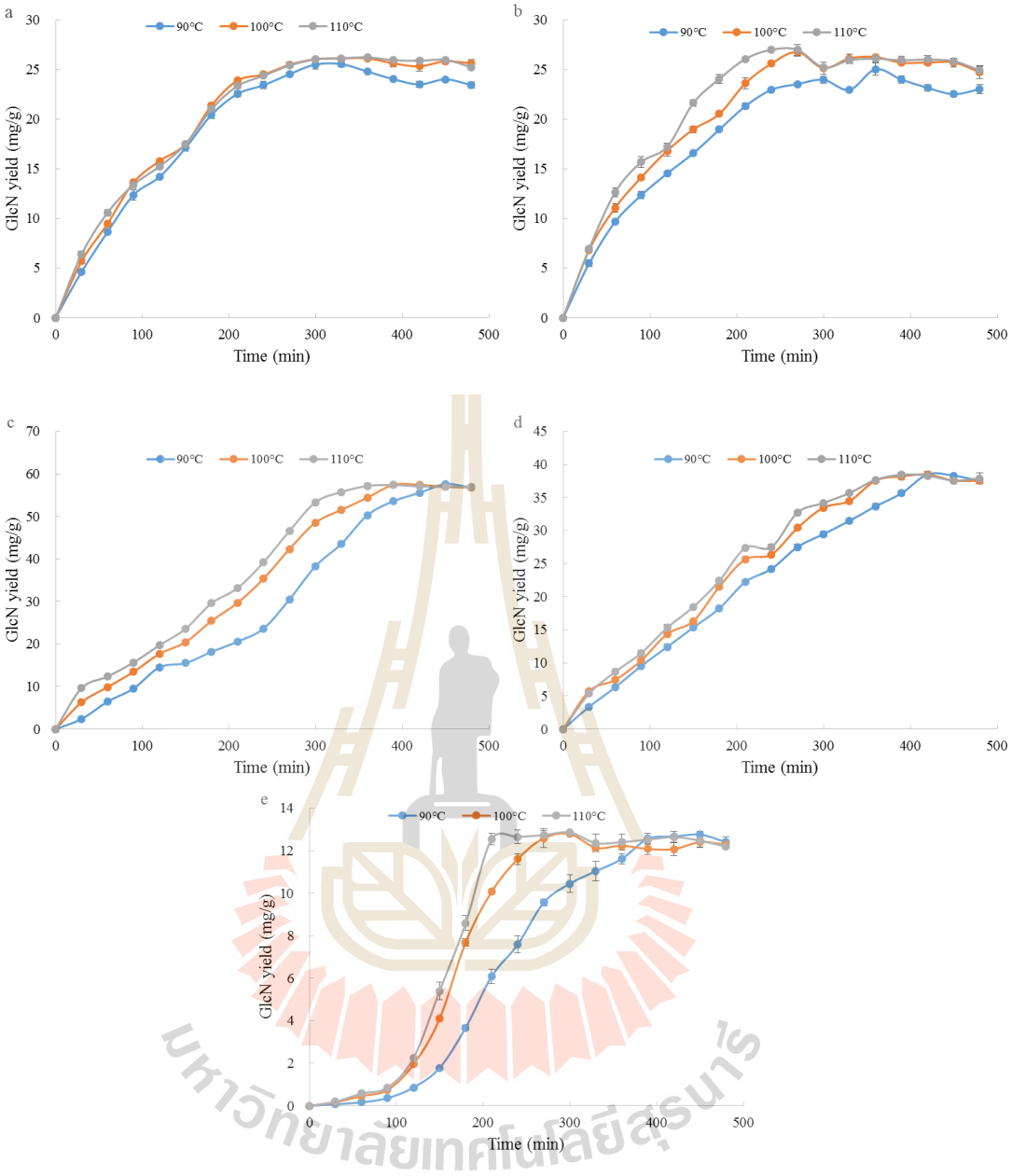


Figure 4.13 Effect of temperature on GlcN yield from mushrooms. a: enoki mushroom, b: oyster mushroom, c: straw mushroom, d: shiitake, e: wood ear mushroom.

As shown in Figure 4.14, using 5.67 M sulfuric acid, the effect of temperature on glucosamine yield was investigated and the temperature efficient (Q_{10}) were list in Table 4.10. There is a general trend of steeper slope with increased hydrolysis temperature, indicting the increasing reaction rate with increased temperature. For oyster mushroom, the Q_{10} factor was 1.2899 between 90°C to 100°C and the Q_{10} factor was 1.6180 between 100°C to 110°C indicating the temperature effect from 100°C to 110°C was more significant for glucosamine production. On the other hand, for enoki mushroom, straw mushroom, shiitake, and wood ear mushroom, the Q_{10} factors between 90°C to 100°C were higher than the values between 100°C to 110°C suggested that the glucosamine yields and the reaction rate raised greatly when the temperature increased from 90°C to 100°C, especially wood ear mushroom.

According to Arrhenius' equation, a plot of the natural logarithm of the rate constants as a function of the inverse of the absolute temperature (Arrhenius plot) can be drawn and the activation energy can be calculated from the slope of the line. The rate constant *k* and activation energy for glucosamine were calculated and summarized in Table 4.10. The activation energies were in the range of 15.91-203.55 kJ/mol. From our preliminary study, activation energy value of 80.35 kJ/mol has been determined for hydrochloric acid hydrolysis of oyster mushroom. This value was much higher than that of sulfuric acid hydrolysis, further confirming that the sulfuric acid was more effective than hydrochloric acid for hydrolysis of mushrooms. Hydrolysis of enoki mushroom exhibited the lowest activation energy, which may indicate that enoki mushroom was easier to hydrolyze than other four mushrooms under the hydrolysis conditions.

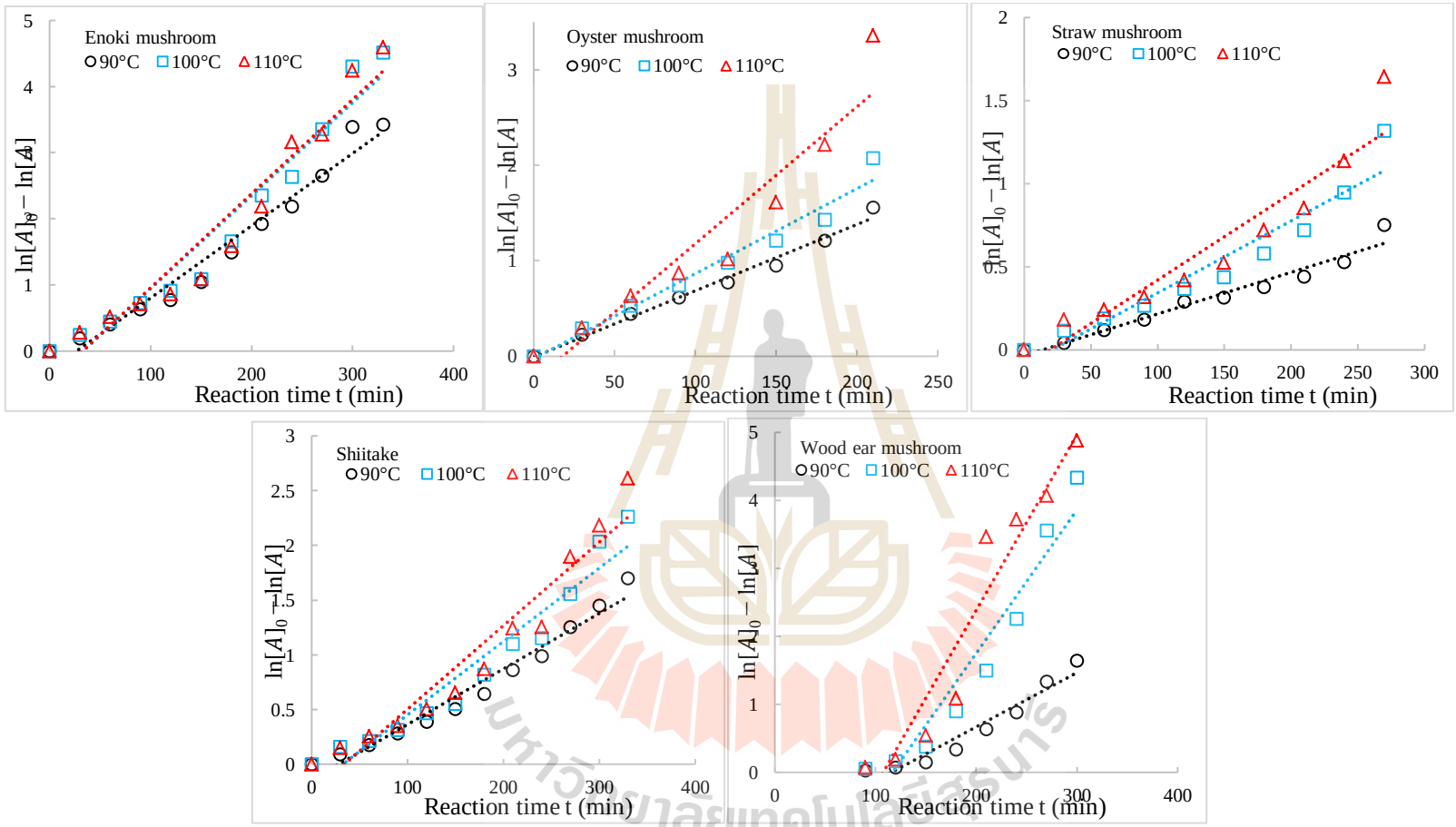


Figure 4.14 Plot of $ln[A]_0$-$ln[A]$ versus reaction time at different temperatures. Reaction conditions: 10 g mushroom sample and 100 mL 5.67 M sulfuric acid. $[A]_0$ and $[A]$ represent the chitin content at reaction time 0 and t, respectively.

Table 4.10 The kinetics parameters for mushroom hydrolysis.

Raw material	Temperature (°C)	K (1/min)	Q_{10}	E_a (kJ/mol)
Enoki mushroom	90	0.0109		15.91
	100	0.0141	1.2936	
	110	0.0143	1.0142	
Oyster mushroom	90	0.0069		42.66
	100	0.0089	1.2899	
	110	0.0144	1.6180	
Straw mushroom	90	0.0025		42.76
	100	0.0043	1.7200	
	110	0.0052	1.2093	
Shiitake	90	0.0051		23.27
	100	0.0067	1.3137	
	110	0.0076	1.1343	
Wood ear mushroom	90	0.0008		203.55
	100	0.0211	26.3750	
	110	0.0258	1.2227	

The activation energies for acid hydrolysis of four mushrooms, namely enoki mushroom, oyster mushroom, straw mushroom, and shiitake, were lower than the activation energy of 78 kJ/mol of chitosan reported by Yan and Evenocheck(2012) (Yan and Evenocheck, 2012). An activation energy value of 203.55 kJ/mol has been determined for hydrolysis of wood ear mushroom, showing a difficult tendency for glucosamine to be liberated. The value was higher than the activation energies for hydrolysis of chitin in hydrochloric acid (Vårum, Ottøy and Smidsrød, 2001, Yan and Evenocheck, 2012). Vårum, Ottøy and Smidsrød (2001) have reported the activation energies for acid hydrolysis of two almost fully de-N-acetylated chitosans were 152.2 and 158.1 kJ/mol, respectively. The activation energies for acid hydrolysis of two partially N-acetylated chitosans were also determined to be 130.4 and 134.3 kJ/mol, respectively. Moreover, at the beginning (up to 1.5 h) of the hydrolysis of wood ear mushroom, the reaction rate was almost zero. This phenomenon may be explained by

the higher amount of glucans and the strong bonding strength the chitin-glucan complex exist in wood ear mushroom (Ivshina, Artamonova, Ivshin and Sharnina, 2009).

4.2.7.3 Glucosamine decomposition

Due to the deamination of glucosamine during acid hydrolysis (Shabrukova, Shestakova, Zainetdinova and Gamayurova, 2002, Yan and Evenocheck, 2012), it is important to minimize the glucosamine decomposition to obtain an accurate result for kinetics study. To monitor the decomposition, glucosamine standard were weighed into digestion flasks and treated with the optimal hydrolysis condition (100°C, 5.67 M H_2SO_4). At the end of each half-hour interval, a sample was taken and analyzed for glucosamine. The results were shown in Figure 4.15.

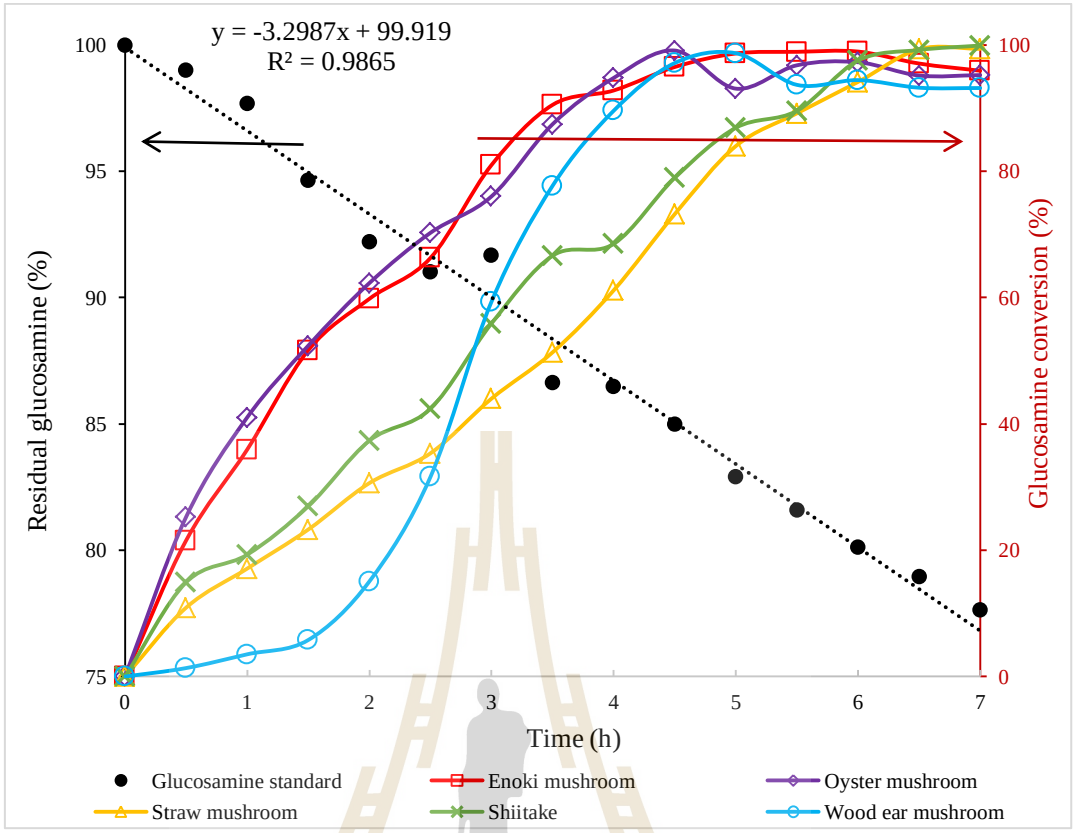


Figure 4.15 Stability of glucosamine standard and glucosamine conversion from five mushrooms under optimal hydrolysis conditions (100°C, 5.67 M H_2SO_4).

The degradation of glucosamine fits a zero-order decomposition model. About 15.01%, 19.86%, and 21.04% loss of glucosamine at hydrolysis time of 4.5, 6, and 6.5h, respectively. On the other hand, after 6 h hydrolysis, about 90% chitin in mushroom could be converted into glucosamine and a loss of 20% glucosamine might be observed according to the decomposition model. To minimize the glucosamine loss during hydrolysis, shorter hydrolysis time, higher temperature, or higher acid concentration might be employed. However, the decomposition kinetics during the mushroom hydrolysis process are complicated by the processes of chitosan depolymerization and deacetylation, and glucosamine generation. The glucosamine

decomposition during mushroom hydrolysis does not appear to have a significant impact. According to the decomposition model, 22% glucosamine was lost after 7 h hydrolysis. However, the glucosamine yields from five mushrooms were above 93%. Additionally, the glucosamine yields (Table 4.9) were greater than 95%, which were experimentally close to the chitin content of five mushrooms, demonstrating that the decomposition of glucosamine during mushroom hydrolysis at the optimal conditions has minimal impact on the accuracy of results.

4.3 Purification and identification of glucosamine and ACE inhibitory peptides

4.3.1 Purification of glucosamine and ACE inhibitory peptides

4.3.1.1 Decoloring process

Decoloring by adsorption using activated bleaching earth, activated carbon or silica-based products is widely used in the removal of pigments (Hameed, Ahmad and Aziz, 2007, Huang, Liu, Liu and Wang, 2007, Hussin, Aroua and Daud, 2011). Figure 4.16 show the removal of pigment from straw mushroom hydrolysate by various decoloring agents. Activated bleaching earth, activated carbon CGC 200C I2 900, activated carbon CGC 200C I2 1000, activated carbon CGC 200C I2 1100, and activated carbon CGC 200U I2 900 showed excellent decoloring efficiency for mushroom hydrolysate because of their high surface areas (powder form).

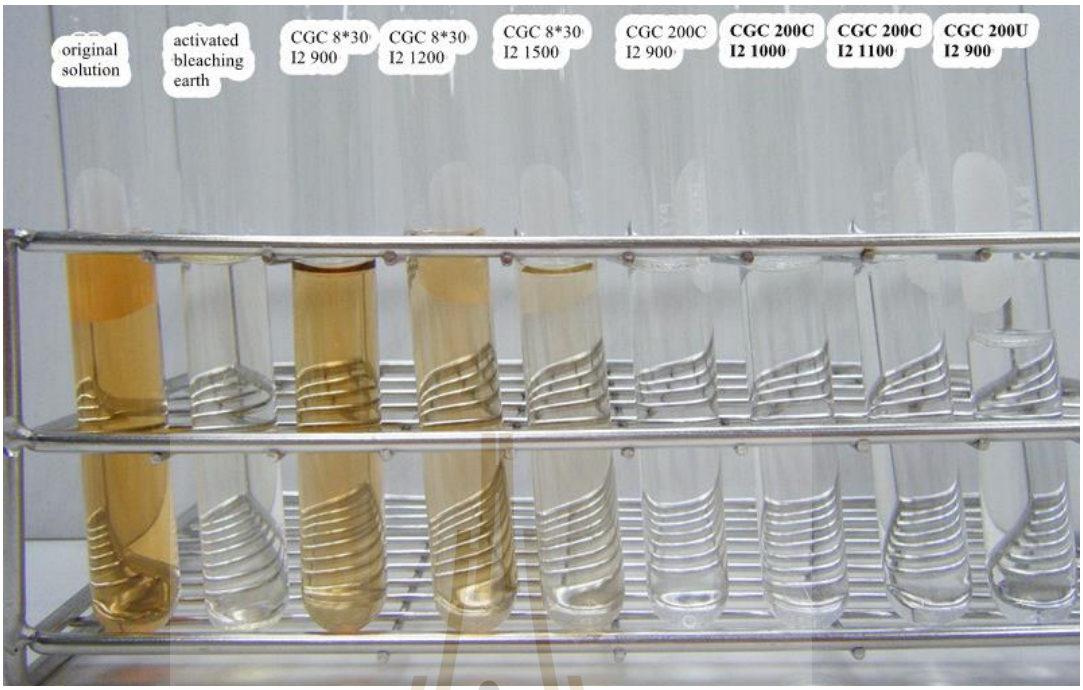


Figure 4.16 Straw mushroom hydrolysate after decoloring process by different decoloring agents.

On the other hand, the retention of target compounds in decoloring agent is a critical problem need to be considered. Table 4.11 summarized the GlcN yields of mushroom hydrolysates after decoloring process. Activated bleaching earth and activated carbon 8*30 I2 900 exhibited lower GlcN retention rate. Activated carbon CGC 200C I2 900, activated carbon CGC 200C I2 1000, activated carbon CGC 200C I2 1100, and activated carbon CGC 200U I2 900 displayed relatively high GlcN retention rate, although their decoloring efficiency were satisfied. Thus, they are not suitable for decoloring of mushroom hydrolysates. Additionally, activated bleaching earth is the most popular adsorbent for decoloring due to its lower purchase cost. Therefore, activated bleaching earth was selected as effective decoloring agent for mushroom hydrolysate decoloring process in this study.

Table 4.11 GlcN yield and efficiency of decoloring agents.

Decoloring agent		Form	GlcN yield (%)	Decoloring efficiency
Activated bleaching earth		Powder	89.30 ± 2.65	****
Activated carbon	CGC 8*30 I2 900	Granular	91.40 ± 1.93	*
	CGC 8*30 I2 1200	Granular	55.25 ± 1.93	**
	CGC 8*30 I2 1500	Granular	33.90 ± 2.54	***
	CGC 200C I2 900	Powder	13.33 ± 1.14	*****
	CGC 200C I2 1000	Powder	14.60 ± 0.82	*****
	CGC 200C I2 1100	Powder	10.19 ± 1.00	*****
	CGC 200U I2 900	Powder	18.38 ± 1.53	*****

Each GlcN yield is mean and S. D. of triplicate measurements.

4.3.1.2 Ultrafiltration

In this study, ultrafiltration was firstly applied to separate mushroom hydrolysate into two fractions with MW distributions of below and above 1kDa. It is well-known that GlcN is a small molecule with a MW of 179.17 g/mol (Henrotin et al., 2013). As expected, the permeate fractions corresponding to MW < 1 kDa maintained more than 95% of GlcN (Table 4.12). The permeate fractions also had higher ACE inhibitory activities than that of the retentate fractions (MW > 1 kDa) (data not shown), confirming that smaller peptides have stronger ACE inhibitory activity as mentioned in previous studies (Chen et al., 2012, Paiva, Lima, Neto and Baptista, 2016, Rui, Boye, Simpson and Prasher, 2013). This result was in accordance with Zhao et al. (2007), who reported that *Acaudina molpadioidea* gelatin hydrolysate was fractionated into three fractions (5-10, 5-1 and < 1 kDa) by ultrafiltration, which

the < 1 kDa fraction showed the most potent ACE inhibitory activity. A similar result was also reported by Chalé et al. (2014) who found that the ACE inhibitory activity of the *Mucuna pruriens* protein hydrolysate with MW below 1 kDa exhibited the strongest ACE inhibitory activity. Additionally, the ACE inhibitory activities of permeate fractions were much higher than that of crude hydrolysates, indicating that the typical MW of the potential ACE inhibitory peptides in hydrolysates were below 1 kDa (Table 4.13). The results showed that ultrafiltration was an effective method for concentrating GlcN and ACE inhibitory peptides in mushroom hydrolysates. Thus, as a simple, fast and reliable processing technique, ultrafiltration could be used for the industrial-scale purification of GlcN and ACE inhibitory peptides. Thus, the permeate fractions were selected for further steps in the present study.

4.3.1.3 Ethanol precipitation

For fractionation and isolation of polysaccharides and proteins from aqueous solutions, organic solvent precipitation is a common method. Among numerous organic solvents, ethanol is the most favorable in the laboratory and industry due to its relatively low cost, low toxicity to human and desirable physicochemical properties (Huang et al., 2013). To separate GlcN and ACE inhibitory peptides, ethanol precipitation was employed. The MW <1 kDa fractions were collected for further isolation of GlcN and ACE inhibitory peptides. When increased the ethanol amount in the system, the amount of available water as solvent was decreased. Therefore, GlcN and other compounds were separated due to their different solubility in water. GlcN was enriched in ethanol-soluble fractions and the purities were improved about 1.85-2.49 times (Table 4.12). The ACE inhibitory activities of ethanol precipitate fractions increased more than 1.5-fold compared to the

crude hydrolysates (Table 4.13). The results evidenced that ethanol precipitation is an efficient process for fractionation of GlcN and ACE inhibitory peptides.

4.3.1.4 Chromatographic purification

On the other hand, during fractionation of GlcN from mushrooms hydrolysates, insoluble precipitated fractions could be collected as byproducts and studied for their ACE inhibitory capacities. Although several ACE inhibitory peptides were identified from water extract of mushrooms, to the best knowledge of the authors, none of the studies have focused on ACE inhibitory peptides from mushroom hydrolysates. The ethanol precipitate fractions were further subjected to DEAE-Sephacel anion exchange and Superdex Peptide 10/300 GL gel filtration chromatography for purification of ACE inhibitory peptides. The chromatographic profiles obtained during various purification steps of ACE inhibitory peptides from five mushroom hydrolysates and their respective ACE inhibitory activity of each purified fraction were shown in Figure 4.17-4.26.

Table 4.12 Summary for GlcN recovery after ultrafiltration and ethanol precipitation.

Purification step	GlcN recovery (%)[a]	GlcN content (%)[b]	Purification fold[c]
Enoki mushroom			
Crude hydrolysate	100.00	10.02 ± 0.59	1.00
Decoloring process	93.55 ± 0.65	10.58 ± 0.34	1.06
Ultrafiltration	90.48 ± 0.73	11.93 ± 0.42	1.19
Ethanol precipitation	74.95 ± 1.19	22.57 ± 0.72	2.25
Oyster mushroom			
Crude hydrolysate	100.00	11.02 ± 0.42	1.00
Decoloring process	94.35 ± 0.22	11.72 ± 0.37	1.06
Ultrafiltration	92.85 ± 0.20	12.82 ± 0.65	1.16
Ethanol precipitation	75.08 ± 1.52	20.42 ± 0.25	1.85
Straw mushroom			
Crude hydrolysate	100.00	11.42 ± 0.82	1.00
Decoloring process	89.30 ± 2.65	11.53 ± 0.36	1.01
Ultrafiltration	86.57 ± 0.85	12.74 ± 0.77	1.12
Ethanol precipitation	76.49 ± 1.26	28.39 ± 0.43	2.49
Shiitake			
Crude hydrolysate	100.00	12.97 ± 0.76	1.00
Decoloring process	88.26 ± 0.53	13.24 ± 0.94	1.02
Ultrafiltration	84.61 ± 0.60	14.96 ± 0.28	1.15
Ethanol precipitation	73.75 ± 0.71	25.33 ± 0.55	1.95
Wood ear mushroom			
Crude hydrolysate	100.00	8.29 ± 0.46	1.00
Decoloring process	90.15 ± 1.01	8.34 ± 0.57	1.01
Ultrafiltration	86.43 ± 0.75	9.97 ± 0.61	1.20
Ethanol precipitation	75.33 ± 0.53	16.98 ± 0.85	2.05

All data are means and S. D. of triplicate measurements.

[a] GlcN recovery was calculated as the GlcN mass of the different step divided by the GlcN mass of crude mushroom hydrolysate.

[b] GlcN content was calculated as the GlcN mass divided by the total mass.

[c] GlcN purification fold was calculated as the GlcN content of the different step divided by the GlcN content of crude mushroom hydrolysate.

For enoki mushroom, after DEAE-Sephacel anion exchange chromatography, three fractions designed as F1-F3 were obtained. Although ACE inhibitory activity was widely observed in all the fractions, F3 fraction possessed the strongest ACE inhibitory activity with an IC_{50} value of 697.81 ± 8.55 mg/mL. The F3 fraction was further separated into five fractions designed as F3I-F3V using Superdex Peptide 10/300 GL gel filtration chromatography. The F3-IV and F3-V fractions exhibited the most potent ACE inhibitory activity with IC_{50} values of 23.11 ± 0.32 and 18.04 ± 0.13 mg/mL, respectively.

For oyster mushroom, after DEAE-Sephacel anion exchange chromatography, six fractions designed as F1-F6 were obtained. Although ACE inhibitory activity was widely observed in all the fractions, F4 fraction possessed the strongest ACE inhibitory activity with an IC_{50} value of 2.68 ± 0.08 mg/mL. The F4 fraction was further separated into four fractions designed as F4I-F4IV using Superdex Peptide 10/300 GL gel filtration chromatography. The F4-IV fraction exhibited the most potent ACE inhibitory activity with an IC_{50} value of 0.587 ± 0.03 mg/mL.

For straw mushroom, after DEAE-Sephacel anion exchange chromatography, five fractions designed as F1-F5 were obtained. Although ACE inhibitory activity was widely observed in all the fractions, F3 fraction possessed the strongest ACE inhibitory activity with an IC_{50} value of 35.29 ± 0.82 mg/mL. The F3 fraction was further separated into six fractions designed as F3I-F3VI using Superdex Peptide 10/300 GL gel filtration chromatography. The F3-V fraction exhibited the most potent ACE inhibitory activity with IC_{50} values of 2.93 ± 0.11 mg/mL.

For shiitake, after DEAE-Sephacel anion exchange chromatography, nine fractions designed as F1-F9 were obtained. Although ACE inhibitory activity was

widely observed in all the fractions, F6 fraction possessed the strongest ACE inhibitory activity with an IC_{50} value of 109.52 ± 3.69 mg/mL. The F6 fraction was further separated into five fractions designed as F6I-F6V using Superdex Peptide 10/300 GL gel filtration chromatography. The F6-V fraction exhibited the most potent ACE inhibitory activity with IC_{50} values of 3.09 ± 0.04 mg/mL.

For wood ear mushroom, after DEAE-Sephacel anion exchange chromatography, three fractions designed as F1-F3 were obtained. Although ACE inhibitory activity was widely observed in all the fractions, F3 fraction possessed the strongest ACE inhibitory activity with an IC_{50} value of 44.06±1.01 mg/mL. The F3 fraction was further separated into four fractions designed as F3I-F3IV using Superdex Peptide 10/300 GL gel filtration chromatography. The F3IV fraction exhibited the most potent ACE inhibitory activity with IC_{50} values of 4.84 ± 0.06 mg/mL.

The purification performances were summarized in Table 4.13. The IC_{50} values decreased by 70-175 folds after the four purification steps, indicating 70-175 times improvement in purities of peptides. The gel filtration chromatography step had the highest purification performance, with a decrease of IC_{50} value by 10-38 times.

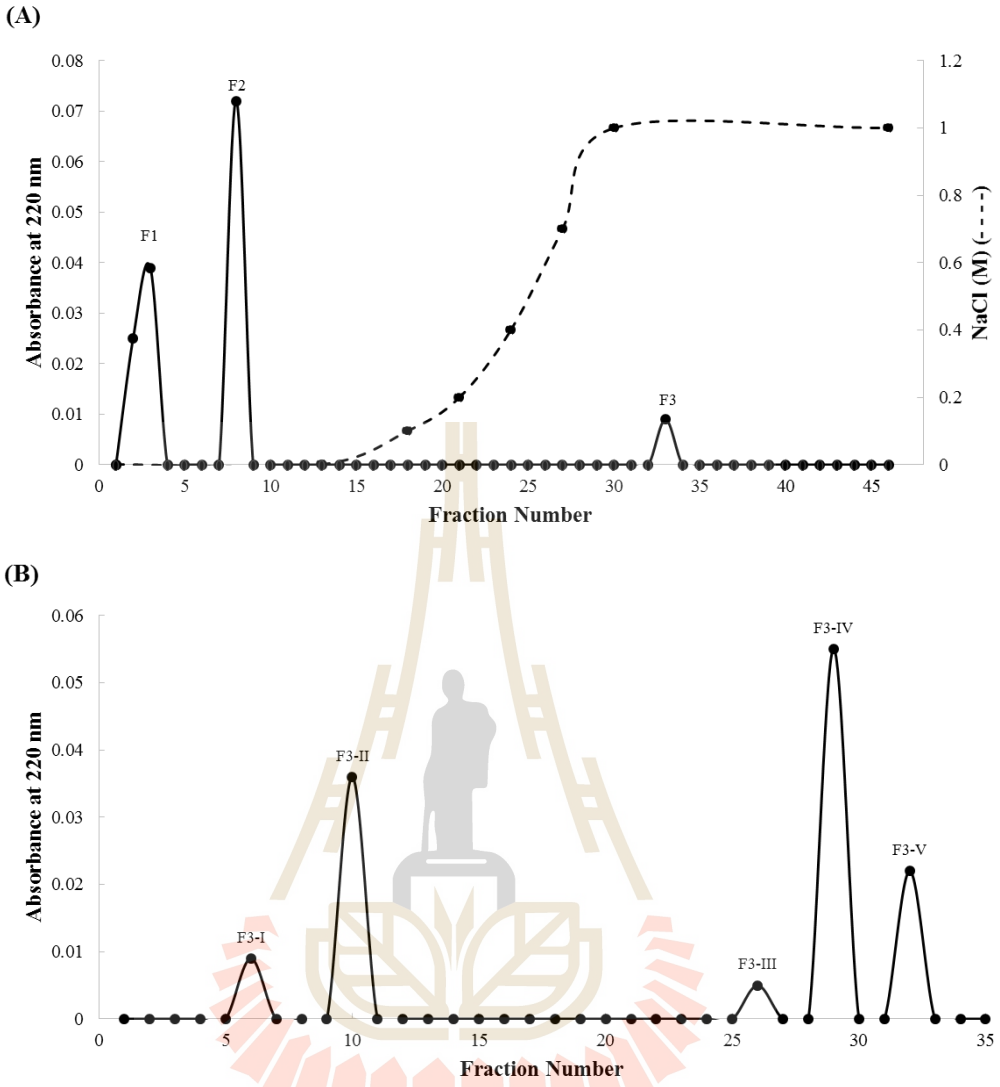


Figure 4.17 Purification chromatograms of ACE inhibitory peptides at various purification steps. (A) HiTrap DEAE Sepharose FF ion exchange chromatogram of the ethanol precipitate from enoki mushroom hydrolysate eluted with a segmented gradient of NaCl (0-1.0 M) in 50 mM Tris-HCl buffer at pH 8.0, (B) Superdex Peptide 10/300 GL gel filtration chromatogram of the F3 fraction eluted with deionized water.

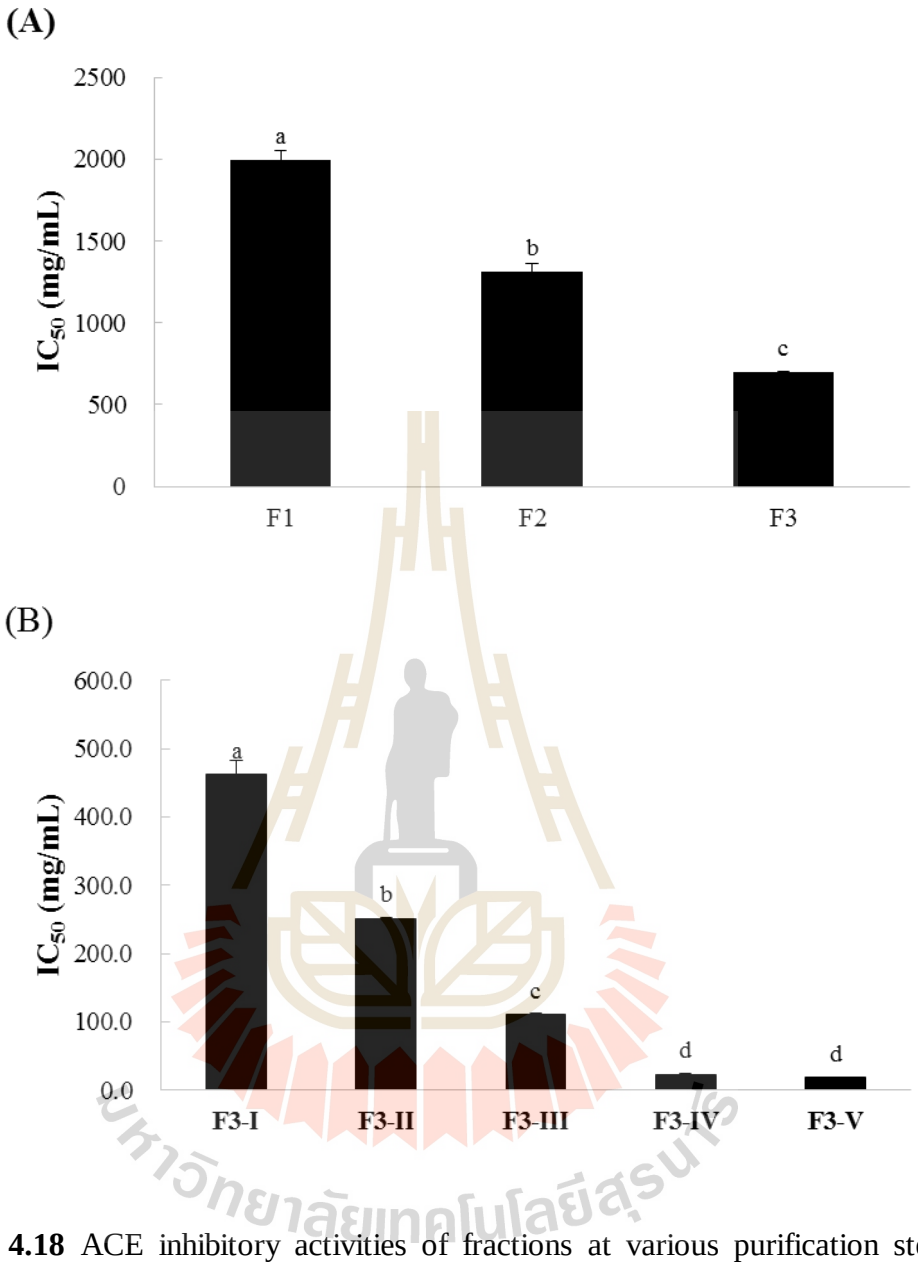


Figure 4.18 ACE inhibitory activities of fractions at various purification steps. (A) HiTrap DEAE Sepharose FF ion exchange chromatogram of the ethanol precipitate from enoki mushroom hydrolysate eluted with a segmented gradient of NaCl (0-1.0 M) in 50 mM Tris-HCl buffer at pH 8.0, (B) Superdex Peptide 10/300 GL gel filtration chromatogram of the F3 fraction eluted with deionized water. The different letters indicate significant differences ($p < 0.05$) between samples when analyzed by one-way ANOVA and post-hoc Tukey's HSD test

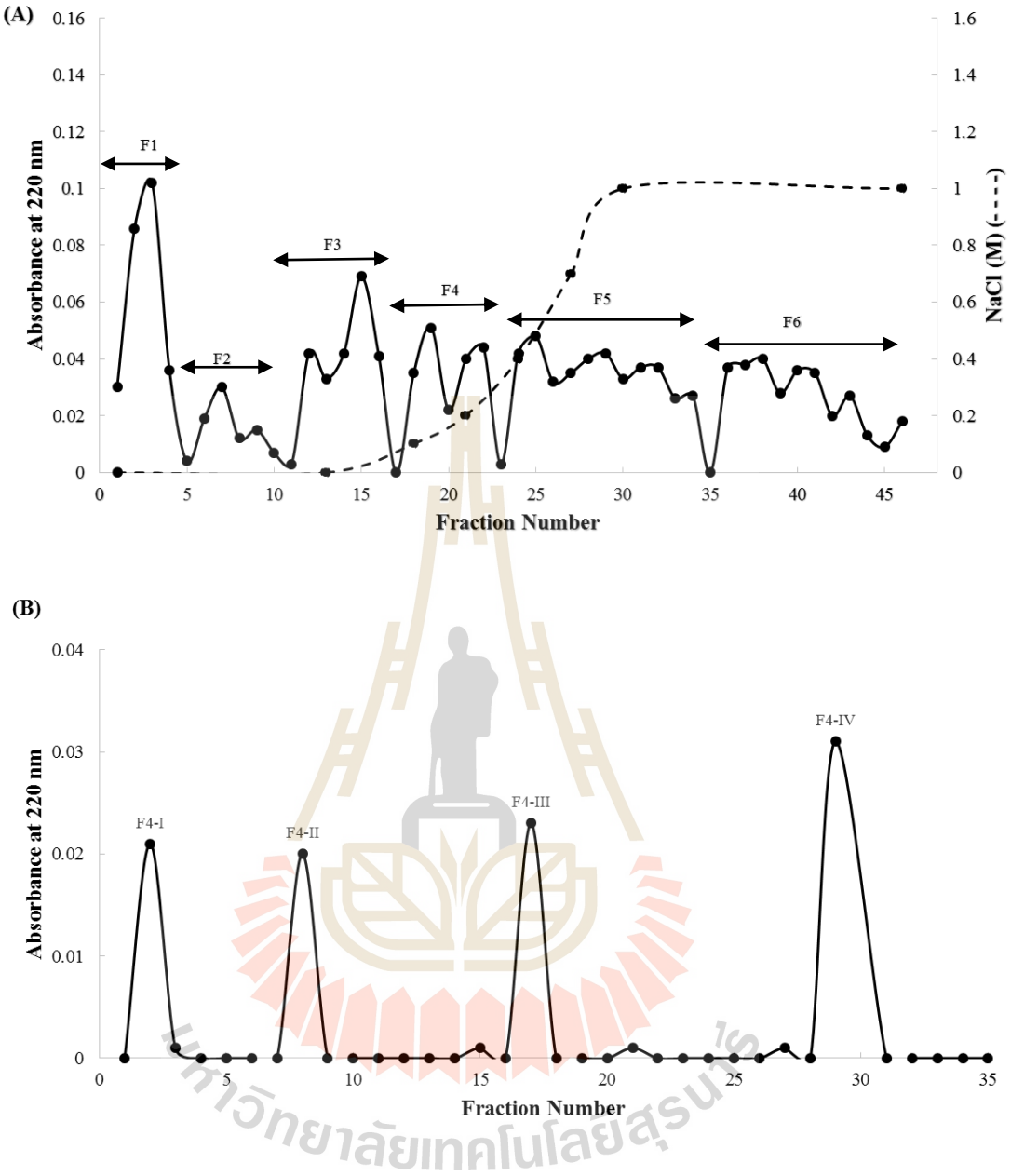


Figure 4.19 Purification chromatograms of ACE inhibitory peptides at various purification steps. (A) HiTrap DEAE Sepharose FF ion exchange chromatogram of the ethanol precipitate from oyster mushroom hydrolysate eluted with a segmented gradient of NaCl (0-1.0 M) in 50 mM Tris-HCl buffer at pH 8.0, (B) Superdex Peptide 10/300 GL gel filtration chromatogram of the F4 fraction eluted with deionized water.

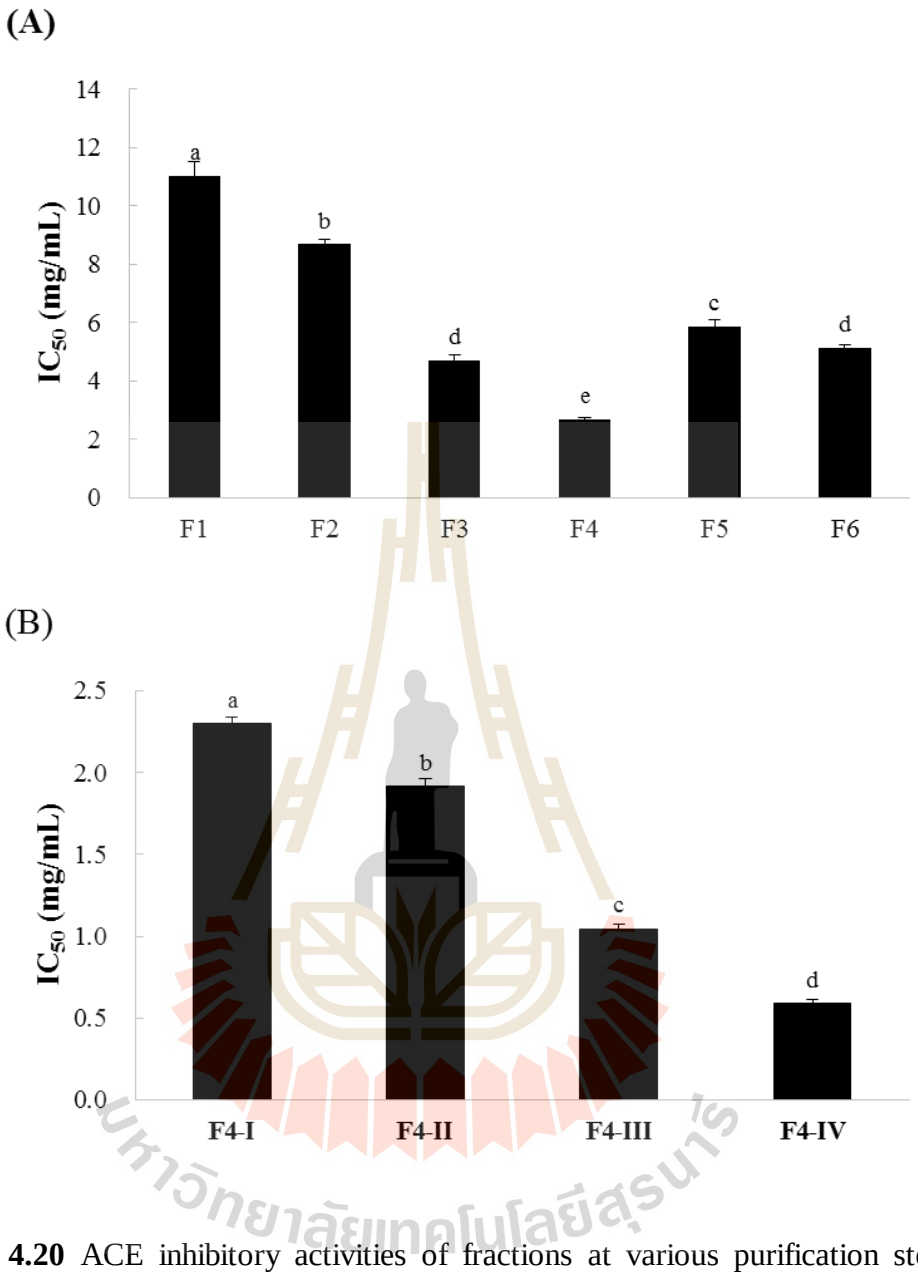


Figure 4.20 ACE inhibitory activities of fractions at various purification steps. (A) HiTrap DEAE Sepharose FF ion exchange chromatogram of the ethanol precipitate from oyster mushroom hydrolysate eluted with a segmented gradient of NaCl (0-1.0 M) in 50 mM Tris-HCl buffer at pH 8.0, (B) Superdex Peptide 10/300 GL gel filtration chromatogram of the F4 fraction eluted with deionized water. The different letters indicate significant differences ($p < 0.05$) between samples when analyzed by one-way ANOVA and post-hoc Tukey's HSD test

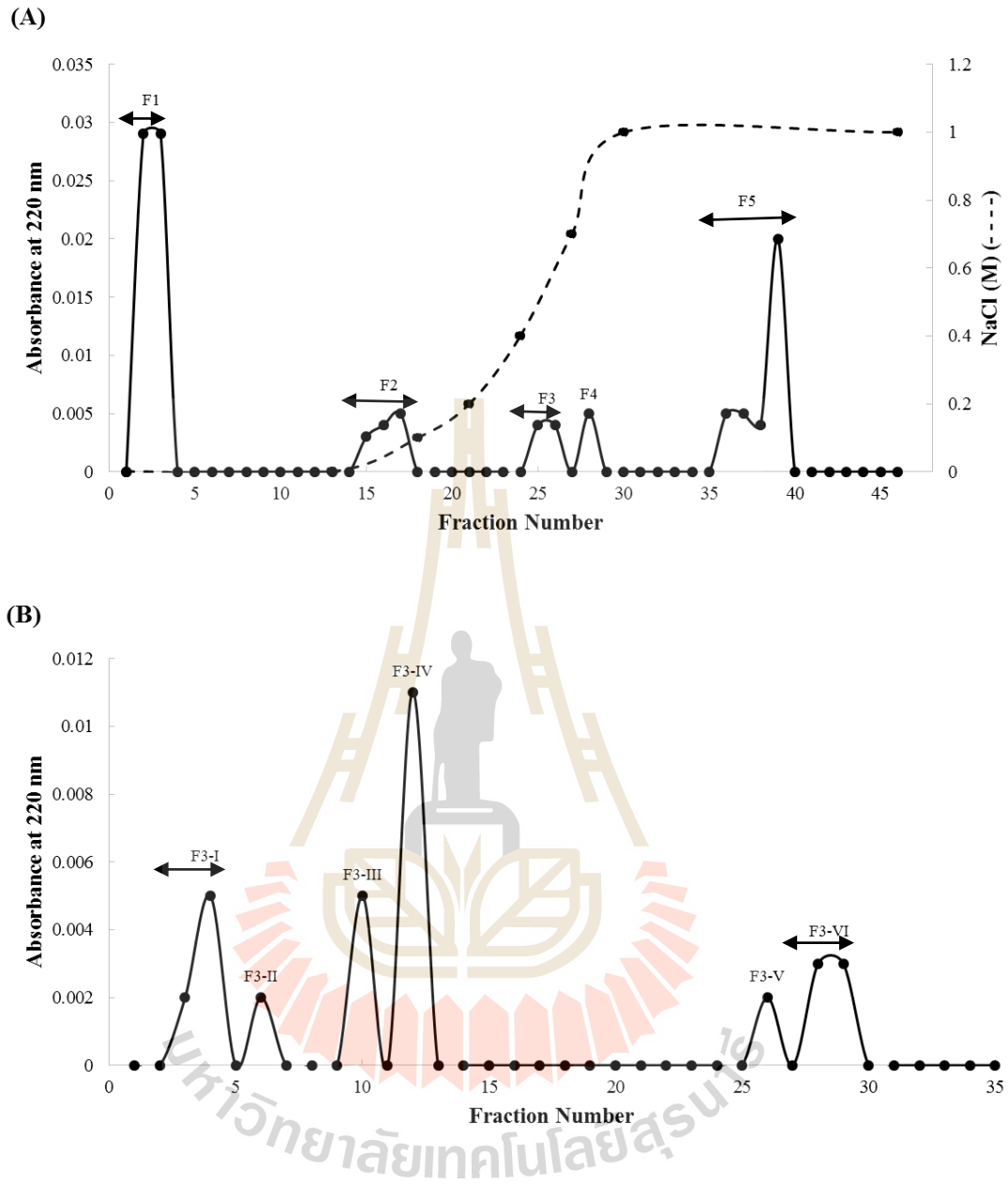


Figure 4.21 Purification chromatograms of ACE inhibitory peptides at various purification steps. (A) HiTrap DEAE Sepharose FF ion exchange chromatogram of the ethanol precipitate from straw mushroom hydrolysate eluted with a segmented gradient of NaCl (0-1.0 M) in 50 mM Tris-HCl buffer at pH 8.0, (B) Superdex Peptide 10/300 GL gel filtration chromatogram of the F3 fraction eluted with deionized water.

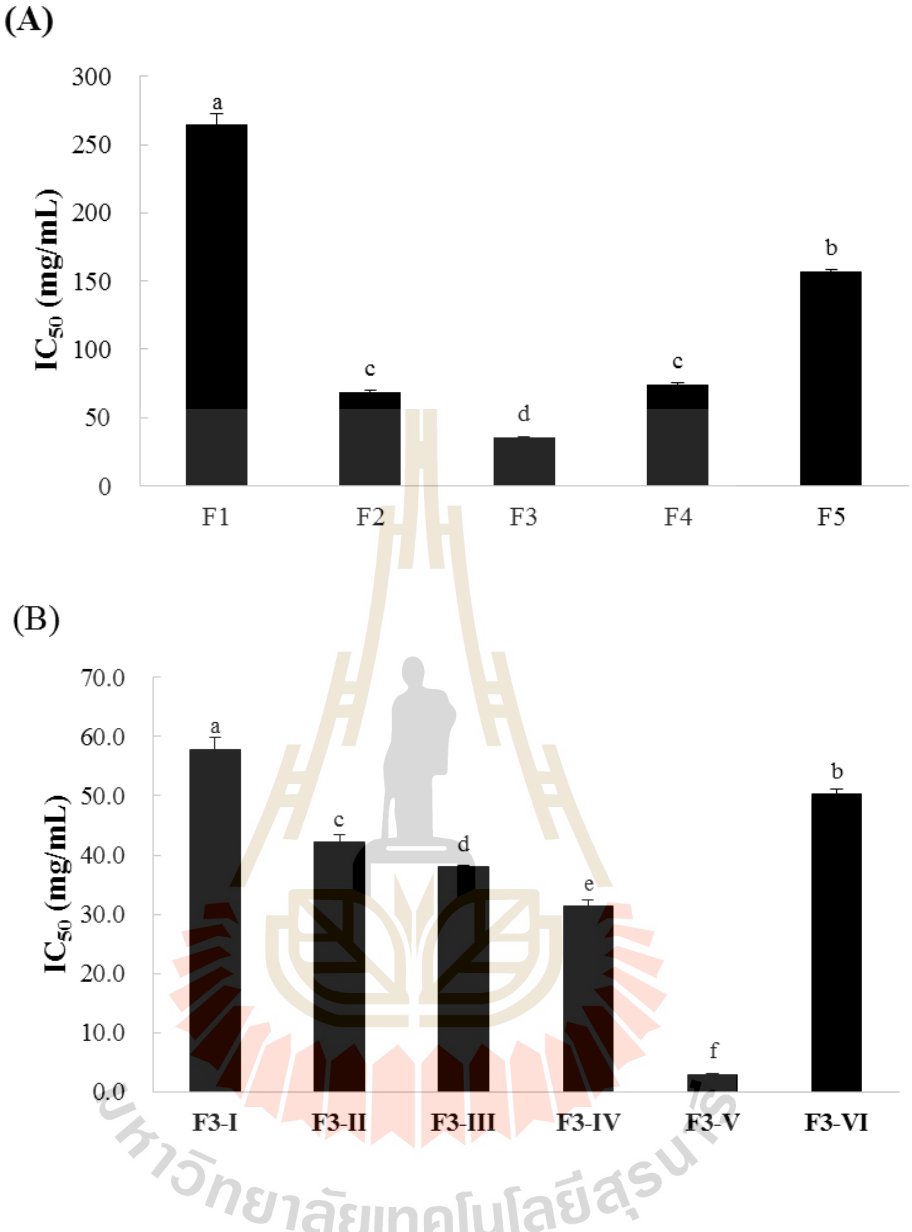


Figure 4.22 ACE inhibitory activities of fractions at various purification steps. (A) HiTrap DEAE Sepharose FF ion exchange chromatogram of the ethanol precipitate from straw mushroom hydrolysate eluted with a segmented gradient of NaCl (0-1.0 M) in 50 mM Tris-HCl buffer at pH 8.0, (B) Superdex Peptide 10/300 GL gel filtration chromatogram of the F3 fraction eluted with deionized water. The different letters indicate significant differences ($p < 0.05$) between samples when analyzed by one-way ANOVA and post-hoc Tukey's HSD test

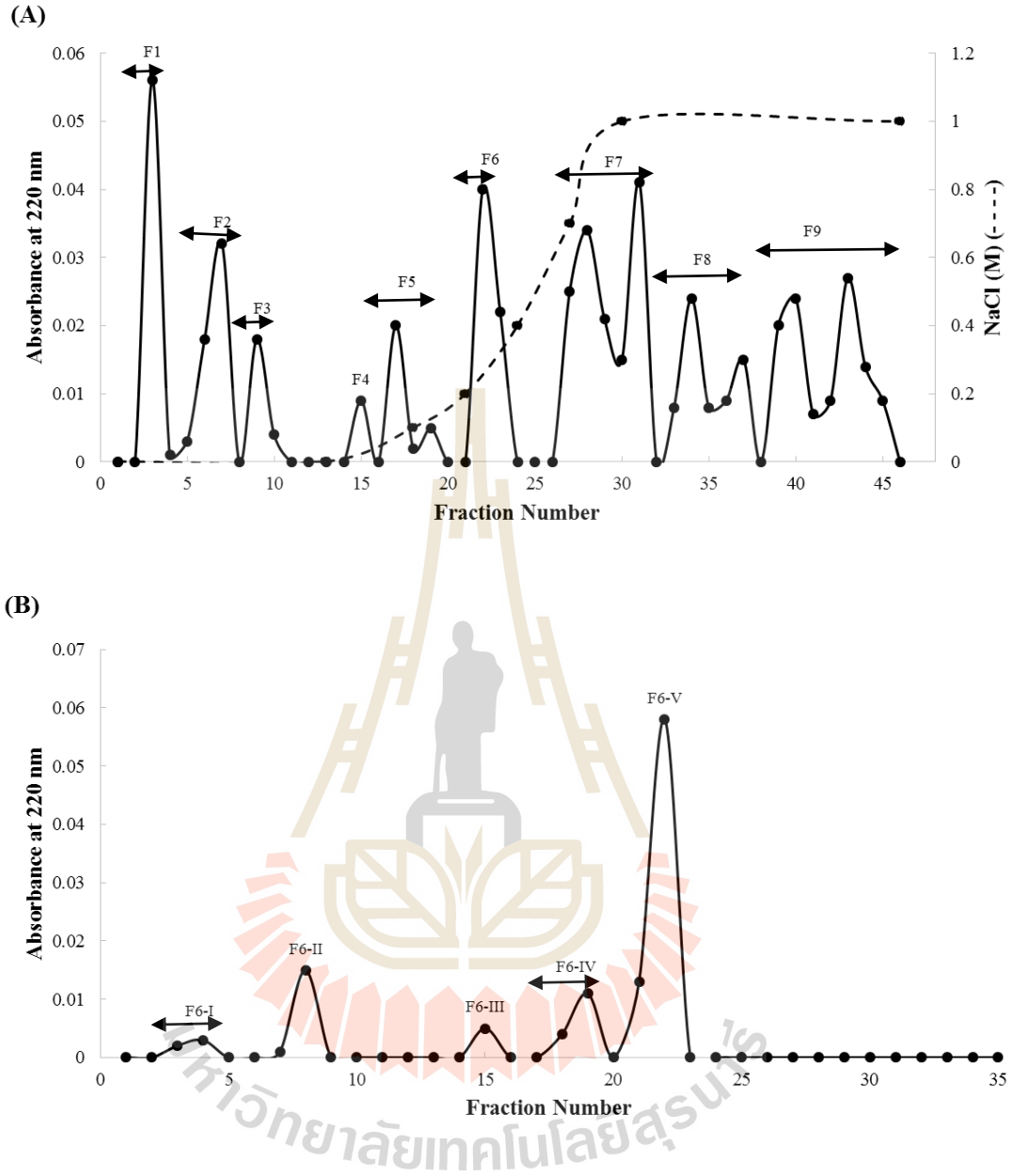


Figure 4.23 Purification chromatograms of ACE inhibitory peptides at various purification steps. (A) HiTrap DEAE Sepharose FF ion exchange chromatogram of the ethanol precipitate from shiitake hydrolysate eluted with a segmented gradient of NaCl (0-1.0 M) in 50 mM Tris-HCl buffer at pH 8.0, (B) Superdex Peptide 10/300 GL gel filtration chromatogram of the F6 fraction eluted with deionized water.

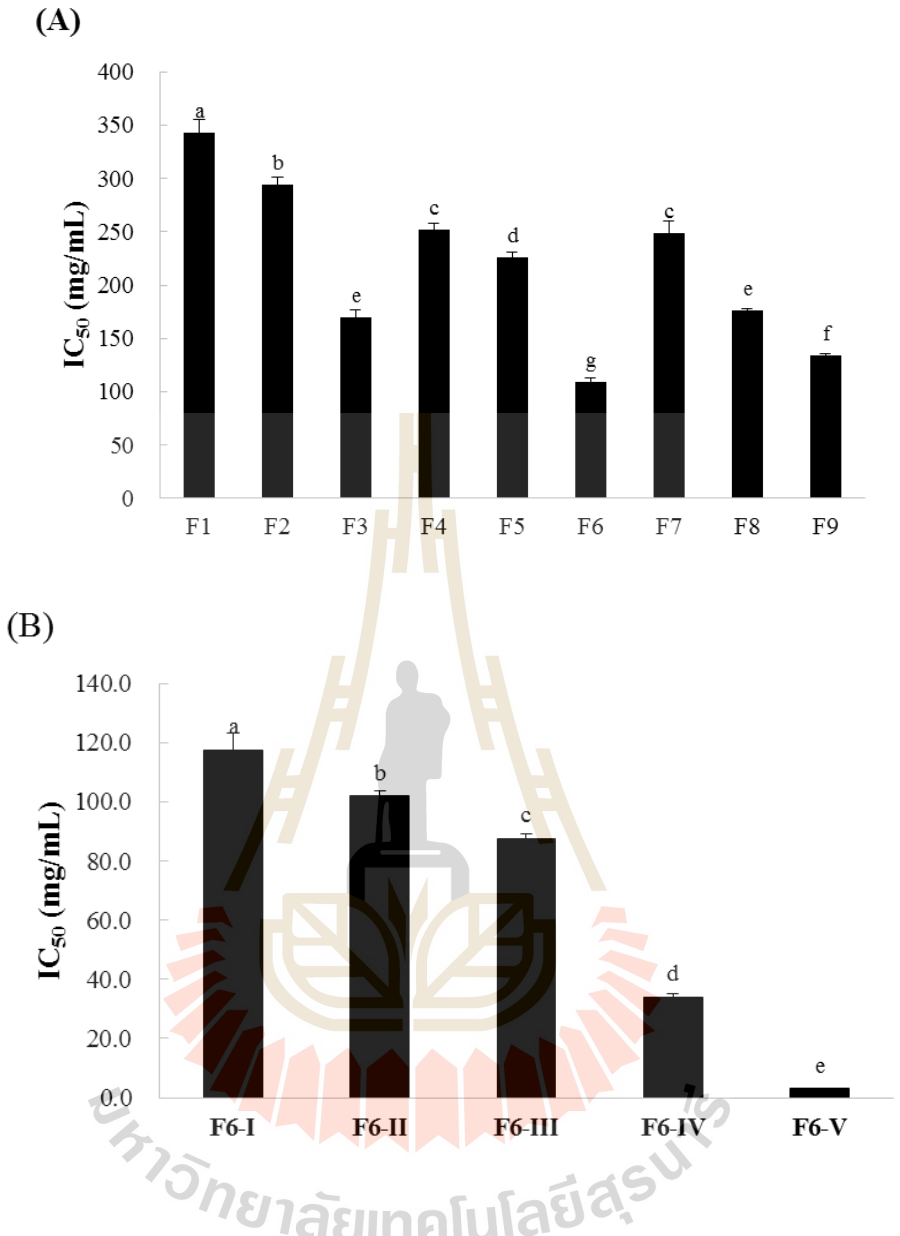


Figure 4.24 ACE inhibitory activities of fractions at various purification steps. (A) HiTrap DEAE Sepharose FF ion exchange chromatogram of the ethanol precipitate from shiitake hydrolysate eluted with a segmented gradient of NaCl (0-1.0 M) in 50 mM Tris-HCl buffer at pH 8.0, (B) Superdex Peptide 10/300 GL gel filtration chromatogram of the F6 fraction eluted with deionized water. The different letters indicate significant differences ($p < 0.05$) between samples when analyzed by one-way ANOVA and post-hoc Tukey's HSD test

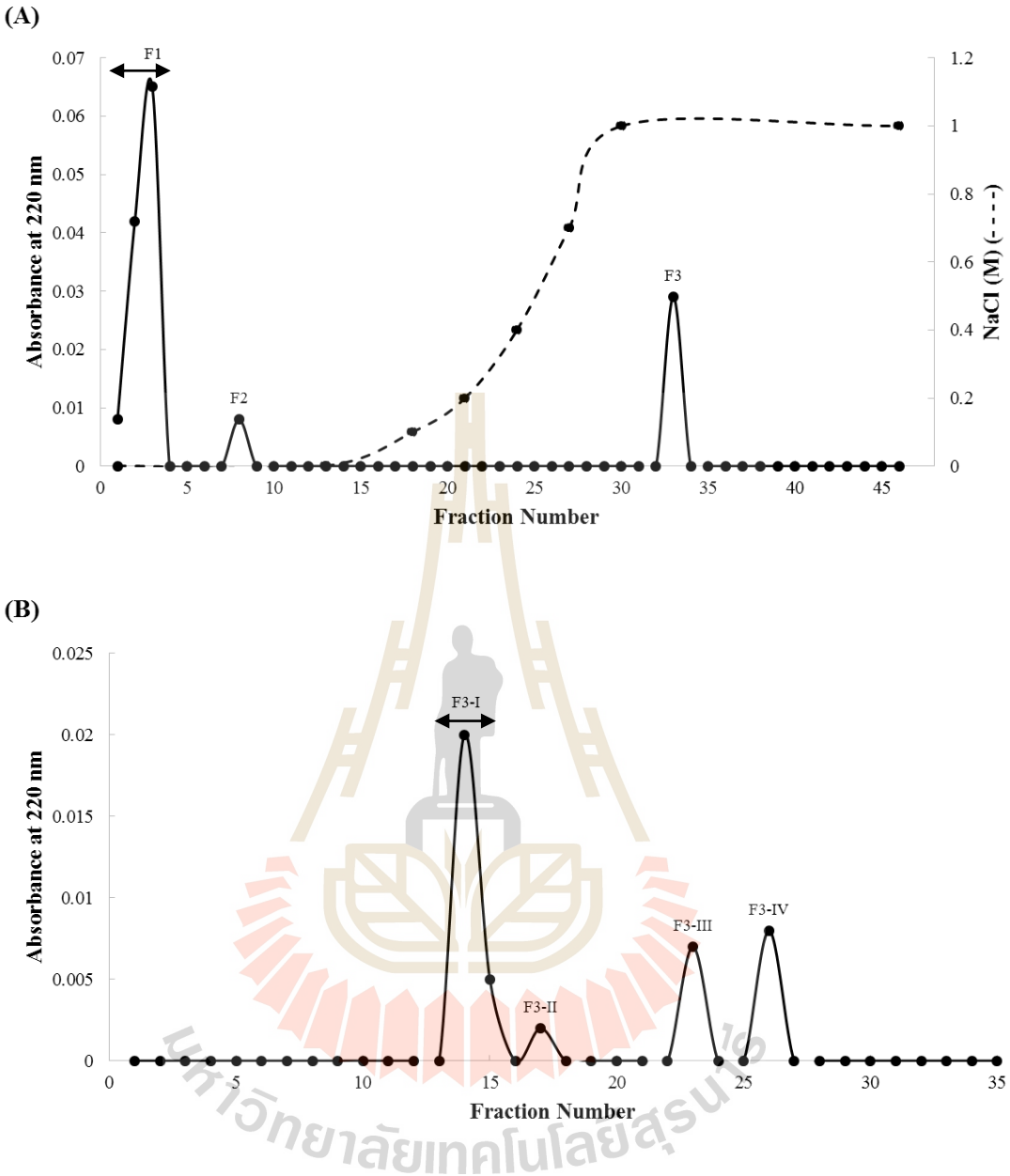


Figure 4.25 Purification chromatograms of ACE inhibitory peptides at various purification steps. (A) HiTrap DEAE Sepharose FF ion exchange chromatogram of the ethanol precipitate from wood ear mushroom hydrolysate eluted with a segmented gradient of NaCl (0-1.0 M) in 50 mM Tris-HCl buffer at pH 8.0, (B) Superdex Peptide 10/300 GL gel filtration chromatogram of the F3 fraction eluted with deionized water.

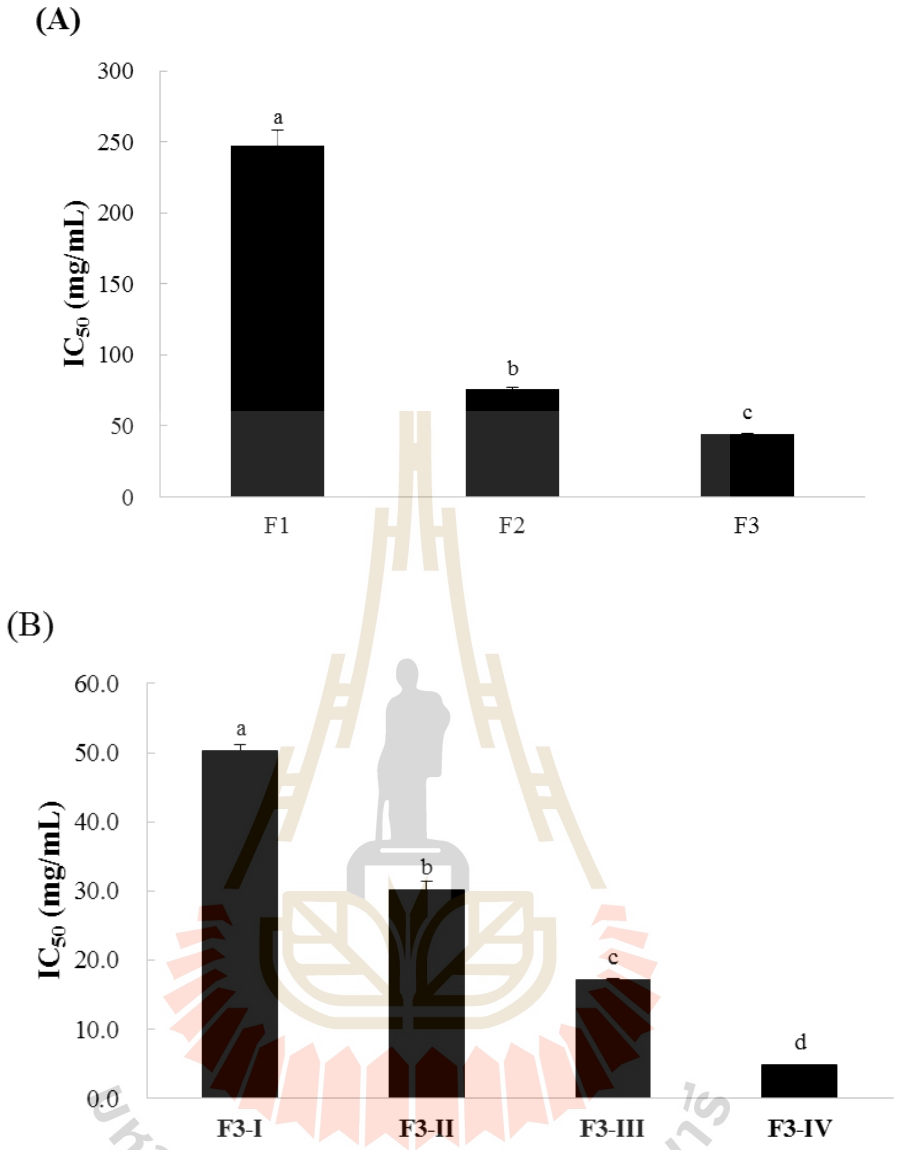


Figure 4.26 ACE inhibitory activities of fractions at various purification steps. (A) HiTrap DEAE Sepharose FF ion exchange chromatogram of the ethanol precipitate from wood ear mushroom hydrolysate eluted with a segmented gradient of NaCl (0-1.0 M) in 50 mM Tris-HCl buffer at pH 8.0, (B) Superdex Peptide 10/300 GL gel filtration chromatogram of the F3 fraction eluted with deionized water. The different letters indicate significant differences ($p < 0.05$) between samples when analyzed by one-way ANOVA and post-hoc Tukey's HSD test

Table 4.13 Summary for IC_{50} value after different purification steps of mushroom hydrolysates.

Purification step	IC_{50} value (mg/mL)[a]	Purification fold[b]
Enoki mushroom		
Crude hydrolysate	2410.88 ± 110.56	1.00
Decoloring process	2399.70 ± 114.95	1.00
Ultrafiltration	1781.87 ± 23.61	1.35
Ethanol precipitation	1774.64 ± 54.33	1.36
DEAE-Sepharose	697.81 ± 8.56	3.45
Superdex Peptide 10/300 GL	18.04 ± 0.13	133.67
Oyster mushroom		
Crude hydrolysate	72.33 ± 1.56	1.00
Decoloring process	64.11 ± 0.88	1.13
Ultrafiltration	32.55 ± 0.45	2.22
Ethanol precipitation	36.45 ± 1.79	1.98
DEAE-Sepharose	2.68 ± 0.08	26.99
Superdex Peptide 10/300 GL	0.59 ± 0.03	122.59
Straw mushroom		
Crude hydrolysate	428.77 ± 10.63	1.00
Decoloring process	427.23 ± 6.90	1.00
Ultrafiltration	190.93 ± 2.54	2.25
Ethanol precipitation	166.94 ± 2.91	2.57
DEAE-Sepharose	35.29 ± 0.82	12.15
Superdex Peptide 10/300 GL	2.93 ± 0.11	146.54
Shiitake		
Crude hydrolysate	539.68 ± 25.34	1.00
Decoloring process	542.60 ± 23.24	0.99
Ultrafiltration	256.36 ± 6.00	2.11
Ethanol precipitation	261.58 ± 4.68	2.06
DEAE-Sepharose	109.52 ± 3.65	4.93
Superdex Peptide 10/300 GL	3.09 ± 0.04	174.54
Wood ear mushroom		
Crude hydrolysate	346.570 ± 15.02	1.00
Decoloring process	338.77 ± 13.84	1.02
Ultrafiltration	122.74 ± 3.69	2.82
Ethanol precipitation	111.32 ± 1.01	3.11
DEAE-Sepharose	44.06 ± 1.01	7.87
Superdex Peptide 10/300 GL	4.84 ± 0.06	71.61

All data are means and S. D. of triplicate measurements.

[a] IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

[b] ACE inhibitory peptides purification fold was calculated as the IC_{50} value of the different step divided by the IC_{50} value of crude mushroom hydrolysate

4.3.2 Characterization of the purified ACE inhibitory peptides

4.3.2.1 Residual activity assay of the purified ACE inhibitory peptides

The processing stability of the purified peptide after various pH and temperature treatments is important in preparing foods with "functional ingredients" (Sheih, Fang and Wu, 2009). To investigate the pH and heat-stability of the purified peptides, the peptides were subjected to incubation at pH 2-12 and temperature 4-100°C for 2 h and measured for residual activity. As shown in Figure 4.27A and Figure 4.27B, these peptides retained at least 80% of their ACE inhibitory activities after various temperature and pH treatments. These results indicated that the mushroom derived ACE inhibitory peptides have satisfactory stability against heat and pH. These results are in accordance with former works that ACE inhibitory peptides derived from soy protein exhibited good resistance to different temperature and pH treatments (Wu and Ding, 2002). Similarly, ACE inhibitory peptides from tuna cooking juice also retained ACE inhibitory activity after various temperature and pH treatments (Hwang, 2010).

In addition to inhibitory activity, bioactive peptides have to resist to the possible gastrointestinal digestion and maintain high bioavailability when they reach the target organs after oral administration (Vásquez-Villanueva, Marina and García, 2015). In order to predict the *in vivo* antihypertensive effects of purified peptides, they were submitted to gastrointestinal proteases digestions with pepsin (simulating stomach digestion) and chymotrypsin (simulating small intestine digestion) using conditions simulating *in vivo* ones. As shown in Figure 4.27C, after and before gastrointestinal proteases digestion, the IC_{50} values of purified peptides showed no

apparent impact of gastrointestinal proteases on their ACE inhibitory activity, which suggested that the purified peptides have strong enzyme-resistant properties against gastrointestinal proteases and that the active sequence of the peptides could not be destroyed in the gastrointestinal tract. Several food-protein derived ACE inhibitory peptides also showed their low susceptibility to hydrolysis by gastrointestinal proteases (Chen et al., 2012, Escudero, Mora and Toldrá, 2014, Sheih, Fang and Wu, 2009). On the contrary, some ACE inhibitory peptides acted as substrates of gastrointestinal proteases, decreasing their inhibitory activity upon digestion (Tsai, Chen and Pan, 2008).

Resistance to different temperature and pH treatments and gastrointestinal proteases are important traits for ACE inhibitory peptides that need to be used in a wind range of food products because the ACE inhibitory activity should not be significantly decreased during food processing, storage, and after oral administration. Overall, the purified peptides from mushrooms were established as a promising candidate for use as functional ingredients in many food products.

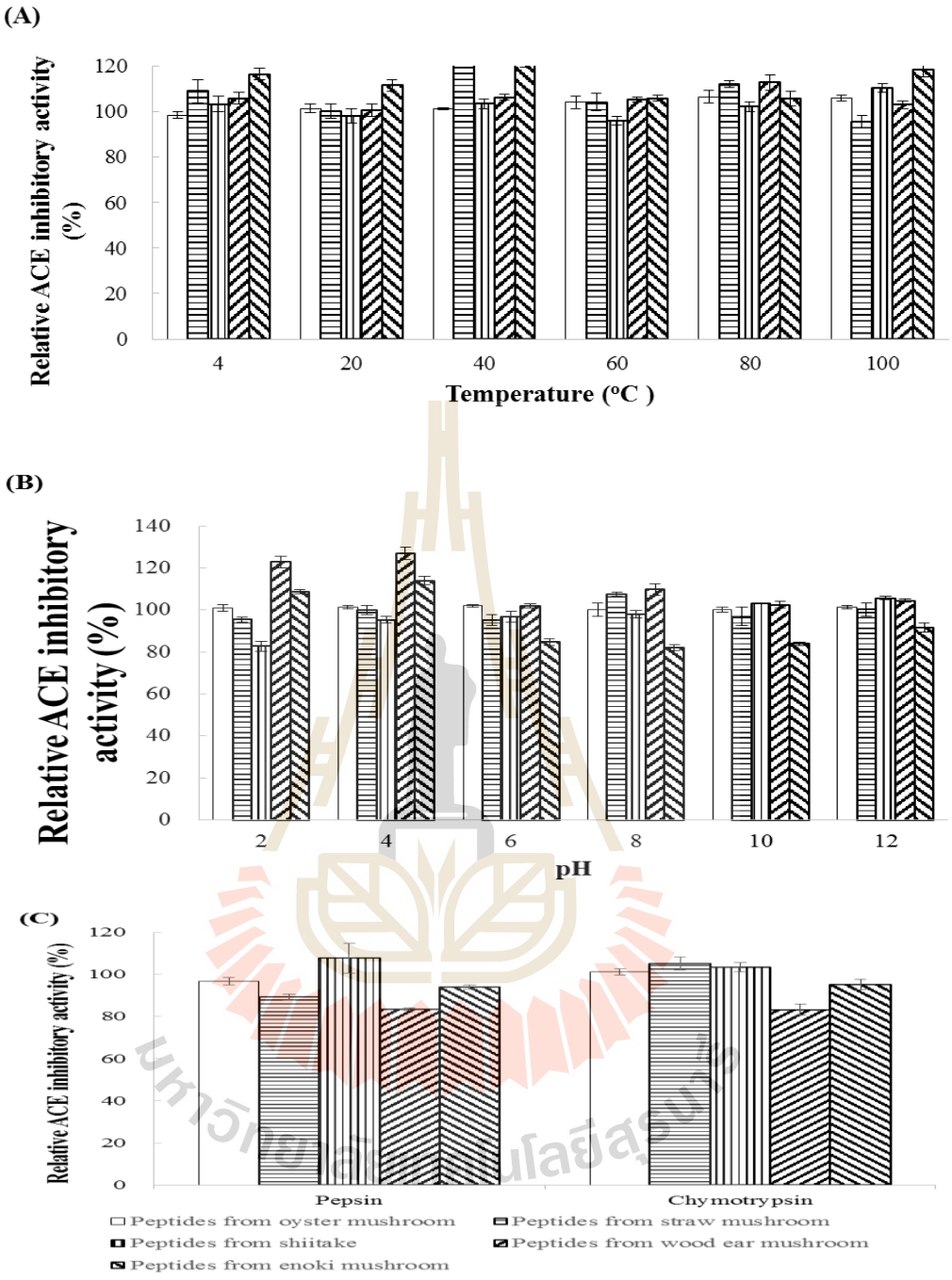


Figure 4.27 Residual activity of the purified peptides after incubation at various (A) temperatures, (B) pHs, and (C) gastrointestinal proteases digestion.

4.3.2.2 Inhibition pattern of the purified ACE inhibitory peptides

As shown in Figure 4.28A, the increased K_m^{app} and constant V_{max}^{app} indicated that the purified peptides from oyster mushroom acted as a competitive ACE inhibitor with a K_i value of 0.24 mg/mL. This revealed that the peptides competed with the substrate for binding to the ACE active sites and, thus, blocked ACE from interacting with substrate (Paiva, Lima, Neto and Baptista, 2016). It also inferred that the ACE inhibition activity was more significant when the ACE inhibitor's amount was increased (Du et al., 2013). On the other hand, since the constant K_m^{app} and decreased V_{max}^{app}, the purified peptides from straw mushroom showed a non-competitive inhibition characteristic with a K_i value of 3.09 mg/mL (Figure 4.28B). The Lineweaver-Burk plot of purified peptides from enoki mushroom also illustrated a non-competitive inhibition pattern with a K_i value of 24.11 mg/mL (Figure 4.28E). The results represented that the peptides from straw mushroom and enoki mushroom could combine with ACE or ACE-substrate complex at a site other than the ACE active sites to produce a dead-end complex (Balti et al., 2015). The non-competitive inhibitor may inhibit ACE by causing a conformational change, which prevented ACE from converting substrate to product, so the inhibitor worked equally well at low and high concentrations of the substrate (Sheih, Fang and Wu, 2009). As for the purified peptides from shiitake, 0.75 mg/mL of peptides displayed a competitive inhibition pattern, however, as the concentration increased, the inhibition pattern shifted to non-competitive (Figure 4.28C). Thus, it can be suggested that the purified peptides from shiitake followed a mixed inhibition pattern. The purified peptides from wood ear mushroom also demonstrated a mixed inhibition pattern (Figure 4.28D). The 0.75 mg/mL concentration of the peptides exhibited a

non-competitive inhibition pattern, however, as the peptides concentration increased, the inhibition pattern changed.

A number of studies have revealed that ACE inhibitory peptides inhibit ACE via competitive, non-competitive, uncompetitive, and mixed inhibition patterns. The competitive ACE inhibitors have been frequently reported, such as VAP from grass carp, PVNNPQIH from small red bean, and AWLHPGAPKVF from *Phascolosoma esculenta* (Chen et al., 2012, Du et al., 2013, Rui, Boye, Simpson and Prasher, 2013). By contrast, some non-competitive or uncompetitive inhibitors have also been found from cuttlefish, *Enteromorpha clathrate*, algae protein waste and tilapia (Balti et al., 2015, Pan, Wang, Jing and Yao, 2016, Sheih, Fang and Wu, 2009, Toopcham, Roytrakul and Yongsawatdigul, 2015). Meanwhile, a few peptides such as peptides isolated from *Stichopus horrens* and hydrolysate of hard clam (*Meretrix lusoria*) exhibited inhibition in a mixed pattern (Forghani et al., 2016, Tsai, Chen and Pan, 2008). The mixed inhibition pattern occurs when more than one inhibition patterns happen at the same time (Forghani et al., 2016).

4.3.2.3 Peptide analysis of the purified ACE inhibitory peptides

The raw data obtained from each MS/MS experiment was processed using MASCOT. No protein identification was obtained from NCBI database search using Mascot. Many peptide matches that are not assigned to proteins hits in the database were detected as shown in Table 4.14. Thirteen potential ACE inhibitory peptides were identified. These peptides are novel ACE inhibitors from mushrooms which have not been reported previously. It is also noted that all reported peptides shared common sequences with ACE inhibitory dipeptides or tripeptides previously reported in BIOPEP database and SATPdb database.

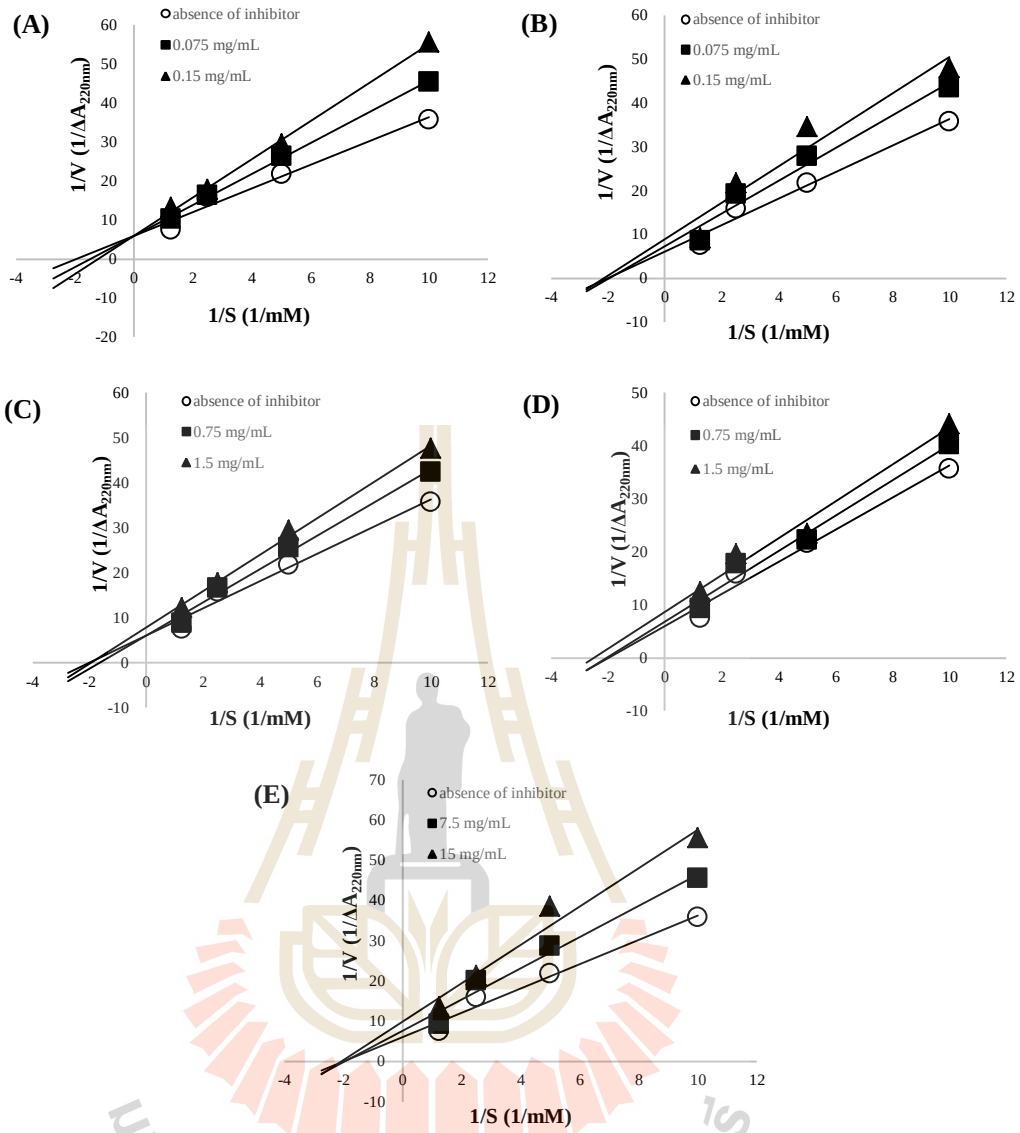


Figure 4.28 Lineweaver-Burk plots of angiotensin-I converting enzyme (ACE) inhibition by the purified peptides. (A) Purified peptides from oyster mushroom, (B) purified peptides from straw mushroom, (C) purified peptides from shiitake, (D) purified peptides from wood ear mushroom, and (E) purified peptides from enoki mushroom. The ACE activities were measured in the absence or presence of the purified peptides. Values are mean ± SD (n = 3).

There are many reported sequences of ACE inhibitory peptides in the range of dipeptide to oligopeptides. In this study, the identified peptides were composed of 7-9 amino acid residues. Their molecular weights were smaller than those of the ACE inhibitory peptides from mushroom *Pleurotus cornucopiae* (1622.85 and 2037.26 Da) (Jang et al., 2011). The low molecular weight peptides are potent ACE inhibitors because the large peptide molecules were restricted from fitting into the active sites of ACE (Lau, Abdullah and Shuib, 2013). It was reported that phenylalanine, tryptophan, tyrosine, or proline at the C-terminal and branched-chain aliphatic amino acids at the N-terminal were preferred for a peptide binding to ACE as a competitive inhibitor (Balti et al., 2015). The purified ACE inhibitory peptide from oyster mushroom, which the amino acid sequence was found to be Leu-Ala-Ser-Leu-Pro-Gly-Asn-Asp-Pro, contained proline in its C-terminal and leucine and alanine present in its N-terminal. This is in accordance with the findings of Balti et al. (2015) that proline at the C-terminal may contribute to ACE inhibitory activity. An antepenultimate aromatic amino acid residue appeared to strengthen the binding of ACE inhibitory peptides and ACE (Choi et al., 2001). The antepenultimate phenylalanine in the peptide from shiitake (Ala-Ser-Pro-Tyr-Ala-Phe-Gly-Leu) may contribute to its higher ACE inhibitory activity. Moreover, in a previous study, proline, lysine or aromatic amino acid residues appeared in most naturally occurring ACE inhibitory peptides (Geng et al., 2016). Proline, phenylalanine, tyrosine and lysine appeared in all peptides identified in this study which in accordance with the characteristic feature of naturally occurring ACE inhibitory peptides.

A predictive software based on novel N to 1 neural network, PeptideRanker (http://bioware.ucd.ie) identifies the potential bioactive sequences among a set of

peptides (Mooney, Haslam, Pollastri and Shields, 2012). Generally, the sequence is considered potentially bioactive when the algorithm assigns a score at least of 0.5. After such filtering of peptide scores, most peptide sequences were rejected and only two peptides (ASPYAFGL and MLCSTTF) were retained in the list of candidates to be bioactive. Additionally, LASLFGNDP with a PeptidRanker score of 0.43 was also selected because its original purified fraction exhibited the highest ACE inhibitory activity. Thus, three peptides (ASPYAFGL, MLCSTTF, and LASLFGNDP) were selected for chemical synthesis. ASPYAFGL exhibited the lowest IC_{50} value of 0.1080 µM followed by MLCSTTF with the IC_{50} value of 0.1524 µM, while the highest IC_{50} value belonged to LASLFGNDP (0.2491 µM). The existence of hydrophobic amino acids (Phe, Gly, Leu) at the C-terminal tripeptide positions and the presence of Ala and Pro at the internal position of peptide ASPYAFGL might positively influence the peptide ACE inhibitory activity (Geng et al., 2016, Pan, Wang, Jing and Yao, 2016). These values are higher than captopril, a standard inhibitor, which has an IC_{50} of 0.014 µM, but significantly better than results reported for ACE inhibitory peptides isolated from mushrooms. Peptide WALKGYK from *Tricholoma matsutake* exhibited an IC_{50} value of 0.40 µM (Geng et al., 2016). Peptide AHEPVK and GPSMR from *Pleurotus cystidiosus* O.K. Miller showed IC_{50} values of 62.8 µM and 277.5 µM, respectively (Lau, Abdullah and Shuib, 2013). Peptide LSMGSASLSP isolated from *Hypsizygus marmoreus* inhibited 50% of the ACE activity at a concentration of 334.92 µM (Kang et al., 2013). Peptide RLPSEFDLSAFLRA and RLSGQTIEVTSEYLFRH from *Pleurotus cornucopiae* displayed IC_{50} values of 283.45 µM and 559.58 µM, respectively (Jang et al., 2011). These results suggested that the three peptides

identified in this study could be applied as a good alternative antihypertensive ingredient in functional foods, dietary supplements, nutraceuticals or pharmaceuticals.

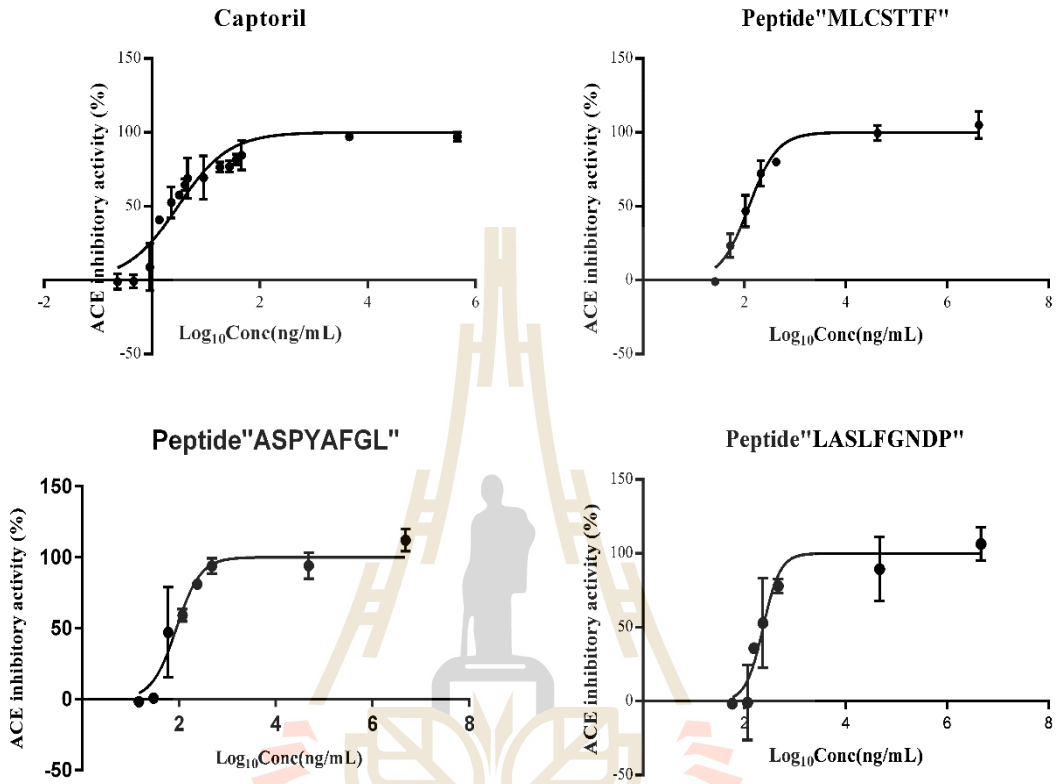


Figure 4.29 The IC_{50} values of Captoril and three synthesized ACE inhibitory peptides. Each point represents the mean of three experiments, and the vertical bars represent the SD.

Structure-activity relationships among different ACE inhibitory peptides indicate that the degree of their ACE capacities is strongly affected by the C-terminal tripeptide sequence (Geng et al., 2016, Pan, Wang, Jing and Yao, 2016). This might explain why peptide ASPYAFGL with Gly, Leu at its C-terminal tripeptide positions showed the highest ACE inhibitory activity. In this regard, peptides GL, YGL, PGL, and DGL bearing a GL dipeptide at the C-terminal showed ACE inhibitory activity (Byun and Kim, 2002, Cheung et al., 1980, Wu, Aluko and Nakai, 2006). To achieve a better understanding of the structure-activity relationship, the antihypertensive activities of ASPYAFGL, ASPYAFG, ASPYAF, and ASPYA, were predicted by a web server known as AHTpin (http://crdd.osdd.net/raghava/ahtpin/). The SVM scores of these four peptides were 0.81, 0.39, 0.27, and 0.16, respectively. The peptide ASPYAFGL exhibited the most potent antihypertensive activity with an SVM score of 0.81. When amino acid at C-terminal (Phe, Gly, and Leu) was removed, the shortened peptide showed a decreasing antihypertensive activity. These results further proved that Phe, Gly, and Leu might play an important role in ACE inhibitory activity of peptide ASPYAFGL. The antepenultimate amino acid (Pro) in peptide ASPYAFGL was replaced, based on the SVM scores, ASWYAFGL (SVM score: 0.82) displayed similar higher antihypertensive activity. Therefore, it is concluded that the presence of Trp and Pro at the antepenultimate position of peptide might positively influence the inhibitory activity.

Table 4.14 Amino acid sequence of peptides purified from five mushrooms.

Mushroom	Sequence	Mass (Da)	PeptidRanker score [a]	ACE inhibitory sequences reported in the literature [b]
Enoki mushroom	GCGPESEAN	862.31	0.19	GP, EA
	YRAPSTNG	863.38	0.25	RA, AP, ST, NG
	ENNYALAA	863.39	0.16	NY, YA, AL, LA, LAA, AA
	MPLLRPAA	867.37	0.37	PL, RP, LRP, AA, LL, PA, LLR, MP
	PVCHGRVT	867.38	0.31	HG, GR
Oyster mushroom	LASLFGNDP	933.44	0.43	LA, ASL, SL, LF, FG, DP
Straw mushroom	EVNILAF	804.43	0.30	EV, IL, LA, AF
	KNVTDSF	808.37	0.16	SF
	MLCSTTF	802.40	0.50	ST, TF
Shiitake	VTGGPAAPR	824.55	0.23	TG, GG, GP, GGP, GPA, PA, AA, AAP, AP, PR
	ASPYAFGL	824.56	0.93	ASP, PY, YA, AF, FG, GL
Wood ear mushroom	TVLICVGPF	947.52	0.38	LI, VL, VG, VGP, GP, PF
	MTEFVTRP	979.48	0.21	TE, FV, VTR, RP

[a] Peptide Ranker: http://bioware.ucd.ie/~compass/biowareweb/Server_pages/peptideranker.php

[b] BIOPEP database: http://www.uwm.edu.pl/biochemia/index.php/pl/biopep. SATPdb database: http://crdd.osdd.net/raghava/satpdb/.

4.4 Storage stability of neutralized mushroom hydrolysates

Storage stability test was conducted to monitor the stability of GlcN and ACE inhibitory peptides in the liquid and powdered samples over the storage period under different temperatures (-20, 4, and 26°C).

Table 4.15 showed the remained GlcN content and ACE inhibitory activity during the storage period in enoki mushroom hydrolysate. It was observed that the sample form (liquid or powdered) did not have a significant impact on the GlcN stability. However, the ACE inhibitory peptides stability in powdered sample was much better than that of the liquid sample. At room temperature (26°C), both GlcN content and ACE inhibitory activity decreased over the course of 60 days, especially the liquid samples. There were significant loss of GlcN and ACE inhibitory activity from 30^{th} to 45^{th} day storage. On the other hand, at refrigeration temperature (-20°C and 4°C), their stabilities were not statistically different from those in the beginning control sample ($p < 0.05$). These results suggested that the powdered sample of enoki mushroom hydrolysate should be stored at refrigeration temperature to avoid the loss of bioactive compounds including GlcN and ACE inhibitory peptides.

Table 4.16 recorded the remained GlcN content and ACE inhibitory activity during the storage period in oyster mushroom hydrolysate. It was also observed that the sample form (liquid or powdered) did not have a significant impact on the GlcN stability. However, the ACE inhibitory peptides stability in powdered sample was much better than that of the liquid sample. The effects of storage temperature and time on the stability of GlcN and ACE inhibitory peptides were not significant ($p < 0.05$). These results suggested that the GlcN and ACE inhibitory peptides in oyster mushroom hydrolysate was relatively stable.

Table 4.17 exhibited the stability of GlcN and ACE inhibitory peptides in straw mushroom hydrolysate during the storage period. It was observed that the sample form (liquid or powdered) did not have a significant impact on the ACE inhibitory peptides stability. However, the GlcN stability in powdered sample was much better than that of the liquid sample. The effect of storage temperature was not significant ($p < 0.05$). Moreover, there was a significant loss of GlcN from 15th day storage. Further study is needed for the mechanism of GlcN degradation and improvement of GlcN stability.

The stabilities of GlcN and ACE inhibitory peptides in shiitake hydrolysate were displayed in Table 4.18. Both GlcN content and ACE inhibitory activity of liquid samples were lower than that of powdered samples. The effects of storage temperature and time on the stability of GlcN and ACE inhibitory peptides were not significant ($p < 0.05$). These results suggested that the shiitake hydrolysate, especially powdered form, exhibited relatively great stability.

Table 4.19 was the remained GlcN content and ACE inhibitory activity in wood ear mushroom hydrolysate during the storage period. Both GlcN content and ACE inhibitory activity of liquid samples were lower than that of powdered samples. The effects of storage temperature and time on the stability of GlcN and ACE inhibitory peptides were not significant ($p < 0.05$). However, there was a significant loss of GlcN in liquid sample at room temperature from 30th to 45th day storage.

Table 4.15 Changes in GlcN concentration and ACE inhibitory activity of enoki mushroom hydrolysate during storage at different temperatures.

Temperature (°C)	Time (Days)	Remained GlcN (%)		Relative ACE inhibitory activity (%)	
		Liquid sample	Powdered sample	Liquid sample	Powdered sample
-20	0	100.00 ± 4.21 [a]	100.00 ± 4.39 [a]	100.00 ± 2.56 [a]	100.00 ± 1.16 [a]
	15	98.47 ± 2.42 [a]	98.60 ± 1.17 [a]	97.00 ± 2.67 [a]	101.18 ± 4.51 [a]
	30	98.16 ±2.92 [a]	99.81 ± 2.37 [a]	101.62 ± 4.00 [a]	100.46 ± 2.10 [a]
	45	97.97 ± 3.99 [a]	97.94 ± 1.93 [a]	99.31 ± 4.81 [a]	100.47 ± 3.30 [a]
	60	97.74 ± 2.25 [a]	98.00 ± 3.26 [a]	96.56 ± 3.49 [a]	99.67 ± 3.71 [a]
4	0	100.00 ± 4.21 [a]	100.00 ± 4.39 [a]	100.00 ± 2.56 [a]	100.00 ± 1.16 [a]
	15	98.95 ± 1.87 [a]	97.09 ± 2.62 [a]	100.06 ± 6.08 [a]	105.23 ± 5.05 [a]
	30	98.17 ± 2.20 [a]	96.06 ± 1.86 [a]	101.55 ± 6.10 [a]	101.72 ± 2.29 [a]
	45	95.35 ± 5.02 [a]	99.04 ± 0.68 [a]	95.49 ± 3.35 [ab]	103.17 ± 2.76 [a]
	60	93.73 ± 5.53 [a]	95.92 ± 1.47 [a]	86.12 ± 5.79 [b]	99.41 ± 0.80 [a]
26	0	100.00 ± 4.21 [a]	100.00 ± 4.39 [a]	100.00 ± 2.56 [a]	100.00 ± 1.16 [a]
	15	96.98 ± 2.14 [a]	97.18 ± 1.82 [ab]	95.76 ± 6.93 [ab]	99.86 ± 0.78 [a]
	30	95.24 ± 1.51 [a]	99.18 ± 3.71 [ab]	82.67 ± 6.42 [bc]	99.19 ± 4.74 [a]
	45	82.44 ± 4.04 [b]	94.60 ± 3.72 [ab]	76.82 ± 2.12 [c]	97.57 ± 1.93 [a]
	60	74.58 ± 5.30 [b]	89.47 ± 4.28 [b]	76.81 ± 5.13 [c]	101.37± 2.49 [a]

All data are means and S. D. of triplicate measurements.

The different superscript letters indicate significant differences ($p < 0.05$) between samples when analyzed by one-way ANOVA and post-hoc Tukey's HSD test.

Table 4.16 Changes in GlcN concentration and ACE inhibitory activity of oyster mushroom hydrolysate during storage at different temperatures.

Temperature (°C)	Time (Days)	Remained GlcN (%)		Relative ACE inhibitory activity (%)	
		Liquid sample	Powdered sample	Liquid sample	Powdered sample
-20	0	100.00 ± 2.55 [a]	100.00 ± 1.22 [a]	100.00 ± 0.55 [a]	100.00 ± 2.44 [a]
	15	98.94 ± 1.14 [a]	98.44 ± 0.91 [a]	99.87 ± 2.19 [a]	98.40 ± 7.23 [a]
	30	99.11 ± 1.93 [a]	100.17 ± 4.26 [a]	99.45 ± 0.73 [a]	90.48 ± 2.01 [a]
	45	99.27 ± 0.98 [a]	99.14 ± 2.63 [a]	98.81 ± 0.62 [a]	99.16 ± 0.38 [a]
	60	98.79 ± 0.71 [a]	99.56 ± 3.11 [a]	98.90 ± 1.12 [a]	98.97 ± 9.28 [a]
4	0	100.00 ± 2.55 [a]	100.00 ± 1.22 [a]	100.00 ± 0.55 [a]	100.00 ± 2.44 [ab]
	15	99.74 ± 0.45 [a]	100.53 ± 0.69 [a]	99.64 ± 0.54 [a]	90.81 ± 3.49 [b]
	30	98.93 ± 2.51 [a]	100.50 ± 0.91 [a]	99.09 ± 0.11 [a]	99.94 ± 4.15 [ab]
	45	100.39 ± 0.69 [a]	101.13 ± 0.81 [a]	99.41 ± 0.61 [a]	101.49 ± 3.16 [a]
	60	98.73 ± 1.31 [a]	100.70 ± 1.10 [a]	99.18 ± 0.59 [a]	94.05 ± 5.52 [ab]
26	0	100.00 ± 2.55 [a]	100.00 ± 1.22 [a]	100.00 ± 0.55 [a]	100.00 ± 2.44 [a]
	15	99.17 ± 3.76 [a]	101.29 ± 1.00 [a]	99.78 ± 0.58 [ab]	93.94 ± 6.25 [a]
	30	100.05 ± 1.73 [a]	98.55 ± 3.80 [a]	99.39 ± 0.52 [ab]	99.92 ± 3.71 [a]
	45	100.51 ± 1.50 [a]	100.78 ± 2.15 [a]	99.31 ± 0.97 [ab]	93.77 ± 8.90 [a]
	60	95.89 ± 3.13 [a]	98.24 ± 2.39 [a]	98.10 ± 0.47 [b]	96.18 ± 4.66 [a]

All data are means and S. D. of triplicate measurements.

The different superscript letters indicate significant differences ($p < 0.05$) between samples when analyzed by one-way ANOVA and post-hoc Tukey's HSD test.

Table 4.17 Changes in GlcN concentration and ACE inhibitory activity of straw mushroom hydrolysate during storage at different temperatures.

Temperature (°C)	Time (Days)	Remained GlcN (%)		Relative ACE inhibitory activity (%)	
		Liquid sample	Powdered sample	Liquid sample	Powdered sample
-20	0	100.00 ± 3.24 [a]	100.00 ± 0.85 [a]	100.00 ± 0.64 [a]	100.00 ± 2.01 [a]
	15	100.73 ± 2.87 [a]	99.54 ± 1.88 [a]	101.82 ± 0.96 [a]	99.63 ± 4.89 [a]
	30	98.57 ± 3.81 [a]	99.37 ± 1.26 [a]	99.85 ± 3.49 [a]	100.03 ± 3.99 [a]
	45	96.39 ± 1.09 [a]	99.44 ± 1.27 [a]	97.46 ± 2.30 [a]	95.50 ±6 .81 [a]
	60	96.56 ± 4.97 [a]	98.75 ± 3.80 [a]	99.01 ± 2.51 [a]	98.66 ± 6.17 [a]
4	0	100.00 ± 3.24 [a]	100.00 ± 0.85 [a]	100.00 ± 0.64 [a]	100.00 ± 2.01 [a]
	15	97.53 ± 4.60 [a]	99.41 ± 0.26 [a]	99.04 ± 4.27 [a]	100.09 ± 2.42 [a]
	30	94.63 ± 2.14 [a]	99.71 ± 0.35 [a]	98.98 ± 6.08 [a]	98.68 ± 0.07 [a]
	45	95.16 ± 1.36 [a]	99.38 ± 0.03 [a]	95.73 ± 1.31 [a]	98.96 ± 0.15 [a]
	60	93.32 ± 1.61 [a]	99.68 ± 0.45 [a]	94.80 ± 2.24 [a]	99.71 ± 1.02 [a]
26	0	100.00 ± 3.24 [a]	100.00 ± 0.85 [ab]	100.00 ± 0.64 [a]	100.00 ± 2.01 [a]
	15	94.47 ± 1.76 [b]	98.80 ± 0.67 [b]	97.66 ± 1.89 [a]	99.18 ± 0.67 [a]
	30	92.17 ± 1.66 [b]	99.61 ± 0.95 [ab]	97.53 ± 2.82 [a]	98.70 ± 0.47 [a]
	45	92.23 ± 0.72 [b]	100.90 ± 0.41 [a]	94.54 ± 5.21 [a]	96.97 ± 3.48 [a]
	60	93.24 ± 1.55 [b]	99.14 ± 0.75 [ab]	94.36 ± 4.10 [a]	98.94 ± 1.25 [a]

All data are means and S. D. of triplicate measurements.

The different superscript letters indicate significant differences ($p < 0.05$) between samples when analyzed by one-way ANOVA and post-hoc Tukey's HSD test.

Table 4.18 Changes in GlcN concentration and ACE inhibitory activity of shiitake hydrolysate during storage at different temperatures.

Temperature (°C)	Time (Days)	Remained GlcN (%)		Relative ACE inhibitory activity (%)	
		Liquid sample	Powdered sample	Liquid sample	Powdered sample
-20	0	100.00 ± 1.83 [a]	100.00 ± 2.11 [a]	100.00 ± 1.49 [a]	100.00 ± 0.93 [a]
	15	99.35 ± 0.27 [a]	99.47 ± 4.23 [a]	98.48 ± 9.51 [a]	99.72 ± 1.25 [a]
	30	98.51 ± 1.33 [a]	99.81 ± 1.58 [a]	97.57 ± 1.74 [a]	99.58 ± 2.29 [a]
	45	97.88 ± 1.37 [a]	99.83 ± 1.84 [a]	98.22 ± 1.45 [a]	98.62 ± 1.86 [a]
	60	97.54 ± 2.01 [a]	99.04 ± 2.11 [a]	96.81 ± 5.08 [a]	99.68 ± 4.32 [a]
4	0	100.00 ± 1.83 [a]	100.00 ± 2.11 [a]	100.00 ± 1.49 [a]	100.00 ± 0.93 [a]
	15	98.70 ± 0.88 [a]	99.63 ± 1.45 [a]	99.13 ± 3.79 [a]	98.63 ± 1.32 [a]
	30	98.56 ± 1.15 [a]	98.90 ± 2.13 [a]	95.33 ± 4.77 [a]	99.94 ± 2.61 [a]
	45	97.47 ± 2.14 [a]	98.07 ± 2.98 [a]	96.42 ± 2.82 [a]	99.35 ± 2.18 [a]
	60	97.40 ± 1.83 [a]	98.50 ± 1.72 [a]	94.07 ± 4.18 [a]	98.71 ± 1.69 [a]
26	0	100.00 ± 1.83 [a]	100.00 ± 2.11 [a]	100.00 ± 1.49 [a]	100.00 ± 0.93 [a]
	15	98.09 ± 0.63 [a]	99.05 ± 3.36 [a]	95.62 ± 4.45 [a]	99.35 ± 0.90 [a]
	30	97.05 ± 0.41 [a]	100.34 ± 2.11 [a]	96.86 ± 5.09 [a]	99.75 ± 2.50 [a]
	45	97.68 ± 0.31 [a]	99.84 ± 1.96 [a]	93.89 ± 5.69 [a]	98.99 ± 2.93 [a]
	60	96.67 ± 2.68 [a]	99.54 ± 2.17 [a]	93.50 ± 6.03 [a]	97.86 ± 1.23 [a]

All data are means and S. D. of triplicate measurements.

The different superscript letters indicate significant differences ($p < 0.05$) between samples when analyzed by one-way ANOVA and post-hoc Tukey's HSD test.

Table 4.19 Changes in GlcN concentration and ACE inhibitory activity of wood ear mushroom hydrolysate during storage at different temperatures.

Temperature (°C)	Time (Days)	Remained GlcN (%)		Relative ACE inhibitory activity (%)	
		Liquid sample	Powdered sample	Liquid sample	Powdered sample
-20	0	100.00 ± 0.71 [a]	100.00 ± 3.09 [a]	100.00 ± 1.53 [a]	100.00 ± 1.28 [a]
	15	99.06 ± 1.20 [a]	100.96 ± 3.64 [a]	99.47 ± 1.04 [a]	99.39 ± 3.52 [a]
	30	97.08 ± 1.46 [a]	99.53 ± 4.32 [a]	98.73 ± 1.62 [a]	99.50 ± 1.50 [a]
	45	97.13 ± 1.58 [a]	99.95 ± 3.12 [a]	96.95 ± 1.51 [a]	99.51 ± 2.72 [a]
	60	98.04 ± 0.92 [a]	99.56 ± 4.43 [a]	96.96 ± 3.96 [a]	99.57 ± 2.76 [a]
4	0	100.00 ± 0.71 [a]	100.00 ± 3.09 [a]	100.00 ± 1.53 [a]	100.00 ± 1.28 [a]
	15	99.74 ± 1.60 [a]	99.01 ± 2.17 [a]	98.75 ± 1.29 [a]	99.92 ± 0.72 [a]
	30	97.13 ± 3.16 [a]	100.68 ± 3.37 [a]	97.38 ± 3.50 [a]	99.05 ± 1.80 [a]
	45	96.27 ± 2.39 [a]	98.61 ± 5.05 [a]	96.02 ± 1.96 [a]	100.33 ± 1.81 [a]
	60	95.13 ± 3.87 [a]	100.03 ± 3.56 [a]	96.08 ± 4.70 [a]	98.79 ± 1.01 [a]
26	0	100.00 ± 0.71 [a]	100.00 ± 3.09 [a]	100.00 ± 1.53 [a]	100.00 ± 1.28 [a]
	15	98.45 ± 1.50 [a]	100.87 ± 3.53 [a]	97.89 ± 1.88 [a]	100.39 ± 1.56 [a]
	30	94.60 ± 3.40 [ab]	99.96 ± 2.84 [a]	96.64 ± 3.66 [a]	99.03 ± 1.56 [a]
	45	90.90 ± 2.73 [b]	99.94 ± 3.28 [a]	94.08 ± 5.05 [a]	98.81 ± 3.51 [a]
	60	89.38 ± 2.89 [b]	97.95 ± 2.69 [a]	93.12 ± 5.01 [a]	98.58 ± 2.25 [a]

All data are means and S. D. of triplicate measurements.

The different superscript letters indicate significant differences ($p < 0.05$) between samples when analyzed by one-way ANOVA and post-hoc Tukey's HSD test.

CHAPTER V

CONCLUSION

Mushrooms have been proven to be excellent foods for well-balanced and healthy diets. Thus, in this study, five mushrooms were investigated for their potential to produce hydrolysates containing GlcN and ACE inhibitory peptides with multifunctional activities. Sulfuric acid was found to be more effective than hydrochloric acid for GlcN production. Then, the developed mathematical model identified the optimum conditions (sulfuric acid, 5.67 M; hydrolysis temperature, 100°C; and time, 4.03 h). The highest GlcN yield (56.81±3.57 mg/g), which was dependent on the chitin content of raw material, was obtained from straw mushroom. Acid hydrolysis kinetics of five mushrooms fitted well with the first-order reaction model with activation energies ranging from 15.91 to 203.55 kJ/mol. The relatively low activation energy for hydrolysis of straw mushroom indicated that it was energy-saving. Thus, sulfuric acid hydrolysis of straw mushroom for GlcN production could be considered as an efficient process for the future industrial application. Subsequently, GlcN and ACE inhibitory peptides were successfully purified from these hydrolysates using decoloration, ultrafiltration and ethanol precipitation, and sequential chromatographic methods. Three novel potential ACE inhibitory peptides were identified as ASPYAFGL, MLCSTTF, and LASLFGNDP with IC_{50} values of 0.1080, 0.1524, and 0.2491 μM, respectively. These results suggested that mushrooms

can be a good source of raw materials for the production of GlcN and ACE inhibitory peptides.

Sulfuric acid hydrolysis, an efficient method developed in this study, has the advantages of simple plant, easy operation, low-energy consumption, and low operation cost for industrial application. Compared with crustacean shells, the traditional and current commercial source of GlcN, the supply of mushrooms is stable and unlimited since they can be cultivated economically throughout the year in a short period without geographical and seasonal restrictions. Additionally, mushrooms are relatively consistent in composition and are not associated with inorganic materials; thus, no demineralization treatment is required and the heavy metal hazard can be avoided. Moreover, GlcN and ACE inhibitory peptides from mushrooms are suitable for shellfish-allergic consumers. Particularly, more and more consumers are choosing a vegetarian lifestyle for environmental, health, religious or ethical reasons. Thus, these products from mushrooms could meet the increasing demand of vegetarian market and criteria of vegetarian labeling regulations, such as V-Label developed by the European Vegetarian Union. Therefore, mushrooms' potential as an economical and low-cost source of GlcN and ACE inhibitory peptides should be encouraged.

REFERENCES

Adejumo, T. and Awosanya, O. (2005). Proximate and mineral composition of four edible mushroom species from South Western Nigeria. **African Journal of Biotechnology**. 4(10):1084-1088.

Aghazadeh-Habashi, A., Carran, J., Anastassiades, T. and Jamali, F. (2005). High performance liquid chromatographic determination of N-butyryl glucosamine in rat plasma. **Journal of Chromatography B**. 819(1):91-96.

Aghazadeh-Habashi, A., Sattari, S., Pasutto, F. and Jamali, F. (2002). High performance liquid chromatographic determination of glucosamine in rat plasma. **Journal of Pharmacy and Pharmaceutical Sciences**. 5(2):176-180.

Agrahar-Murugkar, D. and Subbulakshmi, G. (2005). Nutritional value of edible wild mushrooms collected from the Khasi hills of Meghalaya. **Food Chemistry**. 89(4):599-603.

Aguilar, R., Ramırez, J., Garrote, G. and Vázquez, M. (2002). Kinetic study of the acid hydrolysis of sugar cane bagasse. **Journal of Food Engineering**. 55(4):309-318.

Ahmad, A., Alkharfy, K. M., Wani, T. A. and Raish, M. (2015). Application of Box–Behnken design for ultrasonic-assisted extraction of polysaccharides from *Paeonia emodi*. **International Journal of Biological Macromolecules**. 72:990-997.

Ahn, C.-B., Jeon, Y.-J., Kim, Y.-T. and Je, J.-Y. (2012). Angiotensin I converting enzyme (ACE) inhibitory peptides from salmon byproduct protein hydrolysate

by Alcalase hydrolysis. **Process Biochemistry**. 47(12):2240-2245.

Aihara, K., Kajimoto, O., Hirata, H., Takahashi, R. and Nakamura, Y. (2005). Effect of powdered fermented milk with *Lactobacillus helveticus* on subjects with high-normal blood pressure or mild hypertension. **Journal of the American College of Nutrition**. 24(4):257-265.

Ajavakom, A., Supsvetson, S., Somboot, A. and Sukwattanasinitt, M. (2012). Products from microwave and ultrasonic wave assisted acid hydrolysis of chitin. **Carbohydrate Polymers**. 90(1):73-77.

Akamatsu, S. and Mitsuhashi, T. (2012). Development of a simple capillary electrophoretic determination of glucosamine in nutritional supplements using in-capillary derivatisation with *o*-phthalaldehyde. **Food Chemistry**. 130(4):1137-1141.

Akıllıoğlu, H. G. and Karakaya, S. (2009). Effects of heat treatment and *in vitro* digestion on the angiotensin converting enzyme inhibitory activity of some legume species. **European Food Research and Technology**. 229(6):915-921.

Ali, A. A., Lewis, S. M., Badgley, H. L., Allaben, W. T. and Leakey, J. E. A. (2011). Oral glucosamine increases expression of transforming growth factor β1 (TGFβ1) and connective tissue growth factor (CTGF) mRNA in rat cartilage and kidney: Implications for human efficacy and toxicity. **Archives of Biochemistry and Biophysics**. 510(1):11-18.

Allan, G. G. and Peyron, M. (1995). Molecular weight manipulation of chitosan I: kinetics of depolymerization by nitrous acid. **Carbohydrate Research**. 277(2):257-272.

Altman, R., Asch, E., Bloch, D., Bole, G., Borenstein, D., Brandt, K., Christy, W., Cooke, T., Greenwald, R. and Hochberg, M. (1986). Development of criteria

for the classification and reporting of osteoarthritis: classification of osteoarthritis of the knee. **Arthritis and Rheumatism**. 29(8):1039-1049.

Aluko, R. E. (2015). Antihypertensive peptides from food proteins. **Annual Review of Food Science and Technology**. 6:235-262.

Anderson, J. W., Nicolosi, R. J. and Borzelleca, J. F. (2005). Glucosamine effects in humans: a review of effects on glucose metabolism, side effects, safety considerations and efficacy. **Food and Chemical Toxicology**. 43(2):187-201.

Anderson, T., Zhou, S., Bergerud, B., Fosdick, L. E., Han, T.-J., Vasina, J., Vanderhoff, A., Trinkle, J. R., Ray, B. L. and Bohlmann, J. A. (2012). *U.S. Patent No. 8222232 B2*. Washington, DC: U.S. Patent and Trademark Office.

AOAC. (2000). Official methods of analysis (17th ed.). Gaithersburg, MD, USA: Association of Official Chemists.

Appel, L. J., Frohlich, E. D., Hall, J. E., Pearson, T. A., Sacco, R. L., Seals, D. R., Sacks, F. M., Smith, S. C., Vafiadis, D. K. and Van Horn, L. V. (2011). The importance of population-wide sodium reduction as a means to prevent cardiovascular disease and stroke a call to action from the american heart association. **Circulation**. 123(10):1138-1143.

Appolinaire, K. K., Hubert, K. K., Parfait, K. E. J. and Patrice, K. L. (2015). Nutritional composition of three edible ectomycorrhizal mushrooms from Center of Côte d'Ivoire. **International Journal of Biosciences**. 7(4):120-127.

Aranaz, I., Mengibar, M., Harris, R., Panos, I., Miralles, B., Acosta, N., Galed, G. and Heras, A. (2009). Functional Characterization of Chitin and Chitosan. **Current Chemical Biology**. 3(2):203-230.

Arunkumar, P., Indulekha, S., Vijayalakshmi, S. and Srivastava, R. (2016). Poly (caprolactone) microparticles and chitosan thermogels based injectable

formulation of etoricoxib for the potential treatment of osteoarthritis. **Materials Science and Engineering: C**. 61:534-544.

Ashar, M. N. and Chand, R. (2004). Antihypertensive peptides purified from milks fermented with *Lactobacillus delbrueckii ssp bulgaricus*. **Milchwissenschaft-Milk Science International**. 59(1-2):14-17.

Azuma, K., Osaki, T., Kurozumi, S., Kiyose, M., Tsuka, T., Murahata, Y., Imagawa, T., Itoh, N., Minami, S., Sato, K. and Okamoto, Y. (2015). Anti-inflammatory effects of orally administered glucosamine oligomer in an experimental model of inflammatory bowel disease. **Carbohydrate Polymers**. 115:448-456.

Bütikofer, U., Meyer, J., Sieber, R., Walther, B. and Wechsler, D. (2008). Occurrence of the angiotensin-converting enzyme-inhibiting tripeptides Val-Pro-Pro and Ile-Pro-Pro in different cheese varieties of Swiss origin. **Journal of Dairy Science**. 91(1):29-38.

Bütikofer, U., Meyer, J., Sieber, R. and Wechsler, D. (2007). Quantification of the angiotensin-converting enzyme-inhibiting tripeptides Val-Pro-Pro and Ile-Pro-Pro in hard, semi-hard and soft cheeses. **International Dairy Journal**. 17(8):968-975.

Baba, Y., Noma, H., Nakayama, R. and Matsushita, Y. (2002). Preparation of chitosan derivatives containing methylthiocarbamoyl and phenylthiocarbamoyl groups and their selective adsorption of copper (II) over iron (III). **Analytical Sciences**. 18(3):359-361.

Balti, R., Bougatef, A., Sila, A., Guillochon, D., Dhulster, P. and Nedjar-Arroume, N. (2015). Nine novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (*Sepia officinalis*) muscle protein hydrolysates and

antihypertensive effect of the potent active peptide in spontaneously hypertensive rats. **Food Chemistry**. 170:519-525.

Balti, R., Nedjar-Arroume, N., Bougatef, A., Guillochon, D. and Nasri, M. (2010). Three novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (*Sepia officinalis*) using digestive proteases. **Food Research International**. 43(4):1136-1143.

Banerjee, P. and Shanthi, C. (2012). Isolation of novel bioactive regions from bovine Achilles tendon collagen having angiotensin I-converting enzyme-inhibitory properties. **Process Biochemistry**. 47(12):2335-2346.

Bano, Z., Srinivasan, K. and Srivastava, H. (1963). Amino acid composition of the protein from a mushroom (*Pleurotus sp.*). **Applied and Environmental Microbiology**. 11(3):184-187.

Baron, A., Zhu, J., Zhu, J., Weldon, H., Maianu, L. and Garvey, W. (1995). Glucosamine induces insulin resistance *in vivo* by affecting GLUT 4 translocation in skeletal muscle. Implications for glucose toxicity. **The Journal of Clinical Investigation**. 96(6):2792.

Barros, L., Calhelha, R. C., Vaz, J. A., Ferreira, I. C., Baptista, P. and Estevinho, L. M. (2007). Antimicrobial activity and bioactive compounds of Portuguese wild edible mushrooms methanolic extracts. **European Food Research and Technology**. 225(2):151-156.

Bertrand-Harb, C., Baday, A., Dalgalarrondo, M., Chobert, J. and Haertle, T. (2002). Thermal modifications of structure and codenaturation of α-lactalbumin and β-lactoglobulin induce changes of solubility and susceptibility to proteases. **Nahrung/Food**. 46(4):283-289.

Beyreuther, B., Callizot, N. and Stohr, T. (2007). Antinociceptive efficacy of lacosamide in the monosodium iodoacetate rat model for osteoarthritis pain. **Arthritis Research and Therapy**. 9(1):R14.

Bijlsma, J. W., Berenbaum, F. and Lafeber, F. P. (2011). Osteoarthritis: an update with relevance for clinical practice. **The Lancet**. 377(9783):2115-2126.

Binod, P., Sandhya, C., Suma, P., Szakacs, G. and Pandey, A. (2007). Fungal biosynthesis of endochitinase and chitobiase in solid state fermentation and their application for the production of *N-acetyl*-D-glucosamine from colloidal chitin. **Bioresource Technology**. 98(14):2742-2748.

Bitton, R. (2009). The economic burden of osteoarthritis. **The American Journal of Managed Care**. 15(8 Suppl):S230-235.

Bloom, D. E., Cafiero, E. T., Jané-Llopis, E., Abrahams-Gessel, S., Bloom, L. R., Fathima, S., Feigl, A.B., Gaziano, T., Mowafi, M., Pandya, A., Prettner, K., Rosenberg, L., Seligman, B., Stein, A. Z. and Weinstein, C. (2011). The global economic burden of noncommunicable diseases: PGDA Working Papers, Program on the Global Demography of Aging, http://EconPapers.repec.org/RePEc:gdm:wpaper:8712.

Boa, E. R. (2004). *Wild edible fungi: a global overview of their use and importance to people*. Rome, Italy: Food and Agriculture Organization of the United Nations.

Bombardier, C., Hawker, G. and Mosher, D. (2011). The impact of arthritis in Canada: today and over the next 30 years. **Arthritis Alliance of Canada**. 606:1-52.

Bondeson, D., Mathew, A. and Oksman, K. (2006). Optimization of the isolation of nanocrystals from microcrystalline cellulose by acid hydrolysis. **Cellulose**. 13:171-180.

Bonomi, F., Fiocchi, A., Frøkiær, H., Gaiaschi, A., Iametti, S., Poiesi, C., Rasmussen,

P., Restani, P. and Rovere, P. (2003). Reduction of immunoreactivity of bovine β-lactoglobulin upon combined physical and proteolytic treatment. **Journal of Dairy Research**. 70(1):51-59.

Bouhallab, S. and Touzé, C. (1995). Continuous hydrolysis of caseinomacropeptide in a membrane reactor: kinetic study and gram-scale production of antithrombotic peptides. **Le Lait**. 75(3):251-258.

Braaksma, A. and Schaap, D. J. (1996). Protein analysis of the common mushroom *Agaricus bisporus*. **Postharvest Biology and Technology**. 7:119-127.

Bradley, J. D., Brandt, K. D., Katz, B. P., Kalasinski, L. A. and Ryan, S. I. (1991). Comparison of an antiinflammatory dose of ibuprofen, an analgesic dose of ibuprofen, and acetaminophen in the treatment of patients with osteoarthritis of the knee. **New England Journal of Medicine**. 325:87-91.

Bruyère, O., Pavelka, K., Rovati, L., Gatterova, J., Giacovelli, G., Olejarova, M., Deroisy, R. and Reginster, J.-Y. (2008). Total joint replacement after glucosamine sulphate treatment in knee osteoarthritis: results of a mean 8-year observation of patients from two previous 3-year, randomised, placebo-controlled trials. **Osteoarthritis and Cartilage**. 16:254-260.

Burczyk, J., Śmietana, B., Termińska-Pabis, K., Zych, M. and Kowalowski, P. (1999). Comparison of nitrogen content amino acid composition and glucosamine content of cell walls of various chlorococcalean algae. **Phytochemistry**. 51:491-497.

Byun, H.-G. and Kim, S.-K. (2001). Purification and characterization of angiotensin I converting enzyme (ACE) inhibitory peptides from Alaska pollack (*Theragra chalcogramma*) skin. **Process Biochemistry**. 36:1155-1162.

Byun, H.-G. and Kim, S.-K. (2002). Structure and activity of angiotensin I converting enzyme inhibitory peptides derived from Alaskan pollack skin. **BMB Reports**. 35:239-243.

Calamia, V., Ruiz-Romero, C., Rocha, B., Fernández-Puente, P., Mateos, J., Montell, E., Vergés, J. and Blanco, F. J. (2010). Pharmacoproteomic study of the effects of chondroitin and glucosamine sulfate on human articular chondrocytes. **Arthritis Research and Therapy**. 12(4):R138-R149.

Campo, G. M., Campo, S., Ferlazzo, A. M., Vinci, R. and Calatroni, A. (2001). Improved high-performance liquid chromatographic method to estimate aminosugars and its application to glycosaminoglycan determination in plasma and serum. **Journal of Chromatography B: Biomedical Sciences and Applications**. 765:151-160.

Carroad, P. A. and Tom, R. A. (1978). Bioconversion of shellfish chitin wastes: process conception and selection of microorganisms. **Journal of Food Science**. 43:1158-1161.

Carter, S., Nokes, S. E. and Crofcheck, C. (2004). The influence of environmental temperature and substrate initial moisture content on *Aspergillus niger* growth and phytase production in solid-state cultivation. **Transactions of the ASAE**. 47(3):945-949.

Chalé, F. G. H., Ruiz, J. C. R., Fernández, J. J. A., Ancona, D. A. B. and Campos, M. R. S. (2014). ACE inhibitory, hypotensive and antioxidant peptide fractions from *Mucuna pruriens* proteins. **Process Biochemistry**. 49:1691-1698.

Chang, H.-H., Chien, P.-J., Tong, M.-H. and Sheu, F. (2007). Mushroom immunomodulatory proteins possess potential thermal/freezing resistance, acid/alkali tolerance and dehydration stability. **Food Chemistry**. 105:597-605.

Chang, Y. (2008). Optimum production of GlcN using *Aspergillus sp.*, thesis: Dept. Chemical Engineering and Materials Science, Yuan Ze University, Taiwan.

Chang, Y. F., Sitanggang, A. B. and Wu, H.-S. (2011). Optimizing biotechnological production of glucosamine as food ingredient from *Aspergillus* sp. BCRC 31742. **Journal of Food Technology**. 9(2):75-82.

Chen, J.-K., Shen, C.-R. and Liu, C.-L. (2010). N-acetylglucosamine: production and applications. **Marine Drugs**. 8:2493-2516.

Chen, J., Liu, S., Ye, R., Cai, G., Ji, B. and Wu, Y. (2013a). Angiotensin-I converting enzyme (ACE) inhibitory tripeptides from rice protein hydrolysate: Purification and characterization. **Journal of Functional Foods**. 5:1684-1692.

Chen, J., Wang, Y., Ye, R., Wu, Y. and Xia, W. (2013b). Comparison of analytical methods to assay inhibitors of angiotensin I-converting enzyme. **Food Chemistry**. 141:3329-3334.

Chen, J., Wang, Y., Zhong, Q., Wu, Y. and Xia, W. (2012). Purification and characterization of a novel angiotensin-I converting enzyme (ACE) inhibitory peptide derived from enzymatic hydrolysate of grass carp protein. **Peptides**. 33:52-58.

Chen, X., Liu, L., Li, J., Du, G. and Chen, J. (2012). Improved glucosamine and N-acetylglucosamine production by an engineered *Escherichia coli* via step-wise regulation of dissolved oxygen level. **Bioresource Technology**. 110:534-538.

Chen, Y., Jiang, S., Jin, Y., Yin, Y., Yu, G., Lan, X., Cui, M., Liang, Y., Wong, B. H. C. and Guo, L. (2012). Purification and characterization of an antitumor protein with deoxyribonuclease activity from edible mushroom *Agrocybe aegerita*. **Molecular Nutrition and Food Research**. 56:1729-1738.

Cheung, H.-S., Wang, F.-l., Ondetti, M. A., Sabo, E. F. and Cushman, D. W. (1980). Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Importance of the COOH-terminal dipeptide sequence. **Journal of Biological Chemistry**. 255:401-407.

Cheung, P. C. K. (1996). Dietary Fiber Content and Composition of Some Cultivated Edible Mushroom Fruiting Bodies and Mycelia. **Journal of Agricultural and Food Chemistry**. 44:468-471.

Chicón, R., Belloque, J., Recio, I. and López-Fandiño, R. (2006). Influence of high hydrostatic pressure on the proteolysis of β-lactoglobulin A by trypsin. **Journal of Dairy Research**. 73:121-128.

Chicón, R., López-Fandiño, R., Quirós, A. and Belloque, J. (2006). Changes in chymotrypsin hydrolysis of β-lactoglobulin A induced by high hydrostatic pressure. **Journal of Agricultural and Food Chemistry**. 54:2333-2341.

Chiusaroli, R., Piepoli, T., Zanelli, T., Ballanti, P., Lanza, M., Rovati, L. C. and Caselli, G. (2011). Experimental Pharmacology of Glucosamine Sulfate. **International Journal of Rheumatology**. 2011:8.

Choi, H., Cho, H., Yang, H., Ra, K. and Suh, H. J. (2001). Angiotensin I-converting enzyme inhibitor from *Grifola frondosa*. **Food Research International**. 34:177-182.

Choi, W.-S., Ahn, K.-J., Lee, D.-W., Byun, M.-W. and Park, H.-J. (2002). Preparation of chitosan oligomers by irradiation. **Polymer Degradation and Stability**. 78:533-538.

Cimmino, M., Parisi, M., Moggiana, G., Maio, T. and Mela, G. (2001). Prevalence of self-reported peripheral joint pain and swelling in an Italian population: the Chiavari study. **Clinical and Experimental Rheumatology**. 19:35-40.

Cinq-Mars, C. D., Hu, C., Kitts, D. D. and Li-Chan, E. C. (2007). Investigations into inhibitor type and mode, simulated gastrointestinal digestion, and cell transport of the angiotensin I-converting enzyme-inhibitory peptides in Pacific hake (*Merluccius productus*) fillet hydrolysate. **Journal of Agricultural and Food Chemistry**. 56:410-419.

Clegg, D. O., Reda, D. J., Harris, C. L., Klein, M. A., O'Dell, J. R., Hooper, M. M., Bradley, J. D., Bingham III, C. O., Weisman, M. H. and Jackson, C. G. (2006). Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. **New England Journal of Medicine**. 354:795-808.

Contreras, M. d. M., Carrón, R., Montero, M. J., Ramos, M. and Recio, I. (2009). Novel casein-derived peptides with antihypertensive activity. **International Dairy Journal**. 19:566-573.

Cosio, I. G., Fisher, R. A. and Carroad, P. A. (1982). Bioconversion of Shellfish Chitin Waste: Waste Pretreatment, Enzyme Production, Process Design, and Economic Analysis. **Journal of Food Science**. 47:901-905.

Crestini, C., Kovac, B. and Giovannozzi-Sermanni, G. (1996). Production and isolation of chitosan by submerged and solid-state fermentation from *Lentinus edodes*. **Biotechnology and Bioengineering**. 50:207-210.

Cushman, D. and Cheung, H. (1971). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. **Biochemical Pharmacology**. 20:1637-1648.

da Cruz, J. N., Pimenta, D. C., de Melo, R. L. and Nascimento, J. R. (2016). Isolation and biochemical characterisation of angiotensin-converting enzyme inhibitory peptides derived from the enzymatic hydrolysis of cupuassu seed protein isolate. **Journal of Functional Foods**. 27:104-114.

Dahaghin, S., Bierma-Zeinstra, S. M., Ginai, A., Pols, H., Hazes, J. and Koes, B. (2005). Prevalence and pattern of radiographic hand osteoarthritis and association with pain and disability (the Rotterdam study). **Annals of the Rheumatic Diseases**. 64:682-687.

del Castillo, M. D., Ferrigno, A., Acampa, I., Borrelli, R. C., Olano, A., Martínez-Rodríguez, A. and Fogliano, V. (2007). *In vitro* release of angiotensin-converting enzyme inhibitors, peroxyl-radical scavengers and antibacterial compounds by enzymatic hydrolysis of glycated gluten. **Journal of Cereal Science**. 45:327-334.

Deng, M.-D., Severson, D. K., Grund, A. D., Wassink, S. L., Burlingame, R. P., Berry, A., Running, J. A., Kunesh, C. A., Song, L., Jerrell, T. A. and Rosson, R. A. (2005). Metabolic engineering of *Escherichia coli* for industrial production of glucosamine and N-acetylglucosamine. **Metabolic Engineering**. 7:201-214.

Deng, M.-D., Wassink, S. L. and Grund, A. D. (2006). Engineering a new pathway for N-acetylglucosamine production: Coupling a catabolic enzyme, glucosamine-6-phosphate deaminase, with a biosynthetic enzyme, glucosamine-6-phosphate N-acetyltransferase. **Enzyme and Microbial Technology**. 39:828-834.

Derringer, G. and Suich, R. (1980). Simultaneous optimization of several response variables. **Journal of Quality Technology**. 12:214-219.

Desai, P., Coutinho, E. and Srivastava, S. (2002). Conformational diversity of T-kinin in DMSO, water and HFA. **European Journal of Medicinal Chemistry**. 37:135-146.

DiBonaventura, M. d., Gupta, S., McDonald, M., Sadosky, A., Pettitt, D. and Silverman, S. (2012). Impact of self-rated osteoarthritis severity in an

employed population: cross-sectional analysis of data from the national health and wellness survey. **Health and Quality of Life Outcomes**. 10:1-12.

Dieppe, P. A. and Lohmander, L. S. (2005). Pathogenesis and management of pain in osteoarthritis. **The Lancet**. 365:965-973.

Dıez, V. and Alvarez, A. (2001). Compositional and nutritional studies on two wild edible mushrooms from northwest Spain. **Food Chemistry**. 75:417-422.

Donzelli, B. G. G., Ostroff, G. and Harman, G. E. (2003). Enhanced enzymatic hydrolysis of langostino shell chitin with mixtures of enzymes from bacterial and fungal sources. **Carbohydrate Research**. 338:1823-1833.

Du, L., Fang, M., Wu, H., Xie, J., Wu, Y., Li, P., Zhang, D., Huang, Z., Xia, Y. and Zhou, L. (2013). A novel angiotensin I-converting enzyme inhibitory peptide from *Phascolosoma esculenta* water-soluble protein hydrolysate. **Journal of Functional Foods**. 5:475-483.

EI-Saharty, Y. S. and Bary, A. A. (2002). High-performance liquid chromatographic determination of neutraceuticals, glucosamine sulphate and chitosan, in raw materials and dosage forms. **Analytica Chimica Acta**. 462:125-131.

Eikenes, M., Fongen, M., Roed, L. and Stenstrøm, Y. (2005). Determination of chitosan in wood and water samples by acidic hydrolysis and liquid chromatography with online fluorescence derivatization. **Carbohydrate Polymers**. 61:29-38.

Einbu, A., Grasdalen, H. and Vårum, K. M. (2007). Kinetics of hydrolysis of chitin/chitosan oligomers in concentrated hydrochloric acid. **Carbohydrate Research**. 342:1055-1062.

Einbu, A. and Vårum, K. M. (2008). Characterization of chitin and its hydrolysis to GlcNAc and GlcN. **Biomacromolecules**. 9:1870-1875.

Erdogan, S., Kaya, M. and Akata, I. (2017). Chitin extraction and chitosan production from cell wall of two mushroom species (*Lactarius vellereus* and *Phyllophora ribis*). Paper presented at the AIP Conference Proceedings.

Escudero, E., Aristoy, M.-C., Nishimura, H., Arihara, K. and Toldrá, F. (2012). Antihypertensive effect and antioxidant activity of peptide fractions extracted from Spanish dry-cured ham. **Meat Science**. 91:306-311.

Escudero, E., Mora, L. and Toldrá, F. (2014). Stability of ACE inhibitory ham peptides against heat treatment and in *vitro* digestion. **Food Chemistry**. 161:305-311.

Escudero, E., Sentandreu, M. A., Arihara, K. and Toldrá, F. (2010). Angiotensin I-converting enzyme inhibitory peptides generated from *in vitro* gastrointestinal digestion of pork meat. **Journal of Agricultural and Food Chemistry**. 58:2895-2901.

Esters, V., Angenot, L., Brandt, V., Frédérich, M., Tits, M., Van Nerum, C., Wauters, J.-N. and Hubert, P. (2006). Validation of a high-performance thin-layer chromatography/densitometry method for the quantitative determination of glucosamine in a herbal dietary supplement. **Journal of Chromatography A**. 1112:156-164.

Fan, W., Bohlmann, J. A., Trinkle, J. R., Steinke, J. D., Hwang, K.-O. and Henning, J. P. (2006). *U.S. Patent No. 7049433 B2*. Washington, DC: U.S. Patent and Trademark Office.

Fenton, J., Chlebek-Brown, K., Caron, J. and Orth, M. (2002). Effect of glucosamine on interleukin-1-conditioned articular cartilage. **Equine Veterinary Journal**. 34:219-223.

Ferreira, I. C. F. R., Vaz, J. A., Vasconcelos, M. H. and Martins, A. (2010). Compounds from wild mushrooms with antitumor potential. **Anti-Cancer Agents in**

Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents). 10:424-436.

Ferreira, S. H., Bartelt, D. C. and Greene, L. J. (1970). Isolation of bradykinin potentiating peptides from Bothrops jararaca venom. **Biochemistry**. 9:11.

Ferrer, J., Paez, G., Marmol, Z., Ramones, E., Garcia, H. and Forster, C. F. (1996). Acid hydrolysis of shrimp-shell wastes and the production of single cell protein from the hydrolysate. **Bioresource Technology**. 57:55-60.

FitzGerald, R. J. and Meisel, H. (2000). Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. **British Journal of Nutrition**. 84:33-37.

Fitzgerald, R. J. and Murray, B. A. (2006). Bioactive peptides and lactic fermentations. **International Journal of Dairy Technology**. 59:118-125.

Forchhammer, L., Thorn, M., Met, O., Gad, M., Weidner, M. and Claesson, M. H. (2003). Immunobiological effects of glucosamine *in vitro*. **Scandinavian Journal of Immunology**. 58:404-411.

Forghani, B., Zarei, M., Ebrahimpour, A., Philip, R., Bakar, J., Hamid, A. A. and Saari, N. (2016). Purification and characterization of angiotensin converting enzyme-inhibitory peptides derived from *Stichopus horrens*: Stability study against the ACE and inhibition kinetics. **Journal of Functional Foods**. 20:276-290.

Fosdick, L. E., Bohlmann, J. A., Trinkle, J. R. and Ray, B. L. (2011). *U.S. Patent No. 8034925 B2*. Washington, DC: U.S. Patent and Trademark Office.

Fuglsang, A., Nilsson, D. and Nyborg, N. C. (2002). Cardiovascular effects of fermented milk containing angiotensin-converting enzyme inhibitors evaluated in permanently catheterized, spontaneously hypertensive rats. **Applied and Environmental Microbiology**. 68:3566-3569.

Fuglsang, A., Rattray, F. P., Nilsson, D. and Nyborg, N. C. (2003). Lactic acid bacteria: inhibition of angiotensin converting enzyme *in vitro* and *in vivo*. **Antonie van Leeuwenhoek**. 83:27-34.

Fujita, H., Yamagami, T. and Ohshima, K. (2001). Effects of an ACE-inhibitory agent, katsuobushi oligopeptide, in the spontaneously hypertensive rat and in borderline and mildly hypertensive subjects. **Nutrition Research**. 21:1149-1158.

Furst, D. E. (1994). Are there differences among nonsteroidal antiinflammatory drugs? **Arthritis and Rheumatism**. 37:1-9.

Gómez-Ruiz, J. A., Recio, I. and Belloque, J. (2004). ACE-Inhibitory activity and structural properties of peptide Asp-Lys-Ile-His-Pro [β-CN f (47-51)]. Study of the peptide forms synthesized by different methods. **Journal of Agricultural and Food Chemistry**. 52:6315-6319.

Gómez-Ruiz, J. Á., Taborda, G., Amigo, L., Recio, I. and Ramos, M. (2006). Identification of ACE-inhibitory peptides in different Spanish cheeses by tandem mass spectrometry. **European Food Research and Technology**. 223:595-601.

Gabriel, S. E., Jaakkimainen, L. and Bombardier, C. (1991). Risk for serious gastrointestinal complications related to use of nonsteroidal anti-inflammatory drugs: a meta-analysis. **Annals of Internal Medicine**. 115:787-796.

Gandhi, N. and Laidler, J. K. (2002). *U.S. Patent No. 6486307 B1*. Washington, DC: U.S. Patent and Trademark Office.

García-Moreno, P. J., Espejo-Carpio, F. J., Guadix, A. and Guadix, E. M. (2015). Production and identification of angiotensin I-converting enzyme (ACE)

inhibitory peptides from Mediterranean fish discards. **Journal of Functional Foods**. 18:95-105.

García-Tejedor, A., Sánchez-Rivera, L., Castelló-Ruiz, M., Recio, I., Salom, J. B. and Manzanares, P. (2014). Novel antihypertensive lactoferrin-derived peptides produced by *Kluyveromyces marxianus*: gastrointestinal stability profile and *in vivo* angiotensin I-converting enzyme (ACE) inhibition. **Journal of Agricultural and Food Chemistry**. 62:1609-1616.

Garcia-Mora, P., Peñas, E., Frias, J., Zielinski, H., Wiczkowski, W., Zielinska, D. and Martinez-Villaluenga, C. (2016). High-Pressure-Assisted Enzymatic Release of Peptides and Phenolics Increases Angiotensin Converting Enzyme I Inhibitory and Antioxidant Activities of Pinto Bean Hydrolysates. **Journal of Agricultural and Food Chemistry**. 64:1730-1740.

Geng, X., Tian, G., Zhang, W., Zhao, Y., Zhao, L., Ryu, M., Wang, H. and Ng, T. B. (2015). Isolation of an angiotensin I-converting enzyme inhibitory protein with antihypertensive effect in spontaneously hypertensive rats from the edible wild mushroom *Leucopaxillus tricolor*. **Molecules**. 20:10141-10153.

Geng, X., Tian, G., Zhang, W., Zhao, Y., Zhao, L., Wang, H. and Ng, T. B. (2016). A *Tricholoma matsutake* Peptide with Angiotensin Converting Enzyme Inhibitory and Antioxidative Activities and Antihypertensive Effects in Spontaneously Hypertensive Rats. **Scientific Rports**. 6.

Gibbs, B. F., Zougman, A., Masse, R. and Mulligan, C. (2004). Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. **Food Research International**. 37:123-131.

Gill, I., López-Fandiño, R., Jorba, X. and Vulfson, E. N. (1996). Biologically active peptides and enzymatic approaches to their production. **Enzyme and Microbial**

Technology. 18:162-183.

Girgih, A. T., Udenigwe, C. C., Li, H., Adebiyi, A. P. and Aluko, R. E. (2011). Kinetics of enzyme inhibition and antihypertensive effects of hemp seed (*Cannabis sativa* L.) protein hydrolysates. **Journal of the American Oil Chemists' Society**. 88:1767-1774.

Gobbetti, M., Minervini, F. and Rizzello, C. G. (2004). Angiotensin I-converting-enzyme-inhibitory and antimicrobial bioactive peptides. **International Journal of Dairy Technology**. 57:173-188.

Gouze, J., Bianchi, A., Becuwe, P., Dauca, M., Netter, P., Magdalou, J., Terlain, B. and Bordji, K. (2002). Glucosamine modulates IL-1-induced activation of rat chondrocytes at a receptor level, and by inhibiting the NF-κB pathway. **FEBS Letters**. 510:166-170.

Griffin, M. R., Ray, W. A. and Schaffner, W. (1988). Nonsteroidal anti-inflammatory drug use and death from peptic ulcer in elderly persons. **Annals of Internal Medicine**. 109:359-363.

Grigolon, L., Azevedo, A., Santos, R. and Franco, T. (2001). Enzymatic modification of chitosan by free and immobilized papain. **Chitin Enzymology**. 78e87.

Gropper, S. and Smith, J. (2012). *Advanced nutrition and human metabolism*. Cengage Learning.

Guillemin, F., Rat, A.-C., Roux, C. H., Fautrel, B., Mazieres, B., Chevalier, X., Euller-Ziegler, L., Fardellone, P., Verrouil, E. and Morvan, J. (2012). The KHOALA cohort of knee and hip osteoarthritis in France. **Joint Bone Spine**. 79:597-603.

Hagiwara, S.-y., Takahashi, M., Shen, Y., Kaihou, S., Tomiyama, T., Yazawa, M., Tamai, Y., Sin, Y., Kazusaka, A. and Terazawa, M. (2005). A phytochemical in

the edible Tamogi-take mushroom (*Pleurotus cornucopiae*), D-mannitol, inhibits ACE activity and lowers the blood pressure of spontaneously hypertensive rats. **Bioscience, Biotechnology, and Biochemistry**. 69:1603-1605.

Hameed, B., Ahmad, A. and Aziz, N. (2007). Isotherms, kinetics and thermodynamics of acid dye adsorption on activated palm ash. **Chemical Engineering Journal**. 133:195-203.

Hammad, Y. H., Magid, H. R. and Sobhy, M. M. (2015). Clinical and biochemical study of the comparative efficacy of topical versus oral glucosamine/chondroitin sulfate on osteoarthritis of the knee. **The Egyptian Rheumatologist**. 37:7.

Hata, Y., Yamamoto, M., Ohni, M., Nakajima, K., Nakamura, Y. and Takano, T. (1996). A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. **The American Journal of Clinical Nutrition**. 64:767-771.

Hatanaka, A., Miyahara, H., Suzuki, K. I. and Sato, S. (2009). Isolation and identification of antihypertensive peptides from antarctic krill tail meat hydrolysate. **Journal of Food Science**. 74:5.

Haugen, I. K., Englund, M., Aliabadi, P., Niu, J., Clancy, M., Kvien, T. K. and Felson, D. T. (2011). Prevalence, incidence and progression of hand osteoarthritis in the general population: the Framingham Osteoarthritis Study. **Annals of the Rheumatic Diseases**. 70:1581-1586.

Haugen, I. K., Slatkowsky-Christensen, B., Bøyesen, P., van der Heijde, D. and Kvien, T. K. (2013). Cross-sectional and longitudinal associations between radiographic features and measures of pain and physical function in hand

osteoarthritis. **Osteoarthritis and Cartilage**. 21:1191-1198.

Hawkey, C. (1999). COX-2 inhibitors. **The Lancet**. 353:307-314.

Hayes, M., Stanton, C., Slattery, H., O'Sullivan, O., Hill, C., Fitzgerald, G. and Ross, R. (2007). Casein fermentate of *Lactobacillus animalis* DPC6134 contains a range of novel propeptide angiotensin-converting enzyme inhibitors. **Applied and Environmental Microbiology**. 73:4658-4667.

He, R., Ma, H., Zhao, W., Qu, W., Zhao, J., Luo, L. and Zhu, W. (2011). Modeling the QSAR of ACE-inhibitory peptides with ANN and its applied illustration. **International Journal of Peptides**. 2012.

He, R., Malomo, S. A., Alashi, A., Girgih, A. T., Ju, X. and Aluko, R. E. (2013). Purification and hypotensive activity of rapeseed protein-derived renin and angiotensin converting enzyme inhibitory peptides. **Journal of Functional Foods**. 5:781-789.

Helander, I., Nurmiaho-Lassila, E.-L., Ahvenainen, R., Rhoades, J. and Roller, S. (2001). Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. **International Journal of Food Microbiology**. 71:235-244.

Henrotin, Y., Chevalier, X., Herrero-Beaumont, G., McAlindon, T., Mobasheri, A., Pavelka, K., Schön, C., Weinans, H. and Biesalski, H. (2013). Physiological effects of oral glucosamine on joint health: current status and consensus on future research priorities. **BMC research notes**. 6:1.

Henrotin, Y. and Lambert, C. (2013). Chondroitin and glucosamine in the management of osteoarthritis: an update. **Current Rheumatology Reports**. 15:1-9.

Henrotin, Y., Mobasheri, A. and Marty, M. (2012). Is there any scientific evidence for the use of glucosamine in the management of human osteoarthritis. **Arthritis**

Research and Therapy. 14:201-209.

Henrotin, Y. E. (2012). Interpretation of metaanalyses: pitfalls should be more widely recognized. **The Journal of Rheumatology**. 39:1107-1109.

Hernández-Ledesma, B., Amigo, L., Ramos, M. and Recio, I. (2004a). Angiotensin converting enzyme inhibitory activity in commercial fermented products. Formation of peptides under simulated gastrointestinal digestion. **Journal of Agricultural and Food Chemistry**. 52:1504-1510.

Hernández-Ledesma, B., Amigo, L., Ramos, M. and Recio, I. (2004b). Release of angiotensin converting enzyme-inhibitory peptides by simulated gastrointestinal digestion of infant formulas. **International Dairy Journal**. 14:889-898.

Hernández-Ledesma, B., del Mar Contreras, M. and Recio, I. (2011). Antihypertensive peptides: production, bioavailability and incorporation into foods. **Advances in Colloid and Interface Science**. 165:23-35.

Hernández-Ledesma, B., Ramos, M., Recio, I. and Amigo, L. (2006). Effect of β-lactoglobulin hydrolysis with thermolysin under denaturing temperatures on the release of bioactive peptides. **Journal of Chromatography A**. 1116:31-37.

Hernández-Ledesma, B., Recio, I., Ramos, M. and Amigo, L. (2002). Preparation of ovine and caprine β-lactoglobulin hydrolysates with ACE-inhibitory activity. Identification of active peptides from caprine β-lactoglobulin hydrolysed with thermolysin. **International Dairy Journal**. 12:805-812.

Hernandez-Ledesma, B., Quiros, A., Amigo, L. and Recio, I. (2007). Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin. **International Dairy Journal**. 17:42-49.

Herrera, A., Téllez-Luis, S. J., González-Cabriales, J. J., Ramírez, J. A. and Vázquez,

M. (2004). Effect of the hydrochloric acid concentration on the hydrolysis of sorghum straw at atmospheric pressure. **Journal of Food Engineering**. 63:103-109.

Herrero-Beaumont, G., Ivorra, J. A. R., del Carmen Trabado, M., Blanco, F. J., Benito, P., Martín-Mola, E., Paulino, J., Marenco, J. L., Porto, A., Laffon, A., Araújo, D., Figueroa, M. and Branco, J. (2007). Glucosamine sulfate in the treatment of knee osteoarthritis symptoms: A randomized, double-blind, placebo-controlled study using acetaminophen as a side comparator. **Arthritis and Rheumatism**. 56:555-567.

Himaya, S. W. A., Ngo, D.-H., Ryu, B. and Kim, S.-K. (2012). An active peptide purified from gastrointestinal enzyme hydrolysate of Pacific cod skin gelatin attenuates angiotensin-1 converting enzyme (ACE) activity and cellular oxidative stress. **Food Chemistry**. 132:1872-1882.

Hirota, T., Ohki, K., Kawagishi, R., Kajimoto, Y., Mizuno, S., Nakamura, Y. and Kitakaze, M. (2007). Casein Hydrolysate Containing the Antihypertensive Tripeptides Val-Pro-Pro and Ile-Pro-Pro Improves Vascular Endothelial Function Independent of Blood Pressure-Lowering Effects: Contribution of the Inhibitory Action of Angiotensin-Converting Enzyme. **Hypertension Research**. 30:489-496.

Hochberg, M., Chevalier, X., Henrotin, Y., Hunter, D. and Uebelhart, D. (2013). Symptom and structure modification in osteoarthritis with pharmaceutical-grade chondroitin sulfate: what's the evidence? **Current Medical Research and Opinion**. 29:259-267.

Hochberg, M. C., Altman, R. D., April, K. T., Benkhalti, M., Guyatt, G., McGowan, J., Towheed, T., Welch, V., Wells, G. and Tugwell, P. (2012). American College of

Rheumatology 2012 recommendations for the use of nonpharmacologic and pharmacologic therapies in osteoarthritis of the hand, hip, and knee. **Arthritis Care and Research**. 64:465-474.

Hochberg, M. C. and Dougados, M. (2001). Pharmacological therapy of osteoarthritis. **Best Practice and Research Clinical Rheumatology**. 15:583-593.

Hochberg, M. C., Martel-Pelletier, J., Monfort, J., Möller, I., Castillo, J. R., Arden, N., Berenbaum, F., Blanco, F. J., Conaghan, P. G. and Doménech, G. (2015). Combined chondroitin sulfate and glucosamine for painful knee osteoarthritis: a multicentre, randomised, double-blind, non-inferiority trial versus celecoxib. **Annals of the Rheumatic Diseases**. annrheumdis-2014-206792.

Holmquist, B., Bünning, P. and Riordan, J. F. (1979). A continuous spectrophotometric assay for angiotensin converting enzyme. **Analytical Biochemistry**. 95:540-548.

Hong, H., Park, Y.-K., Choi, M.-S., Ryu, N.-H., Song, D.-K., Suh, S.-I., Nam, K.-Y., Park, G.-Y. and Jang, B.-C. (2009). Differential down-regulation of COX-2 and MMP-13 in human skin fibroblasts by glucosamine-hydrochloride. **Journal of Dermatological Science**. 56:43-50.

Howling, G. I., Dettmar, P. W., Goddard, P. A., Hampson, F. C., Dornish, M. and Wood, E. J. (2001). The effect of chitin and chitosan on the proliferation of human skin fibroblasts and keratinocytes *in vitro*. **Biomaterials**. 22:2959-2966.

Hsieh, J.-W., Wu, H.-S., Wei, Y.-H. and Wang, S. S. (2007). Determination and Kinetics of Producing Glucosamine Using Fungi. **Biotechnology Progress**. 23:1009-1016.

Hua, J., Suguro, S., Hirano, S., Sakamoto, K. and Nagaoka, I. (2005). Preventive actions of a high dose of glucosamine on adjuvant arthritis in rats. **Inflammation**

Research. 54:127-132.

Huang, J., Liu, Y., Liu, Y. and Wang, X. (2007). Effect of attapulgite pore size distribution on soybean oil bleaching. **Journal of the American Oil Chemists' Society**. 84:687-692.

Huang, Q.-L., Siu, K.-C., Wang, W.-Q., Cheung, Y.-C. and Wu, J.-Y. (2013). Fractionation, characterization and antioxidant activity of exopolysaccharides from fermentation broth of a *Cordyceps sinensis* fungus. **Process Biochemistry**. 48:380-386.

Huang, T. m., Deng, C. H., Chen, N. z., Liu, Z. and Duan, G. L. (2006). High performance liquid chromatography for the determination of glucosamine sulfate in human plasma after derivatization with 9-fluorenylmethyl chloroformate. **Journal of Separation Science**. 29:2296-2302.

Hung, P. and Nhi, N. (2012). Nutritional composition and antioxidant capacity of several edible mushrooms grown in the Southern Vietnam. **International Food Research Journal**. 19:611-615.

Hussin, F., Aroua, M. K. and Daud, W. M. A. W. (2011). Textural characteristics, surface chemistry and activation of bleaching earth: A review. **Chemical Engineering Journal**. 170:90-106.

Hwang, J.-S. (2010). Impact of processing on stability of angiotensin I-converting enzyme (ACE) inhibitory peptides obtained from tuna cooking juice. **Food Research International**. 43:902-906.

Hylek, E. M., Heiman, H., Skates, S. J., Sheehan, M. A. and Singer, D. E. (1998). Acetaminophen and other risk factors for excessive warfarin anticoagulation. **TheJournal of the American Medical Association**. 279:657-662.

Ibadallah, B. X., Abdullah, N. and Shuib, A. S. (2015). Identification of Angiotensin-

Converting Enzyme Inhibitory Proteins from Mycelium of *Pleurotus pulmonarius* (Oyster Mushroom). **Planta Med**. 81:123-129.

Ibe, S., Yoshida, K., Kumada, K., Tsurushiin, S., Furusho, T. and Otobe, K. (2009). Antihypertensive effects of natto, a traditional Japanese fermented food, in spontaneously hypertensive rats. **Food Science and Technology Research**. 15:199-202.

Ifuku, S., Nomura, R., Morimoto, M. and Saimoto, H. (2011). Preparation of chitin nanofibers from mushrooms. **Materials**. 4:1417-1425.

Igarashi, M., Kaga, I., Takamori, Y., Sakamoto, K., Miyazawa, K. and Nagaoka, I. (2011). Effects of glucosamine derivatives and uronic acids on the production of glycosaminoglycans by human synovial cells and chondrocytes. **International Journal of Molecular Medicine**. 27(6):821-827.

Igarashi, M., Sakamoto, K. and Nagaoka, I. (2011). Effect of glucosamine, a therapeutic agent for osteoarthritis, on osteoblastic cell differentiation. **International Journal of Molecular Medicine**. 28:373.

Imagawa, K., De Andres, M., Hashimoto, K., Pitt, D., Itoi, E., Goldring, M. B., Roach, H. I. and Oreffo, R. O. (2011). The epigenetic effect of glucosamine and a nuclear factor-kappa B (NF-kB) inhibitor on primary human chondrocytes–implications for osteoarthritis. **Biochemical and Biophysical Research Communications**. 405:362-367.

Intarasirisawat, R., Benjakul, S., Wu, J. and Visessanguan, W. (2013). Isolation of antioxidative and ACE inhibitory peptides from protein hydrolysate of skipjack (*Katsuwana pelamis*) roe. **Journal of Functional Foods**. 5:1854-1862.

Iroyukifujita, H., Eiichiyokoyama, K. and Yoshikawa, M. (2000). Classification and

Antihypertensive Activity of Angiotensin I-Converting Enzyme Inhibitory Peptides Derived from Food Proteins. **Journal of Food Science**. 65:564-569.

Ismail, T. A., Soliman, M. M., Nassan, M. A. and Mohamed, D. I. (2015). Antihypercholesterolemic Effects of Mushroom, Chrysin, Curcumin and Omega-3 in Experimental Hypercholesterolemic Rats. **Journal of Food and Nutrition Research**. 3:77-87.

Ivanovska, N. and Dimitrova, P. (2011). Bone resorption and remodeling in murine collagenase-induced osteoarthritis after administration of glucosamine. **Arthritis Research and Therapy**. 13(2):R44-R56.

Ivshina, T., Artamonova, S., Ivshin, V. and Sharnina, F. (2009). Isolation of the chitin-glucan complex from the fruiting bodies of mycothallus. **Applied Biochemistry and Microbiology**. 45:313-318.

Jáč, P., Los, P., Spáčil, Z., Pospíšilová, M. and Polášek, M. (2008). Fast assay of glucosamine in pharmaceuticals and nutraceuticals by capillary zone electrophoresis with contactless conductivity detection. **Electrophoresis**. 29:3511-3518.

Jaeger, P., Ferguson, R. K., Brunner, H. R., Kirchertz, E. J. and Gavras, H. (1978). Mechanism of blood pressure reduction by teprotide (SQ 20881) in rats. **Kidney International**. 13:289-296.

Jamali, F. and Ibrahim, A. (2010). Improved sensitive high performance liquid chromatography assay for glucosamine in human and rat biological samples with fluorescence detection. **Journal of Pharmacy and Pharmaceutical Sciences**. 13:128-135.

Jang, A., Jo, C. and Lee, M. (2007). Storage stability of the synthetic angiotensin converting enzyme (ACE) inhibitory peptides separated from beef

sarcoplasmic protein extracts at different pH, temperature, and gastric digestion. **Food Science and Biotechnology**. 16:572-575.

Jang, J.-H., Jeong, S.-C., Kim, J.-H., Lee, Y.-H., Ju, Y.-C. and Lee, J.-S. (2011). Characterisation of a new antihypertensive angiotensin I-converting enzyme inhibitory peptide from *Pleurotus cornucopiae*. **Food Chemistry**. 127:412-418.

Jauhiainen, T., Collin, M., Narva, M., Cheng, Z. J., Poussa, T., Vapaatalo, H. and Korpela, R. (2005). Effect of long-term intake of milk peptides and minerals on blood pressure and arterial function in spontaneously hypertensive rats. **Milchwissenschaft-Milk Science International**. 60:358-362.

Je, J.-Y., Park, P.-J., Kwon, J. Y. and Kim, S.-K. (2004). A novel angiotensin I converting enzyme inhibitory peptide from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. **Journal of Agricultural and Food Chemistry**. 52:7842-7845.

Jeong, D., Shin, D. S., Ahn, C., Song, I.-S. and Lee, H.-J. (2007). Expression of antihypertensive peptide, His-His-Leu, as tandem repeats in *Escherichia coli*. **Journal of Microbiology and Biotechnology**. 17:952.

Jia, J., Ma, H., Zhao, W., Wang, Z., Tian, W., Luo, L. and He, R. (2010). The use of ultrasound for enzymatic preparation of ACE-inhibitory peptides from wheat germ protein. **Food Chemistry**. 119:336-342.

Jia, Z. and Shen, D. (2002). Effect of reaction temperature and reaction time on the preparation of low-molecular-weight chitosan using phosphoric acid. **Carbohydrate Polymers**. 49:393-396.

Jiang, T., Wang, Q., Xu, S., Jahangir, M. M. and Ying, T. (2010). Structure and composition changes in the cell wall in relation to texture of shiitake mushrooms (*Lentinula edodes*) stored in modified atmosphere packaging. **Journal**

of the Science of Food and Agriculture. 90:742-749.

Jianguo, B., Degui, W. and Wang, J. (2013). *U.S. Patent No. 8383808 B2*. Washington, DC: U.S. Patent and Trademark Office.

Jiménez-Escrig, A., Alaiz, M., Vioque, J. and Rupérez, P. (2010). Health-promoting activities of ultra-filtered okara protein hydrolysates released by *in vitro* gastrointestinal digestion: identification of active peptide from soybean lipoxygenase. **European Food Research and Technology**. 230:655-663.

Jimsheena, V. and Gowda, L. R. (2010). Arachin derived peptides as selective angiotensin I-converting enzyme (ACE) inhibitors: structure-activity relationship. **Peptides**. 31:1165-1176.

Jordan, K., Arden, N., Doherty, M., Bannwarth, B., Bijlsma, J., Dieppe, P., Gunther, K., Hauselmann, H., Herrero-Beaumont, G. and Kaklamanis, P. (2003). EULAR Recommendations 2003: an evidence based approach to the management of knee osteoarthritis: Report of a Task Force of the Standing Committee for International Clinical Studies Including Therapeutic Trials (ESCISIT). **Annals of the Rheumatic Diseases**. 62:1145-1155.

Joseph, G. B., McCulloch, C. E., Nevitt, M. C., Heilmeier, U., Nardo, L., Lynch, J. A., Liu, F., Baum, T. and Link, T. M. (2015). A reference database of cartilage 3 T MRI T2 values in knees without diagnostic evidence of cartilage degeneration: data from the osteoarthritis initiative. **Osteoarthritis and Cartilage**. 23:897-905.

Juillard, V., Guillot, A., Le Bars, D. and Gripon, J.-C. (1998). Specificity of Milk Peptide Utilization by *Lactococcus lactis*. **Applied and Environmental Microbiology**. 64:1230-1236.

Jung, C.-W., Jo, J.-R., Lee, S.-H., Park, Y.-K., Jung, N.-K., Song, D.-K., Bae, J., Nam,

K.-Y., Ha, J.-S., Park, I.-S., Park, G.-Y., Jang, B.-C. and Park, J.-W. (2012). Anti-cancer properties of glucosamine-hydrochloride in YD-8 human oral cancer cells: Induction of the caspase-dependent apoptosis and down-regulation of HIF-1α. **Toxicology in Vitro**. 26:42-50.

Jung, W.-J., Kuk, J.-H., Kim, K.-Y., Jung, K.-C. and Park, R.-D. (2006). Purification and characterization of exo-β-d-glucosaminidase from Aspergillus fumigatus S-26. **Protein Expression and Purification**. 45:125-131.

Jung, W.-J., Souleimanov, A., Park, R.-D. and Smith, D. L. (2007). Enzymatic production of N-acetyl chitooligosaccharides by crude enzyme derived from Paenibacillus illioisensis KJA-424. **Carbohydrate Polymers**. 67:256-259.

Jung, W.-K., Mendis, E., Je, J.-Y., Park, P.-J., Son, B. W., Kim, H. C., Choi, Y. K. and Kim, S.-K. (2006). Angiotensin I-converting enzyme inhibitory peptide from yellowfin sole (*Limanda aspera*) frame protein and its antihypertensive effect in spontaneously hypertensive rats. **Food Chemistry**. 94:26-32.

Kadkhodaee, R. and Povey, M. J. (2008). Ultrasonic inactivation of Bacillus α-amylase. I. Effect of gas content and emitting face of probe. **Ultrasonics Sonochemistry**. 15:133-142.

Kadnikova, I. A., Costa, R., Kalenik, T. K., Guruleva, O. N. and Yanguo, S. (2015). Chemical Composition and Nutritional Value of the Mushroom Auricularia auricula-judae. **Journal of Food and Nutrition Research**. 3:478-482.

Kalač, P. (2009). Chemical composition and nutritional value of European species of wild growing mushrooms: A review. **Food Chemistry**. 113:9-16.

Kalač, P. (2013). A review of chemical composition and nutritional value of wild‐growing and cultivated mushrooms. **Journal of the Science of Food and**

Agriculture. 93:209-218.

Kang, M.-G., Kim, Y.-H., Bolormaa, Z., Kim, M.-K., Seo, G.-S. and Lee, J.-S. (2013). Characterization of an antihypertensive angiotensin I-converting enzyme inhibitory peptide from the edible mushroom *Hypsizygus marmoreus*. **BioMed Research International**. 2013.

Kapaun, E., Loos, E. and Reisser, W. (1992). Cell wall composition of virus-sensitive symbiotic Chlorella species. **Phytochemistry**. 31:3103-3104.

Kapoor, M., Mineau, F., Fahmi, H., Pelletier, J.-P. and Martel-Pelletier, J. (2012). Glucosamine sulfate reduces prostaglandin E2 production in osteoarthritic chondrocytes through inhibition of microsomal PGE synthase-1. **The Journal of Rheumatology**. 39:635-644.

Kato, H. and Suzuki, T. (1971). Bradykinin-potentiating peptides from the venom of Agkistrodon halys. Isolation of five bradykinin potentiators and the amino acid sequences of two of them, potentiators B and C. **Biochemistry**. 10:972-980.

Khajeh, M. (2011). Response surface modelling of lead pre-concentration from food samples by miniaturised homogenous liquid-liquid solvent extraction: Box-Behnken design. **Food Chemistry**. 129:1832-1838.

Kim, H.-J., Kang, S.-G., Jaiswal, L., Li, J., Choi, J.-H., Moon, S.-M., Cho, J.-Y. and Ham, K.-S. (2016). Identification of four new angiotensin I-converting enzyme inhibitory peptides from fermented anchovy sauce. **Applied Biological Chemistry**. 59:25-31.

Kim, M. M., Mendis, E., Rajapakse, N. and Kim, S.-K. (2007). Glucosamine sulfate promotes osteoblastic differentiation of MG-63 cells via anti-inflammatory effect. **Bioorganic and Medicinal Chemistry Letters**. 17:1938-1942.

Kim, S.-K., Byun, H.-G., Park, P.-J. and Shahidi, F. (2001). Angiotensin I Converting

Enzyme Inhibitory Peptides Purified from Bovine Skin Gelatin Hydrolysate. **Journal of Agricultural and Food Chemistry**. 49:2992-2997.

KIRBAĞ, S. and AKYÜZ, M. (2010). Nutritive Value of Edible Wild and Cultured Mushrooms. **Turkish Journal of Biology**. 34:97-102.

Kloppenburg, M., Maheu, E., Kraus, V. B., Cicuttini, F., Doherty, M., Dreiser, R. L., Henrotin, Y., Jiang, G. L., Mandl, L., Martel-Pelletier, J., Nelson, A. E., Neogi, T., Pelletier, J. P., Punzi, L., Ramonda, R., Simon, L. S. and Wang, S. (2015). OARSI Clinical Trials Recommendations: Design and conduct of clinical trials for hand osteoarthritis. **Osteoarthritis and Cartilage**. 23:772-786.

Ko, S.-C., Kang, N., Kim, E.-A., Kang, M. C., Lee, S.-H., Kang, S.-M., Lee, J.-B., Jeon, B.-T., Kim, S.-K., Park, S.-J., Park, P.-J., Jung, W.-K., Kim, D. and Jeon, Y.-J. (2012a). A novel angiotensin I-converting enzyme (ACE) inhibitory peptide from a marine *Chlorella ellipsoidea* and its antihypertensive effect in spontaneously hypertensive rats. **Process Biochemistry**. 47:2005-2011.

Ko, S.-C., Lee, J.-K., Byun, H.-G., Lee, S.-C. and Jeon, Y.-J. (2012b). Purification and characterization of angiotensin I-converting enzyme inhibitory peptide from enzymatic hydrolysates of *Styela clava* flesh tissue. **Process Biochemistry**. 47:34-40.

Kobayashi, Y., Yamauchi, T., Katsuda, T., Yamaji, H. and Katoh, S. (2008). Angiotensin-I converting enzyme (ACE) inhibitory mechanism of tripeptides containing aromatic residues. **Journal of Bioscience and Bioengineering**. 106:310-312.

Kohlhof, H., Gravius, S., Kohl, S., Ahmad, S. S., Randau, T., Schmolders, J., Rommelspacher, Y., Friedrich, M. and Kaminski, T. P. (2016). Single Molecule

Microscopy Reveals an Increased Hyaluronan Diffusion Rate in Synovial Fluid from Knees Affected by Osteoarthritis. **Scientific Reports**. 6:1-6.

Kokean, Y., Nishii, T., Sakakura, H. and Furuichi, Y. (2005). Effect of frying with edible oil on antihypertensive properties of Hatakeshimeji (*Lyophyllum decastes* Sing.) mushroom. **Food Science and Technology Research**. 11:339-343.

Konieczna-Molenda, A., Fiedorowicz, M., Zhong, W. and Tomasik, P. (2008). Polarized light-stimulated enzymatic hydrolysis of chitin and chitosan. **Carbohydrate Research**. 343:3117-3119.

Konno, S., Chu, K., Feuer, N., Phillips, J. and Choudhury, M. (2015). Potent Anticancer Effects of Bioactive Mushroom Extracts (*Phellinus linteus*) on a Variety of Human Cancer Cells. **Journal of Clinical Medicine Research**. 7:76.

Koo, K.-C., Lee, D.-H., Kim, J.-H., Yu, H.-E., Park, J.-S. and Lee, J.-S. (2006). Production and characterization of antihypertensive angiotensin I-converting enzyme inhibitor from *Pholiota adiposa*. **Journal of Microbiology and Biotechnology**. 16:757-763.

Korhonen, H. and Pihlanto, A. (2003). Food-derived bioactive peptides-opportunities for designing future foods. **Current Pharmaceutical Design**. 9:1297-1308.

Korhonen, H. and Pihlanto, A. (2006). Bioactive peptides: production and functionality. **International Dairy Journal**. 16:945-960.

Kozarski, M., Klaus, A., Niksic, M., Jakovljevic, D., Helsper, J. P. and Van Griensven, L. J. (2011). Antioxidative and immunomodulating activities of polysaccharide extracts of the medicinal mushrooms *Agaricus bisporus*, *Agaricus brasiliensis*, *Ganoderma lucidum* and *Phellinus linteus*. **Food Chemistry**. 129:1667-1675.

Kuk, J. H., Jung, W. J., Jo, G. H., Ahn, J. S., Kim, K. Y. and Park, R. D. (2005a).

Selective preparation of N-acetyl-D-glucosamine and N,N′-diacetylchitobiose from chitin using a crude enzyme preparation from *Aeromonas sp.* **Biotechnology Letters**. 27:7-11.

Kuk, J. H., Jung, W. J., Jo, G. H., Kim, Y. C., Kim, K. Y. and Park, R. D. (2005b). Production of N-acetyl-β-d-glucosamine from chitin by *Aeromonas sp.* GJ-18 crude enzyme. **Applied Microbiology and Biotechnology**. 68:384-389.

Kumar, A. B. V., Gowda, L. R. and Tharanathan, R. N. (2004). Non‐specific depolymerization of chitosan by pronase and characterization of the resultant products. **European Journal of Biochemistry**. 271:713-723.

Kumar, A. V. and Tharanathan, R. (2004). A comparative study on depolymerization of chitosan by proteolytic enzymes. **Carbohydrate Polymers**. 58:275-283.

Kumar, A. V., Varadaraj, M. C., Lalitha, R. G. and Tharanathan, R. (2004). Low molecular weight chitosans: preparation with the aid of papain and characterization. **Biochimica et Biophysica Acta (BBA)-General Subjects**. 1670:137-146.

Kumar, M. N. R. (2000). A review of chitin and chitosan applications. **Reactive and Functional Polymers**. 46:1-27.

Kurtzman Jr, R. (2005). Mushrooms: sources for modern western medicine. **Micologia Aplicada International**. 17:21-33.

Lahogue, V., Réhel, K., Taupin, L., Haras, D. and Allaume, P. (2010). A HPLC-UV method for the determination of angiotensin I-converting enzyme (ACE) inhibitory activity. **Food Chemistry**. 118:870-875.

Largo, R., Alvarez-Soria, M., Dıez-Ortego, I., Calvo, E., Sanchez-Pernaute, O., Egido, J. and Herrero-Beaumont, G. (2003). Glucosamine inhibits IL-1β-induced

NFκB activation in human osteoarthritic chondrocytes. **Osteoarthritis and Cartilage**. 11:290-298.

Larkin, J., Lohr, T. A., Elefante, L., Shearin, J., Matico, R., Su, J. L., Xue, Y., Liu, F., Genell, C., Miller, R. E., Tran, P. B., Malfait, A. M., Maier, C. C. and Matheny, C. J. (2015). Translational development of an ADAMTS-5 antibody for osteoarthritis disease modification. **Osteoarthritis and Cartilage**. 23(8):1254-1266.

Lau, C. C., Abdullah, N. and Shuib, A. S. (2013). Novel angiotensin I-converting enzyme inhibitory peptides derived from an edible mushroom, *Pleurotus cystidiosus* OK Miller identified by LC-MS/MS. **BMC Complementary and Alternative Medicine**. 13:313-322.

Lau, C. C., Abdullah, N., Shuib, A. S. and Aminudin, N. (2014). Novel angiotensin I-converting enzyme inhibitory peptides derived from edible mushroom *Agaricus bisporus* (J.E. Lange) Imbach identified by LC–MS/MS. **Food Chemistry**. 148:396-401.

Lavanya, J. and Subhashini, S. (2013). Therapeutic proteins and peptides from edible and medicinal mushrooms-Review. **European Scientific Journal**. 9(24):162-176.

Lavarack, B., Griffin, G. and Rodman, D. (2002). The acid hydrolysis of sugarcane bagasse hemicellulose to produce xylose, arabinose, glucose and other products. **Biomass and Bioenergy**. 23:367-380.

LeCleir, G. R., Buchan, A. and Hollibaugh, J. T. (2004). Chitinase Gene Sequences Retrieved from Diverse Aquatic Habitats Reveal Environment-Specific Distributions. **Applied and Environmental Microbiology**. 70(12):6977-6983.

Lee, B. C., Kim, M. S., Choi, S. H., Kim, K. Y. and Kim, T. S. (2009). *In vitro* and *in*

vivo antimicrobial activity of water-soluble chitosan oligosaccharides against *Vibrio vulnificus*. **International Journal of Molecular Medicine**. 24:327-333.

Lee, D.-H., Kim, J.-H., Cheong, J.-C., Gong, W.-S., Yoo, Y.-B., Park, J.-S., Yoo, C.-H. and Lee, J.-S. (2003). Screening of mushrooms having angiotensin I-converting enzyme inhibitor. **The Korean Journal of Mycology**. 31:148-154.

Lee, D. H., Kim, J. H., Park, J. S., Choi, Y. J. and Lee, J. S. (2004). Isolation and characterization of a novel angiotensin I-converting enzyme inhibitory peptide derived from the edible mushroom *Tricholoma giganteum*. **Peptides**. 25:621-627.

Lee, J. K., Jeon, J.-K. and Byun, H.-G. (2014). Antihypertensive effect of novel angiotensin I converting enzyme inhibitory peptide from chum salmon (*Oncorhynchus keta*) skin in spontaneously hypertensive rats. **Journal of Functional Foods**. 7:381-389.

Lee, S.-H., Qian, Z.-J. and Kim, S.-K. (2010). A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. **Food Chemistry**. 118:96-102.

Lee, S. H., Nguyen, H. M., Koo, Y.-M. and Ha, S. H. (2008). Ultrasound-enhanced lipase activity in the synthesis of sugar ester using ionic liquids. **Process Biochemistry**. 43:1009-1012.

Lee, Y. H., Woo, J.-H., Choi, S. J., Ji, J. D. and Song, G. G. (2010). Effect of glucosamine or chondroitin sulfate on the osteoarthritis progression: a meta-analysis. **Rheumatology International**. 30:357-363.

Li, F.-J., Yin, L.-J., Cheng, Y.-Q., Yamaki, K., Fan, J.-F. and Li, L.-T. (2009). Comparison of angiotensin I-converting enzyme inhibitor activities of

pre-fermented douchi (a Chinese traditional fermented soybean food) started with various cultures. **International Journal of Food Engineering**. 5(2).

Li, G.-H., Le, G.-W., Shi, Y.-H. and Shrestha, S. (2004). Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. **Nutrition Research**. 24:469-486.

Li, G.-H., Liu, H., Shi, Y.-H. and Le, G.-W. (2005). Direct spectrophotometric measurement of angiotensin I-converting enzyme inhibitory activity for screening bioactive peptides. **Journal of Pharmaceutical and Biomedical Analysis**. 37:219-224.

Li, H., Prairie, N., Udenigwe, C. C., Adebiyi, A. P., Tappia, P. S., Aukema, H. M., Jones, P. J. and Aluko, R. E. (2011). Blood pressure lowering effect of a pea protein hydrolysate in hypertensive rats and humans. **Journal of Agricultural and Food Chemistry**. 59:9854-9860.

Li, N., Ng, T. B., Wong, J. H., Qiao, J. X., Zhang, Y. N., Zhou, R., Chen, R. R. and Liu, F. (2013). Separation and purification of the antioxidant compounds, caffeic acid phenethyl ester and caffeic acid from mushrooms by molecularly imprinted polymer. **Food Chemistry**. 139:1161-1167.

Li, P., Jia, J., Fang, M., Zhang, L., Guo, M., Xie, J., Xia, Y., Zhou, L. and Wei, D. (2014). *In vitro* and *in vivo* ACE inhibitory of pistachio hydrolysates and in silico mechanism of identified peptide binding with ACE. **Process Biochemistry**. 49:898-904.

Li, X.-F., Feng, X.-Q., Yang, S., Wang, T.-P. and Su, Z.-X. (2008). Effects of molecular weight and concentration of chitosan on antifungal activity against *Aspergillus niger*. **Iranian Polymer Journal**. 17:843-852.

Li, Y., Sadiq, F. A., Liu, T., Chen, J. and He, G. (2015). Purification and identification

of novel peptides with inhibitory effect against angiotensin I-converting enzyme and optimization of process conditions in milk fermented with the yeast *Kluyveromyces marxianus*. **Journal of Functional Foods**. 16:278-288.

Liang, T.-W., Chen, Y.-J., Yen, Y.-H. and Wang, S.-L. (2007). The antitumor activity of the hydrolysates of chitinous materials hydrolyzed by crude enzyme from *Bacillus amyloliquefaciens* V656. **Process Biochemistry**. 42:527-534.

Liao, W., Liu, Y., Frear, C. and Chen, S. (2008). Co-production of fumaric acid and chitin from a nitrogen-rich lignocellulosic material-dairy manure-using a pelletized filamentous fungus *Rhizopus oryzae* ATCC 20344. **Bioresource Technology**. 99:5859-5866.

Lien, T. S., Too, J. R., Wu, S. T. and Yu, S. T. (2005). Production of N-Acetylchitooligosaccharides by *Aeromonas sp.* DYU-TOO 7. **Journal of Food Biochemistry**. 29:422-439.

Lignitto, L., Cavatorta, V., Balzan, S., Gabai, G., Galaverna, G., Novelli, E., Sforza, S. and Segato, S. (2010). Angiotensin-converting enzyme inhibitory activity of water-soluble extracts of Asiago d'allevo cheese. **International Dairy Journal**. 20:11-17.

Lin, L., Lv, S. and Li, B. (2012). Angiotensin-I-converting enzyme (ACE)-inhibitory and antihypertensive properties of squid skin gelatin hydrolysates. **Food Chemistry**. 131:225-230.

Lin, Y.-C., Liang, Y.-C., Sheu, M.-T., Lin, Y.-C., Hsieh, M.-S., Chen, T.-F. and Chen, C.-H. (2008). Chondroprotective effects of glucosamine involving the p38 MAPK and Akt signaling pathways. **Rheumatology International**. 28:1009-1016.

Litwic, A., Edwards, M. H., Dennison, E. M. and Cooper, C. (2013). Epidemiology and

burden of osteoarthritis. **British medical bulletin**. 105(1):185-199.

Liu, B., Huang, Q., Cai, H., Guo, X., Wang, T. and Gui, M. (2015). Study of heavy metal concentrations in wild edible mushrooms in Yunnan Province, China. **Food Chemistry**. 188:294-300.

Liu, D., Sun, H., Zhang, L., Li, S. and Qin, Z. (2007). High-level expression of milk-derived antihypertensive peptide in *Escherichia coli* and its bioactivity. **Journal of Agricultural and Food Chemistry**. 55:5109-5112.

Liu, X., Zhang, M., Zhang, C. and Liu, C. (2012). Angiotensin converting enzyme (ACE) inhibitory, antihypertensive and antihyperlipidaemic activities of protein hydrolysates from *Rhopilema esculentum*. **Food Chemistry**. 134:2134-2140.

Loeser, R. F. (2010). Age-related changes in the musculoskeletal system and the development of osteoarthritis. **Clinics in Geriatric Medicine**. 26:371-386.

Loeser, R. F., Goldring, S. R., Scanzello, C. R. and Goldring, M. B. (2012). Osteoarthritis: a disease of the joint as an organ. **Arthritis and Rheumatism**. 64:1697-1707.

Lopez-Fandino, R., Otte, J. and Van Camp, J. (2006). Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. **International Dairy Journal**. 16:1277-1293.

Lv, C., Li, Y., Xu, J., Cao, H., Li, X., Ma, B., Ning, P., Wang, Y., Wang, M., Zhu, S., Ge, Y., Huang, S. and Jin, Y. (2015). Association of SPP1 promoter variants with hip osteoarthritis susceptibility in Chinese population. **Gene**. 564:9-13.

Lv, G., Huo, G. and Fu, X. (2003). Expression of milk-derived antihypertensive peptide in Escherichia coli. **Journal of Dairy Science**. 86:1927-1931.

Ma, L., Rudert, W. A., Harnaha, J., Wright, M., Machen, J., Lakomy, R., Qian, S., Lu,

L., Robbins, P. D. and Trucco, M. (2002). Immunosuppressive effects of glucosamine. **Journal of Biological Chemistry**. 277:39343-39349.

Magaña, A. A., Wrobel, K., Escobosa, A. R. C. and Wrobel, K. (2014). Fast determination of glucosamine in pharmaceutical formulations by high performance liquid chromatography without pre-column derivatization. **Acta Universitaria**. 24:16-22.

Majumder, K. and Wu, J. (2009). Angiotensin I converting enzyme inhibitory peptides from simulated *in vitro* gastrointestinal digestion of cooked eggs. **Journal of Agricultural and Food Chemistry**. 57:471-477.

Malik, S., Singh, M. and Mathur, A. (2013). Antimicrobial activity of food grade glucosamine. **International Journal of Biotechnology and Bioengineering Research.** 4(4):307-312.

Mannoni, A., Briganti, M., Di Bari, M., Ferrucci, L., Serni, U., Masotti, G. and Marchionni, N. (2000). Prevalence of symptomatic hand osteoarthritis in community-dwelling older persons: the ICARe Dicomano study. **Osteoarthritis and Cartilage**. 8:S11-S13.

Manzi, P., Aguzzi, A. and Pizzoferrato, L. (2001). Nutritional value of mushrooms widely consumed in Italy. **Food Chemistry**. 73:321-325.

Manzi, P., Gambelli, L., Marconi, S., Vivanti, V. and Pizzoferrato, L. (1999). Nutrients in edible mushrooms: an inter-species comparative study. **Food Chemistry**. 65:477-482.

Manzi, P., Marconi, S., Aguzzi, A. and Pizzoferrato, L. (2004). Commercial mushrooms: nutritional quality and effect of cooking. **Food Chemistry**. 84:201-206.

Mao, S. and Li, C. (2015). Hypotensive and Angiotensin-Converting Enzyme Inhibitory

Activities of *Eisenia fetida* Extract in Spontaneously Hypertensive Rats. **Evidence-Based Complementary and Alternative Medicine**. 2015.

Mao, X.-Y., Ni, J.-R., Sun, W.-L., Hao, P.-P. and Fan, L. (2007). Value-added utilization of yak milk casein for the production of angiotensin-I-converting enzyme inhibitory peptides. **Food Chemistry**. 103:1282-1287.

Mario, F. D., Rapanà, P., Tomati, U. and Galli, E. (2008). Chitin and chitosan from Basidiomycetes. **International Journal of Biological Macromolecules**. 43:8-12.

Maruyama, S., Mitachi, H., Tanaka, H., Tomizuka, N. and Suzuki, H. (1987). Studies on the Active Site and Antihypertensive Activity of Angiotensin I-Converting Enzyme Inhibitors Derived from Casein. **Agricultural and Biological Chemistry**. 51:1581-1586.

Masuda, O., Nakamura, Y. and Takano, T. (1996). Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides to spontaneously hypertensive rats. **The Journal of nutrition**. 126(12):3063.

Matsufuji, H., Matsui, T., Seki, E., Osajima, K., Nakashima, M. and Osajima, Y. (1994). Angiotensin I-converting enzyme inhibitory peptides in an alkaline protease hydrolyzate derived from sardine muscle. **Bioscience, Biotechnology, and Biochemistry**. 58:2244-2245.

Matsui, T., Matsufuji, H., Seki, E., Osajima, K., Nakashima, M. and Osajima, Y. (1993). Inhibition of angiotensin I-converting enzyme by *Bacillus licheniformis* alkaline protease hydrolyzates derived from sardine muscle. **Bioscience, Biotechnology, and Biochemistry**. 57:922-925.

McAlindon, T., LaValley, M. P., Gulin, J. P. and Felson, D. T. (2000). Glucosamine and chondroitin for treatment of osteoarthritis: A systematic quality assessment and

meta-analysis. **The Journal of the American Medical Association**. 283:1469-1475.

McCarty, M. F. (1996). Glucosamine for wound healing. **Medical Hypotheses**. 47:273-275.

McCarty, M. F. (1997). Glucosamine for psoriasis? **Medical Hypotheses**. 48:437-441.

Mehta, B., Jain, S., Sharma, G., Doshi, A. and Jain, H. (2013). Response Surface Optimization of Osmotic Dehydration Process Parameters for Button Mushroom (" *Agaricus bisporus*")-Part I. **Focusing on Modern Food Industry**. 2:91-102.

Meinita, M. D. N., Hong, Y.-K. and Jeong, G.-T. (2012). Comparison of sulfuric and hydrochloric acids as catalysts in hydrolysis of *Kappaphycus alvarezii* (cottonii). **Bioprocess and Biosystems Engineering**. 35:123-128.

Memarpoor-Yazdi, M., Asoodeh, A. and Chamani, J. (2012). Structure and ACE-inhibitory activity of peptides derived from hen egg white lysozyme. **International Journal of Peptide Research and Therapeutics**. 18:353-360.

Mendis, E., Kim, M.-M., Rajapakse, N. and Kim, S.-K. (2008). Sulfated glucosamine inhibits oxidation of biomolecules in cells via a mechanism involving intracellular free radical scavenging. **European Journal of Pharmacology**. 579:74-85.

Meyer, J., Bütikofer, U., Walther, B., Wechsler, D. and Sieber, R. (2009). Hot topic: Changes in angiotensin-converting enzyme inhibition and concentrations of the tripeptides Val-Pro-Pro and Ile-Pro-Pro during ripening of different Swiss cheese varieties. **Journal of Dairy Science**. 92:826-836.

Miguel, M., Aleixandre, M., Ramos, M. and López-Fandiño, R. (2006). Effect of simulated gastrointestinal digestion on the antihypertensive properties of

ACE-inhibitory peptides derived from ovalbumin. **Journal of Agricultural and Food Chemistry**. 54:726-731.

Miguel, M., Contreras, M. M., Recio, I. and Aleixandre, A. (2009). ACE-inhibitory and antihypertensive properties of a bovine casein hydrolysate. **Food Chemistry**. 112:211-214.

Miguel, M., Gómez‐Ruiz, J. Á., Recio, I. and Aleixandre, A. (2010). Changes in arterial blood pressure after single oral administration of milk‐casein‐derived peptides in spontaneously hypertensive rats. **Molecular Nutrition and Food Research**. 54:1422-1427.

Miguel, M., Muguerza, B., Sánchez, E., Delgado, M., Recio, I., Ramos, M. and Aleixandre, M. (2005). Changes in arterial blood pressure in hypertensive rats caused by long-term intake of milk fermented by *Enterococcus faecalis* CECT 5728. **British Journal of Nutrition**. 94:36-43.

Miguel, M., Recio, I., Ramos, M., Delgado, M. and Aleixandre, M. (2006). Antihypertensive effect of peptides obtained from *Enterococcus faecalis*-fermented milk in rats. **Journal of Dairy Science**. 89:3352-3359.

Miles, P. G. and Chang, S.-T. (2004). Mushrooms: cultivation, nutritional value, medicinal effect, and environmental impact: CRC press.

Minkiewicz, P., Dziuba, J., Iwaniak, A., Dziuba, M. and Darewicz, M. (2008). BIOPEP database and other programs for processing bioactive peptide sequences. **Journal of AOAC International**. 91:965-980.

Miyazawa, N., Okazaki, M. and Ohga, S. (2008). Antihypertensive effect of *Pleurotus nebrodensis* in spontaneously hypertensive rats. **Journal of Oleo Science**. 57:675-681.

Mizuno, S., Nishimura, S., Matsuura, K., Gotou, T. and Yamamoto, N. (2004). Release of short and proline-rich antihypertensive peptides from casein hydrolysate with an *Aspergillus oryzae* protease. **Journal of Dairy Science**. 87:3183-3188.

Mizushima, S., Ohshige, K., Watanabe, J., Kimura, M., Kadowaki, T., Nakamura, Y., Tochikubo, O. and Ueshima, H. (2004). Randomized controlled trial of sour milk on blood pressure in borderline hypertensive men. **American Journal of Hypertension**. 17:701-706.

Mojarrad, J. S., Nemati, M., Valizadeh, H., Ansarin, M. and Bourbour, S. (2007). Preparation of Glucosamine from Exoskeleton of Shrimp and Predicting Production Yield by Response Surface Methodology. **Journal of Agricultural and Food Chemistry**. 55:2246-2250.

Monauni, T., Zenti, M. G., Cretti, A., Daniels, M. C., Targher, G., Caruso, B., Caputo, M., McClain, D., Del Prato, S. and Giaccari, A. (2000). Effects of glucosamine infusion on insulin secretion and insulin action in humans. **Diabetes**. 49:926-935.

Mooney, C., Haslam, N. J., Pollastri, G. and Shields, D. C. (2012). Towards the improved discovery and design of functional peptides: common features of diverse classes permit generalized prediction of bioactivity. **PLOS ONE**. 7(10):e45012.

Mourabet, M., El Rhilassi, A., El Boujaady, H., Bennani-Ziatni, M., El Hamri, R. and Taitai, A. (2012). Removal of fluoride from aqueous solution by adsorption on Apatitic tricalcium phosphate using Box-Behnken design and desirability function. **Applied Surface Science**. 258:4402-4410.

Murray, B. and FitzGerald, R. (2007). Angiotensin converting enzyme inhibitory peptides derived from food proteins: biochemistry, bioactivity and production.

Current Pharmaceutical Design. 13:773-791.

Murray, C. J., Vos, T., Lozano, R., Naghavi, M., Flaxman, A. D., Michaud, C., Ezzati, M., Shibuya, K., Salomon, J. A. and Abdalla, S. (2013). Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. **The Lancet**. 380:2197-2223.

Muzzarelli, R. A. A., Boudrant, J., Meyer, D., Manno, N., DeMarchis, M. and Paoletti, M. G. (2012). Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. **Carbohydrate Polymers**. 87:995-1012.

Nagaoka, I., Igarashi, M., Hua, J., Ju, Y., Yomogida, S. and Sakamoto, K. (2011). Recent aspects of the anti-inflammatory actions of glucosamine. **Carbohydrate Polymers**. 84:825-830.

Naito, K., Watari, T., Furuhata, A., Yomogida, S., Sakamoto, K., Kurosawa, H., Kaneko, K. and Nagaoka, I. (2010). Evaluation of the effect of glucosamine on an experimental rat osteoarthritis model. **Life Sciences**. 86:538-543.

Nakagomi, K., Yamada, R., Ebisu, H., Sadakane, Y., Akizawa, T. and Tanimura, T. (2000). Isolation of Acein-2, a novel angiotensin-I-converting enzyme inhibitory peptide derived from a tryptic hydrolysate of human plasma. **FEBS Letters**. 467:235-238.

Nakahara, T., Sano, A., Yamaguchi, H., Sugimoto, K., Chikata, H., Kinoshita, E. and Uchida, R. (2009). Antihypertensive effect of peptide-enriched soy sauce-like seasoning and identification of its angiotensin I-converting enzyme inhibitory substances. **Journal of Agricultural and Food Chemistry**. 58:821-827.

Nakamura, H. (2011). Application of glucosamine on human disease-Osteoarthritis. **Carbohydrate Polymers**. 84:835-839.

Nakamura, H., Shibakawa, A., Tanaka, M., Kato, T. and Nishioka, K. (2003). Effects of glucosamine hydrochloride on the production of prostaglandin E2, nitric oxide and metalloproteases by chondrocytes and synoviocytes in osteoarthritis. **Clinical and Experimental Rheumatology**. 22:293-299.

Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S. and Takano, T. (1995). Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. **Journal of Dairy Science**. 78:777-783.

Nakamura, Y., Yamamoto, N., Sakai, K. and Takano, T. (1995). Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. **Journal of Dairy Science**. 78:1253-1257.

Nakashima, Y., Arihara, K., Sasaki, A., Mio, H., Ishikawa, S. and Itoh, M. (2002). Antihypertensive activities of peptides derived from porcine skeletal muscle myosin in spontaneously hypertensive rats. **Journal of Food Science**. 67:434-437.

Natesh, R., Schwager, S. L., Sturrock, E. D. and Acharya, K. R. (2003). Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. **Nature**. 421:551-554.

Nitschke, J., Altenbach, H.-J., Malolepszy, T. and Mölleken, H. (2011). A new method for the quantification of chitin and chitosan in edible mushrooms. **Carbohydrate Research**. 346:1307-1310.

No, H., Park, N., Lee, S., Hwang, H. and Meyers, S. (2002). Antibacterial activities of chitosans and chitosan oligomers with different molecular weights on spoilage bacteria isolated from tofu. **Journal of Food Science**. 67:1511-1514.

Oegema, T. R., Deloria, L. B., Sandy, J. D. and Hart, D. A. (2002). Effect of oral glucosamine on cartilage and meniscus in normal and chymopapain - injected knees of young rabbits. **Arthritis and Rheumatism**. 46:2495-2503.

Ofenbeher-Miletić, I., Stanimirović, D. and Stanimirović, S. (1984). On determination of chitin content in mushrooms. **Plant Foods for Human Nutrition**. 34:197-201.

Oh, H.-J., Lee, J. S., Song, D.-K., Shin, D.-H., Jang, B.-C., Suh, S.-I., Park, J.-W., Suh, M.-H. and Baek, W.-K. (2007). D-Glucosamine inhibits proliferation of human cancer cells through inhibition of p70S6K. **Biochemical and Biophysical Research Communications**. 360:840-845.

Okamoto, A., Hanagata, H., Matsumoto, E., Kawamura, Y., Koizumi, Y. and Yanagida, F. (1995). Angiotensin I converting enzyme inhibitory activities of various fermented foods. **Bioscience, Biotechnology, and Biochemistry**. 59:1147-1149.

Oliveria, S. A., Felson, D. T., Reed, J. I., Cirillo, P. A. and Walker, A. M. (1995). Incidence of symptomatic hand, hip, and knee osteoarthritis among patients in a health maintenance organization. **Arthritis and Rheumatism**. 38:1134-1141.

Ondetti, M. A., Williams, N. J., Sabo, E., Pluscec, J., Weaver, E. R. and Kocy, O. (1971). Angiotensin-converting enzyme inhibitors from the venom of *Bothrops jararaca*. Isolation, elucidation of structure, and synthesis. **Biochemistry**. 10:4033-4039.

Oshima, G., Shimabukuro, H. and Nagasawa, K. (1979). Peptide inhibitors of angiotensin I-converting enzyme in digests of gelatin by bacterial collagenase. **Biochimica et Biophysica Acta (BBA)-Enzymology**. 566:128-137.

Ospina Álvarez, S. P., Ramírez Cadavid, D. A., Escobar Sierra, D. M., Ossa Orozco, C. P., Rojas Vahos, D. F., Zapata Ocampo, P. and Atehortúa, L. (2014). Comparison of extraction methods of chitin from Ganoderma lucidum mushroom obtained in submerged culture. **BioMed Research International**. 2014.

Pérez-Vega, J. A., Olivera-Castillo, L., Gómez-Ruiz, J. Á. and Hernández-Ledesma, B. (2013). Release of multifunctional peptides by gastrointestinal digestion of sea cucumber (*Isostichopus badionotus*). **Journal of Functional Foods**. 5:869-877.

Paiva, L., Lima, E., Neto, A. I. and Baptista, J. (2016). Isolation and characterization of angiotensin I-converting enzyme (ACE) inhibitory peptides from *Ulva rigida* C. Agardh protein hydrolysate. **Journal of Functional Foods**. 26:65-76.

Pan, S., Wang, S., Jing, L. and Yao, D. (2016). Purification and characterisation of a novel angiotensin-I converting enzyme (ACE)-inhibitory peptide derived from the enzymatic hydrolysate of *Enteromorpha clathrata* protein. **Food Chemistry**. 211:423-430.

Pan, Y., Dong, S., Hao, Y., Zhou, Y., Ren, X., Wang, J., Wang, W. and Chu, T. (2010). Ultrasonic-assisted extraction process of crude polysaccharides from Yunzhi mushroom and its effect on hydroxyproline and glycosaminoglycan levels. **Carbohydrate Polymers**. 81:93-96.

Park, C., Lee, J., Hong, S.-S., Lee, H.-S. and Kim, S. (1998). High-level expression of the angiotensin-converting-enzyme-inhibiting peptide, YG-1, as tandem multimers in *Escherichia coli*. **Applied Microbiology and Biotechnology**. 50:71-76.

Petiard, V., Michaux, S. and Courtois, D. (2013). *U.S. Patent No. 8378090 B2*. Washington,

DC: U.S. Patent and Trademark Office.

Petrovska, B. B. (2001). Protein fraction in edible Macedonian mushrooms. **European Food Research and Technology**. 212:469-472.

Phat, C., Moon, B. and Lee, C. (2016). Evaluation of umami taste in mushroom extracts by chemical analysis, sensory evaluation, and an electronic tongue system. **Food Chemistry**. 192:1068-1077.

Pichyangkura, R., Kudan, S., Kuttiyawong, K., Sukwattanasinitt, M. and Aiba, S.-i. (2002). Quantitative production of 2-acetamido-2-deoxy-d-glucose from crystalline chitin by bacterial chitinase. **Carbohydrate Research**. 337:557-559.

Pihlanto, A., Virtanen, T. and Korhonen, H. (2010). Angiotensin I converting enzyme (ACE) inhibitory activity and antihypertensive effect of fermented milk. **International Dairy Journal**. 20:3-10.

Piperno, M., Reboul, P., Le Graverand, M. H., Peschard, M., Annefeld, M., Richard, M. and Vignon, E. (2000). Glucosamine sulfate modulates dysregulated activities of human osteoarthritic chondrocytes *in vitro*. **Osteoarthritis and Cartilage**. 8:207-212.

Poolsup, N., Suthisisang, C., Channark, P. and Kittikulsuth, W. (2005). Glucosamine long-term treatment and the progression of knee osteoarthritis: systematic review of randomized controlled trials. **Annals of Pharmacotherapy**. 39:1080-1087.

Prashanth, K. H. and Tharanathan, R. (2007). Chitin/chitosan: modifications and their unlimited application potential-an overview. **Trends in Food Science and Technology**. 18:117-131.

Pripp, A. H., Isaksson, T., Stepaniak, L. and Sørhaug, T. (2004). Quantitative structure-

activity relationship modelling of ACE-inhibitory peptides derived from milk proteins. **European Food Research and Technology**. 219:579-583.

Puchalska, P., García, M. C. and Marina, M. L. (2014). Identification of native angiotensin-I converting enzyme inhibitory peptides in commercial soybean based infant formulas using HPLC-Q-ToF-MS. **Food Chemistry**. 157:62-69.

Pujalte, J. M., Llavore, E. P. and Ylescupidez, F. R. (1980). Double-blind clinical evaluation of oral glucosamine sulphate in the basic treatment of osteoarthrosis. **Current Medical Research and Opinion**. 7:5.

Qi, L., Zhang, S.-F., Zuo, M. and Chen, Y. (2006). Capillary electrophoretic determination of glucosamine in osteoarthritis tablets via microwave-accelerated dansylation. **Journal of Pharmaceutical and Biomedical Analysis**. 41:1620-1624.

Qin, C., Du, Y., Xiao, L., Li, Z. and Gao, X. (2002). Enzymic preparation of water-soluble chitosan and their antitumor activity. **International Journal of Biological Macromolecules**. 31:111-117.

Quang, D. N., Hashimoto, T. and Asakawa, Y. (2006). Inedible mushrooms: a good source of biologically active substances. **The Chemical Record**. 6:79-99.

Quirós, A., Chichón, R., Recio, I. and López-Fandiño, R. (2007). The use of high hydrostatic pressure to promote the proteolysis and release of bioactive peptides from ovalbumin. **Food Chemistry**. 104:1734-1739.

Quirós, A., Contreras, M. d. M., Ramos, M., Amigo, L. and Recio, I. (2009). Stability to gastrointestinal enzymes and structure-activity relationship of β-casein-peptides with antihypertensive properties. **Peptides**. 30:1848-1853.

Quirós, A., Ramos, M., Muguerza, B., Delgado, M. A., Miguel, M., Aleixandre, A. and Recio, I. (2007). Identification of novel antihypertensive peptides in milk fermented

with *Enterococcus faecalis*. **International Dairy Journal**. 17:33-41.

Racine, J. and Aaron, R. (2013). Pathogenesis and epidemiology of osteoarthritis. **Rhode Island medical journal (2013)**. 96:19-22.

Rafi, M. M., Yadav, P. N. and Rossi, A. O. (2007). Glucosamine inhibits LPS‐induced COX‐2 and iNOS expression in mouse macrophage cells (RAW 264.7) by inhibition of p38‐MAP kinase and transcription factor NF-κB. **Molecular Nutrition and Food Research**. 51:587-593.

Rao, S., Su, Y., Li, J., Xu, Z. and Yang, Y. (2009). Design and expression of recombinant antihypertensive peptide multimer gene in *Escherichia coli* BL21. **Journal of Microbiology and Biotechnology**. 19:1620-1627.

Ravens, D. (1960). The chemical reactivity of poly (ethylene terephthalate): Heterogeneous hvdrolysis by hydrochloric acid. **Polymer**. 1:375-383.

Reginster, J.-Y., Bruyere, O., Lecart, M.-P. and Henrotin, Y. (2003). Naturocetic (glucosamine and chondroitin sulfate) compounds as structure-modifying drugs in the treatment of osteoarthritis. **Current opinion in rheumatology**. 15:651-655.

Reginster, J. Y., Deroisy, R., Rovati, L. C., Lee, R. L., Lejeune, E., Bruyere, O., Giacovelli, G., Henrotin, Y., Dacre, J. E. and Gossett, C. (2001). Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. **The Lancet**. 357:251-256.

Rho, S. J., Lee, J.-S., Chung, Y. I., Kim, Y.-W. and Lee, H. G. (2009). Purification and identification of an angiotensin I-converting enzyme inhibitory peptide from fermented soybean extract. **Process Biochemistry**. 44:490-493.

Rojas-Ronquillo, R., Cruz-Guerrero, A., Flores-Nájera, A., Rodríguez-Serrano, G.,

Gómez-Ruiz, L., Reyes-Grajeda, J. P., Jiménez-Guzmán, J. and García-Garibay, M. (2012). Antithrombotic and angiotensin-converting enzyme inhibitory properties of peptides released from bovine casein by *Lactobacillus casei* Shirota. **International Dairy Journal**. 26:147-154.

Roseman, S., Li, X. and Comb, D. (2010). *U.S. Patent No. 9267141 B2*. Washington, DC: U.S. Patent and Trademark Office.

Rosmiza, M., Davies, W., Jabil, M. and Mazdi, M. (2016). Prospects for increasing commercial mushroom production in Malaysia: challenges and opportunities. **Mediterranean Journal of Social Sciences**. 7:406.

Rovati, L. C., Girolami, F., D'Amato, M. and Giacovelli, G. (2015). Effects of Glucosamine Sulfate on the use of Rescue Non-Steroidal Anti-Inflammatory Drugs in Knee Osteoarthritis: Results From The Pharmaco-Epidemiology Of Gonarthrosis (PEGAsus) Study. Paper presented at the Seminars in Arthritis and Rheumatism.

Rozendaal, R. M., Koes, B. W., Van Osch, G. J., Uitterlinden, E. J., Garling, E. H., Willemsen, S. P., Ginai, A. Z., Verhaar, J. A., Weinans, H. and Bierma-Zeinstra, S. M. (2008). Effect of glucosamine sulfate on hip osteoarthritis: a randomized trial. **Annals of Internal Medicine**. 148:268-277.

Rui, X., Boye, J. I., Simpson, B. K. and Prasher, S. O. (2013). Purification and characterization of angiotensin I-converting enzyme inhibitory peptides of small red bean (*Phaseolus vulgaris*) hydrolysates. **Journal of Functional Foods**. 5:1116-1124.

Ruiz-Ruiz, J., Dávila-Ortíz, G., Chel-Guerrero, L. and Betancur-Ancona, D. (2013). Angiotensin I-Converting Enzyme Inhibitory and Antioxidant Peptide

Fractions from Hard-to-Cook Bean Enzymatic Hydrolysates. **Journal of Food Biochemistry**. 37:26-35.

Ruiz, J. Á. G., Ramos, M. and Recio, I. (2004). Angiotensin converting enzyme-inhibitory activity of peptides isolated from Manchego cheese. Stability under simulated gastrointestinal digestion. **International Dairy Journal**. 14:1075-1080.

Ruiz, R. and Date, T. E. (1996). Determination of carbohydrates in biomass by high performance liquid chromatography. Paper presented at the Laboratory Analytical Procedure No. 002, National Renewable Research Laboratory.

Runhaar, J., Deroisy, R., van Middelkoop, M., Barretta, F., Barbetta, B., Oei, E. H., Vroegindeweij, D., Giacovelli, G., Bruyère, O. and Rovati, L. C. (2015). The Role of Diet and Exercise and of Glucosamine Sulfate in the Prevention of Knee Osteoarthritis: Further Results from the Prevention of Knee Osteoarthritis in Overweight Females (PROOF) Study. Paper presented at the Seminars in Arthritis and Rheumatism.

Russell, A. L. and McCarty, M. F. (2000). Glucosamine for migraine prophylaxis? **Medical Hypotheses**. 55:195-198.

Saeman, J. F. (1945). Kinetics of wood saccharification-hydrolysis of cellulose and decomposition of sugars in dilute acid at high temperature. **Industrial and Engineering Chemistry**. 37:43-52.

Saiga, A., Okumura, T., Makihara, T., Katsuda, S.-I., Morimatsu, F. and Nishimura, T. (2006). Action mechanism of an angiotensin I-converting enzyme inhibitory peptide derived from chicken breast muscle. **Journal of Agricultural and Food Chemistry**. 54:942-945.

Saito, T., Nakamura, T., Kitazawa, H., Kawai, Y. and Itoh, T. (2000). Isolation and

structural analysis of antihypertensive peptides that exist naturally in Gouda cheese. **Journal of Dairy Science**. 83:1434-1440.

Sal'nikova, S., Drogovoz, S. and Zupanets, I. (1990). The liver-protective properties of D-glucosamine. **Farmakologiia i Toksikologiia**. 53:33.

Salmon, J., Rat, A., Sellam, J., Michel, M., Eschard, J., Guillemin, F., Jolly, D. and Fautrel, B. (2016). Economic impact of lower-limb osteoarthritis worldwide: a systematic review of cost-of-illness studies. **Osteoarthritis and Cartilage**. 24:1500-1508.

Samaranayaka, A. G., Kitts, D. D. and Li-Chan, E. C. (2010). Antioxidative and angiotensin-I-converting enzyme inhibitory potential of a Pacific hake (*Merluccius productus*) fish protein hydrolysate subjected to simulated gastrointestinal digestion and Caco-2 cell permeation. **Journal of Agricultural and Food Chemistry**. 58:1535-1542.

Samuels, J., Krasnokutsky, S. and Abramson, S. B. (2008). A tale of three tissues. **Bulletin of the NYU Hospital for Joint Diseases**. 66:244-250.

Sangsawad, P., Roytrakul, S. and Yongsawatdigul, J. (2017). Angiotensin converting enzyme (ACE) inhibitory peptides derived from the simulated *in vitro* gastrointestinal digestion of cooked chicken breast. **Journal of Functional Foods**. 29:77-83.

Sanmee, R., Dell, B., Lumyong, P., Izumori, K. and Lumyong, S. (2003). Nutritive value of popular wild edible mushrooms from northern Thailand. **Food Chemistry**. 82:527-532.

Sarzi-Puttini, P., Cimmino, M. A., Scarpa, R., Caporali, R., Parazzini, F., Zaninelli, A., Atzeni, F. and Canesi, B. (2005). Osteoarthritis: an overview of the disease and its treatment strategies. Paper presented at the Seminars in Arthritis and Rheumatism.

Sashiwa, H., Fujishima, S., Yamano, N., Kawasaki, N., Nakayama, A., Muraki, E., Hiraga, K., Oda, K. and Aiba, S.-i. (2002). Production of N-acetyl-d-glucosamine from α-chitin by crude enzymes from *Aeromonas hydrophila* H-2330. **Carbohydrate Research**. 337:761-763.

Sashiwa, H., Fujishima, S., Yamano, N., Kawasaki, N., Nakayama, A., Muraki, E., Sukwattanasinitt, M., Pichyangkura, R. and Aiba, S.-i. (2003). Enzymatic production of N-acetyl-d-glucosamine from chitin. Degradation study of N-acetylchitooligosaccharide and the effect of mixing of crude enzymes. **Carbohydrate Polymers**. 51:391-395.

Segura Campos, M. R., Chel Guerrero, L. A. and Betancur Ancona, D. A. (2010). Angiotensin‐I converting enzyme inhibitory and antioxidant activities of peptide fractions extracted by ultrafiltration of cowpea *Vigna unguiculata* hydrolysates. **Journal of the Science of Food and Agriculture**. 90:2512-2518.

Seo, W.-G., Pae, H.-O., Kim, N.-Y., Oh, G.-S., Park, I.-S., Kim, Y.-H., Kim, Y.-M., Lee, Y.-h., Jun, C.-D. and Chung, H.-T. (2000). Synergistic cooperation between water-soluble chitosan oligomers and interferon-γ for induction of nitric oxide synthesis and tumoricidal activity in murine peritoneal macrophages. **Cancer Letters**. 159:189-195.

Seppo, L., Jauhiainen, T., Poussa, T. and Korpela, R. (2003). A fermented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects. **The American Journal of Clinical Nutrition**. 77:326-330.

Serra, C. P., Côrtes, S. d. F., Lombardi, J. A., De Oliveira, A. B. and Braga, F. C. (2005). Validation of a colorimetric assay for the in vitro screening of inhibitors

of angiotensin-converting enzyme (ACE) from plant extracts. **Phytomedicine**. 12:424-432.

Serrato-Joya, O., Jiménez-Islas, H., Botello-Álvarez, E., Rico-Martínez, R. and Navarrete-Bolaños, J. (2006). Production of β-cryptoxanthin, a provitamin-A precursor, by Flavobacterium lutescens. **Journal of Food Science**. 71:E314-E319.

Shabrukova, N. V., Shestakova, L. M., Zainetdinova, D. R. and Gamayurova, V. S. (2002). Research of acid hydrolyses of chitin-glucan and chitosan-glucan complexes. **Chemistry and Computational Simulation. Butlerov Communications**. 2:57-59.

Shah, H., Khalil, I. A. and Jabeen, S. (1997). Nutritional composition and protein quality of Pleurotus mushroom. **Sarhad Journal of Agriculture (Pakistan)**. 13(6):621-626.

Shahidi, F., Arachchi, J. K. V. and Jeon, Y.-J. (1999). Food applications of chitin and chitosans. **Trends in Food Science and Technology**. 10:37-51.

Shalaby, S. M., Zakora, M. and Otte, J. (2006). Performance of two commonly used angiotensin-converting enzyme inhibition assays using FA-PGG and HHL as substrates. **Journal of Dairy Research**. 73:178-186.

Shao, Y., Alluri, R., Mummert, M., Koetter, U. and Lech, S. (2004). A stability-indicating HPLC method for the determination of glucosamine in pharmaceutical formulations. **Journal of Pharmaceutical and Biomedical Analysis**. 35:625-631.

Sharif, B., Kopec, J., Bansback, N., Rahman, M. M., Flanagan, W. M., Wong, H., Fines, P. and Anis, A. (2015). Projecting the Direct Cost Burden of

Osteoarthritis in Canada Using a Microsimulation Model. **Osteoarthritis and Cartilage**. 23(10):1654-1663.

Sharma, A. and Arora, S. (2013). Dermal delivery of glucosamine sulphate: formulation, characterization and performance evaluation. **World Journal of Pharmacy and Pharmaceutical Sciences**. 2(6):6448-6462.

Sheih, I.-C., Fang, T. J. and Wu, T.-K. (2009). Isolation and characterisation of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide from the algae protein waste. **Food Chemistry**. 115:279-284.

Shikhman, A. R., Kuhn, K., Alaaeddine, N. and Lotz, M. (2001). N-acetylglucosamine prevents IL-1β-mediated activation of human chondrocytes. **The Journal of Immunology**. 166:5155-5160.

Shimizu, M. (2004). Food - derived peptides and intestinal functions. **Biofactors**. 21:43-47.

Shin, Z.-I., Yu, R., Park, S.-A., Chung, D. K., Ahn, C.-W., Nam, H.-S., Kim, K.-S. and Lee, H. J. (2001). His-His-Leu, an angiotensin I converting enzyme inhibitory peptide derived from Korean soybean paste, exerts antihypertensive activity *in vivo*. **Journal of Agricultural and Food Chemistry**. 49:3004-3009.

Simon, L., Hatoum, H., Bittman, R., Archambault, W. and Polisson, R. (1996). Risk factors for serious nonsteroidal-induced gastrointestinal complications: regression analysis of the MUCOSA trial. **Family Medicine**. 28:204-210.

Singh, S., Chaudhary, K., Dhanda, S. K., Bhalla, S., Usmani, S. S., Gautam, A., Tuknait, A., Agrawal, P., Mathur, D. and Raghava, G. P. (2016). SATPdb: a database of structurally annotated therapeutic peptides. **Nucleic Acids Research**. 44:D1119-D1126.

Sipola, M., Finckenberg, P., Korpela, R., Vapaatalo, H. and Nurminen, M.-L. (2002). Effect of long-term intake of milk products on blood pressure in hypertensive rats. **Journal of Dairy Research**. 69:103-111.

Sitanggang, A., Wu, H.-S. and Wang, S. (2009). Determination of fungal glucosamine using HPLC with 1-napthyl isothiocyanate derivatization and microwave heating. **Biotechnology and Bioprocess Engineering**. 14:819-827.

Sitanggang, A. B., Wu, H.-S., Wang, S. S. and Ho, Y.-C. (2010). Effect of pellet size and stimulating factor on the glucosamine production using *Aspergillus* sp. BCRC 31742. **Bioresource Technology**. 101:3595-3601.

Skelley, A. M. and Mathies, R. A. (2006). Rapid on-column analysis of glucosamine and its mutarotation by microchip capillary electrophoresis. **Journal of Chromatography A**. 1132:304-309.

Smacchi, E. and Gobbetti, M. (1998). Peptides from several Italian cheeses inhibitory to proteolytic enzymes of lactic acid bacteria, *Pseudomonas fluorescens* ATCC 948 and to the angiotensin I-converting enzyme. **Enzyme and Microbial Technology**. 22:687-694.

So, P. B. T., Rubio, P., Lirio, S., Macabeo, A. P., Huang, H.-Y., Corpuz, M. J.-A. T. and Villaflores, O. B. (2016). *In vitro* angiotensin I converting enzyme inhibition by a peptide isolated from *Chiropsalmus quadrigatus* Haeckel (box jellyfish) venom hydrolysate. **Toxicon**. 119:77-83.

Spahn, G., Kirschbaum, S. and Kahl, E. (2006). Factors that influence high tibial osteotomy results in patients with medial gonarthritis: a score to predict the results. **Osteoarthritis and Cartilage**. 14:190-195.

Sparringa, R. and Owens, J. (1999). Glucosamine content of tempe mould, Rhizopus oligosporus. **International Journal of Food Microbiology**. 47:153-157.

Stankiewicz, B. A., Mastalerz, M., Hof, C. H. J., Bierstedt, A., Flannery, M. B., Briggs, D. E. G. and Evershed, R. P. (1998). Biodegradation of the chitin-protein complex in crustacean cuticle. **Organic Geochemistry**. 28:67-76.

Sukwattanasinitt, M., Zhu, H., Sashiwa, H. and Aiba, S.-i. (2002). Utilization of commercial non-chitinase enzymes from fungi for preparation of 2-acetamido-2-deoxy-d-glucose from β-chitin. **Carbohydrate Research**. 337:133-137.

Sunday, E. A., Israel, A. U. and Odey, M. T. (2016). Proximate Analysis and Mineral Element Composition of False Yam (*Icacina Trichantha*) Tuber and Oyster Mushroom (*Pleurotus Ostreatus*). **Equatorial Journal of Chemical Sciences.** 1(1):125-135.

Suxia, R., Haiyan, X., Jinling, Z., Shunqing, L., Xiaofeng, H. and Tingzhou, L. (2012). Furfural production from rice husk using sulfuric acid and a solid acid catalyst through a two-stage process. **Carbohydrate Research**. 359:1-6.

Suzuki, K., Okawa, Y., Hashimoto, K., Suzuki, S. and Suzuki, M. (1984). Protecting effect of chitin and chitosan on experimentally induced murine candidiasis. **Microbiology and Immunology**. 28:903-912.

Talpur, N. A., Echard, B. W., Fan, A. Y., Jaffari, O., Bagchi, D. and Preuss, H. G. (2002). Antihypertensive and metabolic effects of whole Maitake mushroom powder and its fractions in two rat strains. **Molecular and Cellular Biochemistry**. 237:129-136.

Taniguchi, S., Ryu, J., Seki, M., Sumino, T., Tokuhashi, Y. and Esumi, M. (2012). Long‐term oral administration of glucosamine or chondroitin sulfate reduces destruction of cartilage and up‐regulation of MMP‐3 mRNA in a model of

spontaneous osteoarthritis in Hartley guinea pigs. **Journal of Orthopaedic Research**. 30:673-678.

Tekko, I. A., Bonner, M. C. and Williams, A. C. (2006). An optimized reverse-phase high performance liquid chromatographic method for evaluating percutaneous absorption of glucosamine hydrochloride. **Journal of Pharmaceutical and Biomedical Analysis**. 41:385-392.

Tellez-Luis, S. J., Ramırez, J. and Vázquez, M. (2002). Mathematical modelling of hemicellulosic sugar production from sorghum straw. **Journal of Food Engineering**. 52:285-291.

Thakral, R., Debnath, U. K. and Dent, C. (2007). Role of glucosamine in osteoarthritis. **Current Orthopaedics**. 21:386-389.

Thamthiankul, S., Suan-Ngay, S., Tantimavanich, S. and Panbangred, W. (2001). Chitinase from Bacillus thuringiensis subsp. pakistani. **Applied Microbiology and Biotechnology**. 56:395-401.

Tiraloche, G., Girard, C., Chouinard, L., Sampalis, J., Moquin, L., Ionescu, M., Reiner, A., Poole, A. R. and Laverty, S. (2005). Effect of oral glucosamine on cartilage degradation in a rabbit model of osteoarthritis. **Arthritis and Rheumatism**. 52:1118-1128.

Tom, R. A. and Carroad, P. A. (1981). Effect of Reaction Conditions on Hydrolysis of Chitin by Serratia marcescens QMB 1466 chitinase. **Journal of Food Science**. 46:646-647.

Tomatsu, M., Shimakage, A., Shinbo, M., Yamada, S. and Takahashi, S. (2013). Novel angiotensin I-converting enzyme inhibitory peptides derived from soya milk. **Food Chemistry**. 136:612-616.

Toopcham, T., Roytrakul, S. and Yongsawatdigul, J. (2015). Characterization and identification of angiotensin I-converting enzyme (ACE) inhibitory peptides derived from tilapia using *Virgibacillus halodenitrificans* SK1-3-7 proteinases. **Journal of Functional Foods**. 14:435-444.

Tovar-Pérez, E., Guerrero-Legarreta, I., Farrés-González, A. and Soriano-Santos, J. (2009). Angiotensin I-converting enzyme-inhibitory peptide fractions from albumin 1 and globulin as obtained of amaranth grain. **Food Chemistry**. 116:437-444.

Towheed, T., Anastassiades, T., Shea, B., Houpt, J., Welch, V. and Hochberg, M. (2000). Glucosamine therapy for treating osteoarthritis. **The Cochrane Library**: **Cochrane Database Systematic Reviews.** 1:CD002946.

Tsai, J.-S., Chen, J.-L. and Pan, B. S. (2008). ACE-inhibitory peptides identified from the muscle protein hydrolysate of hard clam (*Meretrix lusoria*). **Process Biochemistry**. 43:743-747.

Tshinyangu, K. K. and Hennebert, G. L. (1996). Protein and chitin nitrogen contents and protein content in *Pleurotus ostreatus* var. *columbinus*. **Food Chemistry**. 57:223-227.

Tuomilehto, J., Lindström, J., Hyyrynen, J., Korpela, R., Karhunen, M., Mikkola, L., Jauhiainen, T., Seppo, L. and Nissinen, A. (2004). Effect of ingesting sour milk fermented using *Lactobacillus helveticus* bacteria producing tripeptides on blood pressure in subjects with mild hypertension. **Journal of Human Hypertension**. 18:795-802.

Udenigwe, C. C., Lin, Y.-S., Hou, W.-C. and Aluko, R. E. (2009). Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. **Journal of Functional Foods**. 1:199-207.

Ueno, K., Mizuno, S. and Yamamoto, N. (2004). Purification and characterization of an endopeptidase that has an important role in the carboxyl terminal processing of antihypertensive peptides in *Lactobacillus helveticus* CM4. **Letters in Applied Microbiology**. 39:313-318.

Vårum, K., Ottøy, M. and Smidsrød, O. (2001). Acid hydrolysis of chitosans. **Carbohydrate Polymers**. 46:89-98.

Vásquez-Villanueva, R., Marina, M. L. and García, M. C. (2015). Revalorization of a peach (*Prunus persica* (L.) Batsch) byproduct: Extraction and characterization of ACE-inhibitory peptides from peach stones. **Journal of Functional Foods**. 18:137-146.

Vermeirssen, V., Van Camp, J., Decroos, K., Van Wijmelbeke, L. and Verstraete, W. (2003). The impact of fermentation and in vitro digestion on the formation of angiotensin-I-converting enzyme inhibitory activity from pea and whey protein. **Journal of Dairy Science**. 86:429-438.

Vermeirssen, V., Van Camp, J. and Verstraete, W. (2004). Bioavailability of angiotensin I converting enzyme inhibitory peptides. **British Journal of Nutrition**. 92:357-366.

Veronese, N., Stubbs, B., Noale, M., Solmi, M., Luchini, C., Smith, T. O., Cooper, C., Guglielmi, G., Reginster, J.-Y. and Rizzoli, R. (In press, Available online 8 October 2016). Adherence to a Mediterranean diet is associated with lower prevalence of osteoarthritis: Data from the osteoarthritis initiative. **Clinical Nutrition**. https://doi.org/10.1016/j.clnu.2016.09.035.

Vetter, J. (2007). Chitin content of cultivated mushrooms *Agaricus bisporus*, *Pleurotus ostreatus* and *Lentinula edodes*. **Food Chemistry**. 102:6-9.

Viitanen, R., Vapaatalo, H. and Valjakka, J. (2014). Does the cis/trans configuration of peptide bonds in bioactive tripeptides play a role in ACE-1 enzyme inhibition? **Biologics: Targets and Therapy**. 8:59-65.

Vinci, J. M., Horwitz, D., Zusman, R. M., Pisano, J., Catt, K. J. and Keiser, H. R. (1979). The effect of converting enzyme inhibition with SQ 20881 on plasma and urinary kinins, prostaglandin E and angiotensin II in hypertensive man. **Hypertension**. 1:416-426.

Vishu, K. A., Varadaraj, M., Gowda, L. and Tharanathan, R. (2005). Characterization of chito-oligosaccharides prepared by chitosanolysis with the aid of papain and Pronase, and their bactericidal action against *Bacillus cereus* and *Escherichia coli*. **Biochemical Journal**. 391:167-175.

Vlad, S. C., LaValley, M. P., McAlindon, T. E. and Felson, D. T. (2007). Glucosamine for pain in osteoarthritis: why do trial results differ? **Arthritis and Rheumatism**. 56:2267-2277.

Volpi, N. (2009). Capillary electrophoresis determination of glucosamine in nutraceutical formulations after labeling with anthranilic acid and UV detection. **Journal of Pharmaceutical and Biomedical Analysis**. 49:868-871.

Wandel, S., Jüni, P., Tendal, B., Nüesch, E., Villiger, P. M., Welton, N. J., Reichenbach, S. and Trelle, S. (2010). Effects of glucosamine, chondroitin, or placebo in patients with osteoarthritis of hip or knee: network meta-analysis. **The British Medical Journal.** 341:c4675.

Wang, C., Tian, J. and Wang, Q. (2011). ACE inhibitory and antihypertensive properties of apricot almond meal hydrolysate. **European Food Research and Technology**. 232:549-556.

Wang, S.-H. and Chen, J.-C. (2005). The protective effect of chitin and chitosan against *Vibrio alginolyticus* in white shrimp *Litopenaeus vannamei*. **Fish and Shellfish Immunology**. 19:191-204.

Wang, S.-L., Lin, C.-L., Liang, T.-W., Liu, K.-C. and Kuo, Y.-H. (2009). Conversion of squid pen by *Serratia ureilytica* for the production of enzymes and antioxidants. **Bioresource Technology**. 100:316-323.

Wang, S. X., Laverty, S., Dumitriu, M., Plaas, A. and Grynpas, M. D. (2007). The effects of glucosamine hydrochloride on subchondral bone changes in an animal model of osteoarthritis. **Arthritis and Rheumatism**. 56:1537-1548.

Wang, X., Chen, X., Chen, L., Wang, B., Peng, C., He, C., Tang, M., Zhang, F., Hu, J. and Li, R. (2008). Optimizing high‐performance liquid chromatography method for quantification of glucosamine using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization in rat plasma: Application to a pharmacokinetic study. **Biomedical Chromatography**. 22:1265-1271.

Wasser, S. P. (2010). Medicinal mushroom science: history, current status, future trends, and unsolved problems. **International Journal of Medicinal Mushrooms**. 12(1):1-16.

Wen, Z. H., Tang, C. C., Chang, Y. C., Huang, S. Y., Hsieh, S. P., Lee, C. H., Huang, G. S., Ng, H. F., Neoh, C. A., Hsieh, C. S., Chen, W. F. and Jean, Y. H. (2010). Glucosamine sulfate reduces experimental osteoarthritis and nociception in rats: association with changes of mitogen-activated protein kinase in chondrocytes. **Osteoarthritis and Cartilage**. 18:1192-1202.

WHO. (2014). Global status report on noncommunicable diseases 2014: World Health Organization.

Wieland, H. A., Michaelis, M., Kirschbaum, B. J. and Rudolphi, K. A. (2005). Osteoarthritis-an untreatable disease? **Nature Reviews Drug Discovery**. 4:331-344.

Wluka, A. E., Ding, C., Wang, Y., Jones, G., Urquhart, D. M. and Cicuttini, F. M. (2014). Aspirin is associated with reduced cartilage loss in knee osteoarthritis: Data from a cohort study. **Osteoarthritis and Cartilage**. 22:S400.

Wolinsky, I. and Driskell, J. A. (2004). Nutritional ergogenic aids: CRC Press.

Wong, J. H., Ng, T., Cheung, R. C., Ye, X., Wang, H., Lam, S., Lin, P., Chan, Y., Fang, E. F. and Ngai, P. H. (2010). Proteins with antifungal properties and other medicinal applications from plants and mushrooms. **Applied Microbiology and Biotechnology**. 87:1221-1235.

Woolf, A. D. and Pfleger, B. (2003). Burden of major musculoskeletal conditions. **Bulletin of the World Health Organization**. 81:646-656.

Wu, J., Aluko, R. E. and Muir, A. D. (2002). Improved method for direct high-performance liquid chromatography assay of angiotensin-converting enzyme-catalyzed reactions. **Journal of Chromatography A**. 950:125-130.

Wu, J., Aluko, R. E. and Nakai, S. (2006). Structural requirements of angiotensin I-converting enzyme inhibitory peptides: quantitative structure-activity relationship study of di-and tripeptides. **Journal of Agricultural and Food Chemistry**. 54:732-738.

Wu, J. and Ding, X. (2002). Characterization of inhibition and stability of soy-protein-derived angiotensin I-converting enzyme inhibitory peptides. **Food Research International**. 35:367-375.

Wu, Q., Jia, J., Yan, H., Du, J. and Gui, Z. (2015). A novel angiotensin-I converting enzyme (ACE) inhibitory peptide from gastrointestinal protease hydrolysate of silkworm pupa (*Bombyx mori*) protein: Biochemical characterization and molecular docking study. **Peptides**. 68:17-24.

Wu, T., Zivanovic, S., Draughon, F. A. and Sams, C. E. (2004). Chitin and chitosan value-added products from mushroom waste. **Journal of Agricultural and Food Chemistry**. 52:7905-7910.

Wu, Y., Hussain, M. and Fassihi, R. (2005). Development of a simple analytical methodology for determination of glucosamine release from modified release matrix tablets. **Journal of Pharmaceutical and Biomedical Analysis**. 38:263-269.

Wyvratt, M. J. and Patchett, A. A. (1985). Recent developments in the design of angiotensin-converting enzyme inhibitors. **Medicinal Research Reviews**. 5:483-531.

Xie, G.-p., Jiang, N., Wang, S.-n., Qi, R.-z., Wang, L., Zhao, P.-r., Liang, L. and Yu, B. (2015). *Eucommia ulmoides Oliv.* bark aqueous extract inhibits osteoarthritis in a rat model of osteoarthritis. **Journal of Ethnopharmacology**. 162:148-154.

Xing, R., Liu, S., Guo, Z., Yu, H., Li, C., Ji, X., Feng, J. and Li, P. (2006). The antioxidant activity of glucosamine hydrochloride *in vitro*. **Bioorganic and Medicinal Chemistry**. 14:1706-1709.

Xing, R., Liu, S., Yu, H., Guo, Z., Wang, P., Li, C., Li, Z. and Li, P. (2005). Salt-assisted acid hydrolysis of chitosan to oligomers under microwave irradiation. **Carbohydrate Research**. 340:2150-2153.

Xu, X., Yan, H., Chen, J. and Zhang, X. (2011). Bioactive proteins from mushrooms. **Biotechnology Advances**. 29:667-674.

Yahaya, N. F. M., Rahman, M. A. and Abdullah, N. (2014). Therapeutic potential of mushrooms in preventing and ameliorating hypertension. **Trends in Food Science and Technology**. 39:104-115.

Yamamoto, N., Maeno, M. and Takano, T. (1999). Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by *Lactobacillus helveticus* CPN4. **Journal of Dairy Science**. 82:1388-1393.

Yan, P. S. and Gao, X. J. (2012). Inhibitory activity of extracts from mycelia materials of several mushrooms on the angiotensin I-converting enzyme. Paper presented at the Advanced Materials Research.

Yan, X. and Evenocheck, H. M. (2012). Chitosan analysis using acid hydrolysis and HPLC/UV. **Carbohydrate Polymers**. 87:1774-1778.

Yan, Y., Wanshun, L., Baoqin, H., Changhong, W., Chenwei, F., Bing, L. and Liehuan, C. (2007). The antioxidative and immunostimulating properties of d-glucosamine. **International Immunopharmacology**. 7:29-35.

Ye, M., Liu, J.-k., Lu, Z.-x., Zhao, Y., Liu, S.-f., Li, L.-l., Tan, M., Weng, X.-x., Li, W. and Cao, Y. (2005). Grifolin, a potential antitumor natural product from the mushroom Albatrellus confluens, inhibits tumor cell growth by inducing apoptosis in vitro. **FEBS Letters**. 579:3437-3443.

Yelin, E. (1998). The economics of osteoarthritis. **Osteoarthritis**. 20:23.

Yelin, E., Murphy, L., Cisternas, M. G., Foreman, A. J., Pasta, D. J. and Helmick, C. G. (2007). Medical care expenditures and earnings losses among persons with arthritis and other rheumatic conditions in 2003, and comparisons with 1997. **Arthritis and Rheumatism**. 56:1397-1407.

Yetilmezsoy, K., Demirel, S. and Vanderbei, R. J. (2009). Response surface modeling of Pb(II) removal from aqueous solution by *Pistacia vera* L.: Box-Behnken

experimental design. **Journal of Hazardous Materials**. 171:551-562.

Yoshioka, T., Motoki, T. and Okuwaki, A. (2001). Kinetics of hydrolysis of poly (ethylene terephthalate) powder in sulfuric acid by a modified shrinking-core model. **Industrial and Engineering Chemistry Research**. 40:75-79.

You, S. J. and Wu, J. (2011). Angiotensin‐I Converting Enzyme Inhibitory and Antioxidant Activities of Egg Protein Hydrolysates Produced with Gastrointestinal and Nongastrointestinal Enzymes. **Journal of Food Science**. 76:C801-C807.

Yu, K., Kim, Y., Shin, K., Kim, J. and Suh, H. J. (2005). Macrophage-stimulating activity of exo-biopolymer from cultured rice bran with Monascus pilosus. **Applied Biochemistry and Biotechnology**. 126:35-48.

Zhang, B.-z., Yan, P.-s., Chen, H. and He, J. (2012). Optimization of production conditions for mushroom polysaccharides with high yield and antitumor activity. **Carbohydrate Polymers**. 87:2569-2575.

Zhang, C., Cao, W., Hong, P., Ji, H., Qin, X. and He, J. (2009). Angiotensin I‐converting enzyme inhibitory activity of *Acetes chinensis* peptic hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. **International Journal of Food Science and Technology**. 44:2042-2048.

Zhang, G.-X., Yu, S., Gran, B. and Rostami, A. (2005). Glucosamine abrogates the acute phase of experimental autoimmune encephalomyelitis by induction of Th2 response. **The Journal of Immunology**. 175:7202-7208.

Zhang, L.-j., Huang, T.-m., Fang, X.-l., Li, X.-n., Wang, Q.-s., Zhang, Z.-w. and Sha, X.-y. (2006). Determination of glucosamine sulfate in human plasma by precolumn derivatization using high performance liquid chromatography with

fluorescence detection: Its application to a bioequivalence study. **Journal of Chromatography B**. 842:8-12.

Zhang, M., Haga, A., Sekiguchi, H. and Hirano, S. (2000). Structure of insect chitin isolated from beetle larva cuticle and silkworm (Bombyx mori) pupa exuvia. **International Journal of Biological Macromolecules**. 27:99-105.

Zhang, M., Tan, T., Yuan, H. and Rui, C. (2003). Insecticidal and fungicidal activities of chitosan and oligo-chitosan. **Journal of Bioactive and Compatible Polymers**. 18:391-400.

Zhang, W., Doherty, M., Arden, N., Bannwarth, B., Bijlsma, J., Gunther, K.-P., Hauselmann, H. J., Herrero-Beaumont, G., Jordan, K. and Kaklamanis, P. (2005). EULAR evidence based recommendations for the management of hip osteoarthritis: report of a task force of the EULAR Standing Committee for International Clinical Studies Including Therapeutics (ESCISIT). **Annals of the Rheumatic Diseases**. 64:669-681.

Zhang, W., Doherty, M., Leeb, B., Alekseeva, L., Arden, N., Bijlsma, J., Dinçer, F., Dziedzic, K., Häuselmann, H. and Herrero-Beaumont, G. (2007a). EULAR evidence based recommendations for the management of hand osteoarthritis: report of a Task Force of the EULAR Standing Committee for International Clinical Studies Including Therapeutics (ESCISIT). **Annals of the Rheumatic Diseases**. 66:377-388.

Zhang, W., Moskowitz, R., Nuki, G., Abramson, S., Altman, R., Arden, N., Bierma-Zeinstra, S., Brandt, K., Croft, P. and Doherty, M. (2007b). OARSI recommendations for the management of hip and knee osteoarthritis, part I: critical appraisal of existing treatment guidelines and systematic review of current research evidence. **Osteoarthritis and Cartilage**. 15:981-1000.

Zhang, W., Moskowitz, R., Nuki, G., Abramson, S., Altman, R., Arden, N., Bierma-Zeinstra, S., Brandt, K., Croft, P. and Doherty, M. (2008). OARSI recommendations for the management of hip and knee osteoarthritis, Part II: OARSI evidence-based, expert consensus guidelines. **Osteoarthritis and Cartilage**. 16:137-162.

Zhang, W., Nuki, G., Moskowitz, R., Abramson, S., Altman, R., Arden, N., Bierma-Zeinstra, S., Brandt, K., Croft, P. and Doherty, M. (2010). OARSI recommendations for the management of hip and knee osteoarthritis: part III: Changes in evidence following systematic cumulative update of research published through January 2009. **Osteoarthritis and Cartilage**. 18:476-499.

Zhao, Y., Li, B., Liu, Z., Dong, S., Zhao, X. and Zeng, M. (2007). Antihypertensive effect and purification of an ACE inhibitory peptide from sea cucumber gelatin hydrolysate. **Process Biochemistry**. 42:1586-1591.

Zhou, J. Z., Waszkuc, T. and Mohammed, F. (2004). Single Laboratory Validation of a Method for Determination of Glucosamine in RawMaterials and Dietary Supplements Containing Glucosamine Sulfate and/or Glucosamine Hydrochloride by High-Performance Liquid Chromatography with FMOC-Su Derivatization. **Journal of AOAC International**. 87:1083-1092.

Zhou, J. Z., Waszkuc, T. and Mohammed, F. (2005). Determination of glucosamine in raw materials and dietary supplements containing glucosamine sulfate and/or glucosamine hydrochloride by high-performance liquid chromatography with FMOC-Su derivatization: Collaborative study. **Journal of AOAC International**. 88:1048.

Zhou, L.-B. and Chen, B. (2011). Bioactivities of water-soluble polysaccharides from

Jisongrong mushroom: anti-breast carcinoma cell and antioxidant potential. **International Journal of Biological Macromolecules**. 48:1-4.

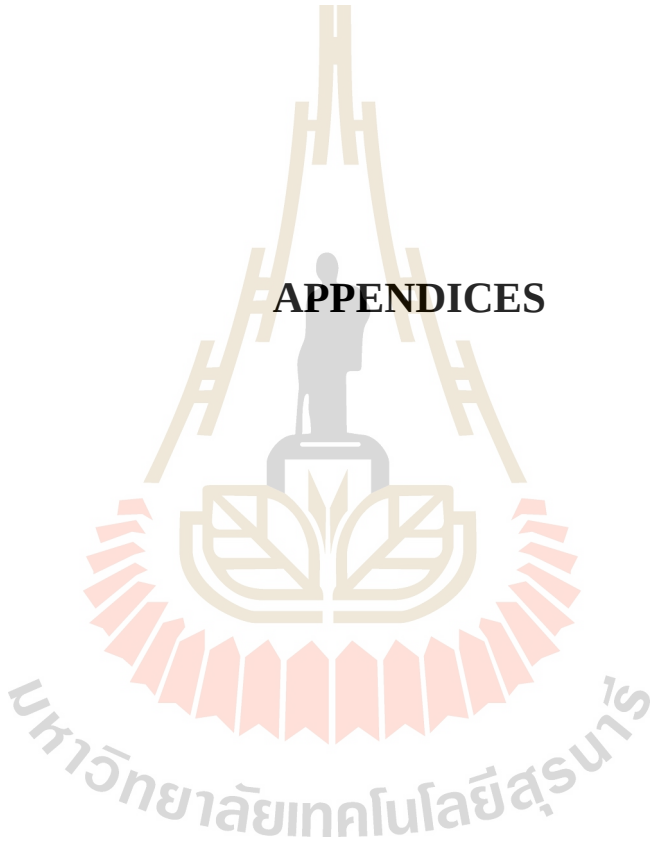
Zhu, X., Cai, J., Yang, J. and Su, Q. (2005). Determination of glucosamine in impure chitin samples by high-performance liquid chromatography. **Carbohydrate Research**. 340:1732-1738.

Zivanovic, S., Basurto, C., Chi, S., Davidson, P. and Weiss, J. (2004). Molecular weight of chitosan influences antimicrobial activity in oil-in-water emulsions. **Journal of Food Protection**. 67:952-959.

Zivanovic, S. and Buescher, R. (2004). Changes in Mushroom Texture and Cell Wall Composition Affected by Thermal Processing. **Journal of Food Science**. 69:SNQ44-SNQ49.

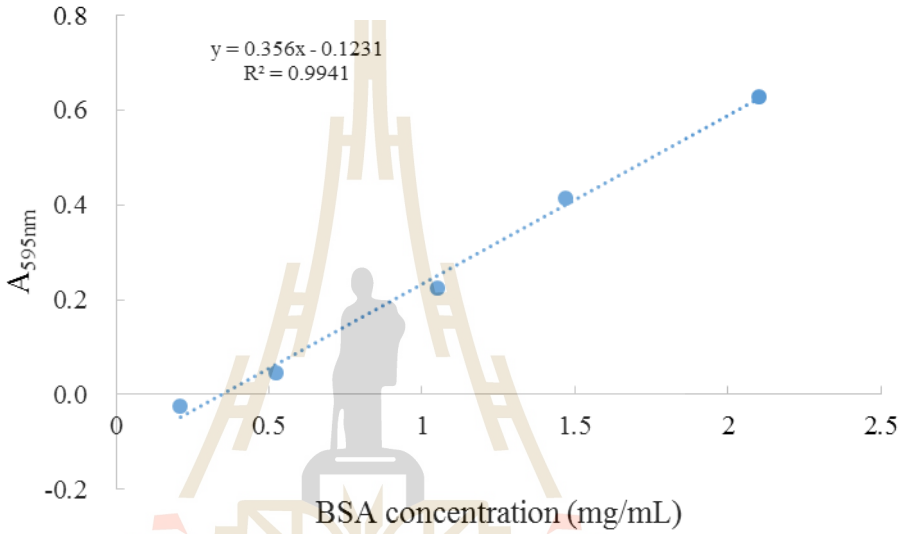
Zivanovic, S., Busher, R. W. and Kim, K. S. (2000). Textural Changes in Mushrooms (*Agaricus bisporus*) Associated with Tissue Ultrastructure and Composition. **Journal of Food Science**. 65:1404-1408.

APPENDICES



APPENDIX A

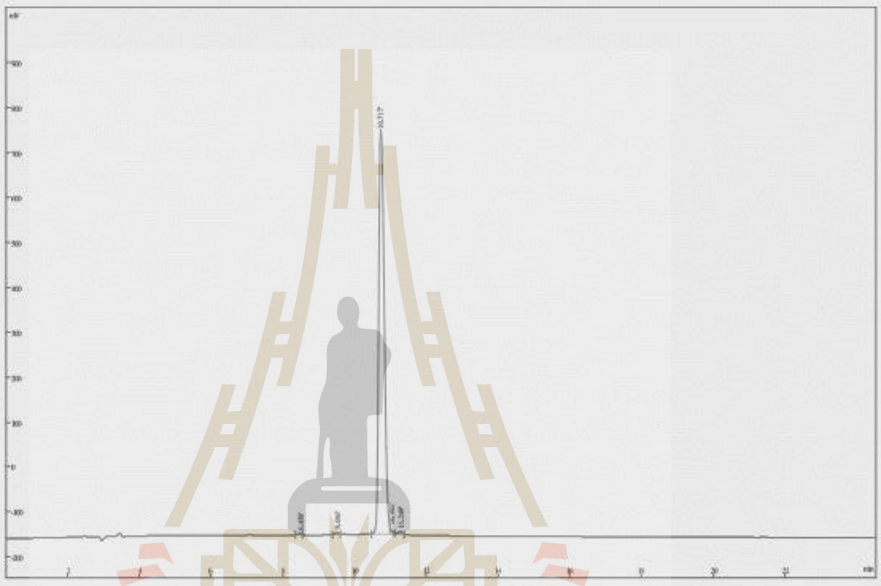
BRADFORD STANDARD CURVE



APPENDIX B

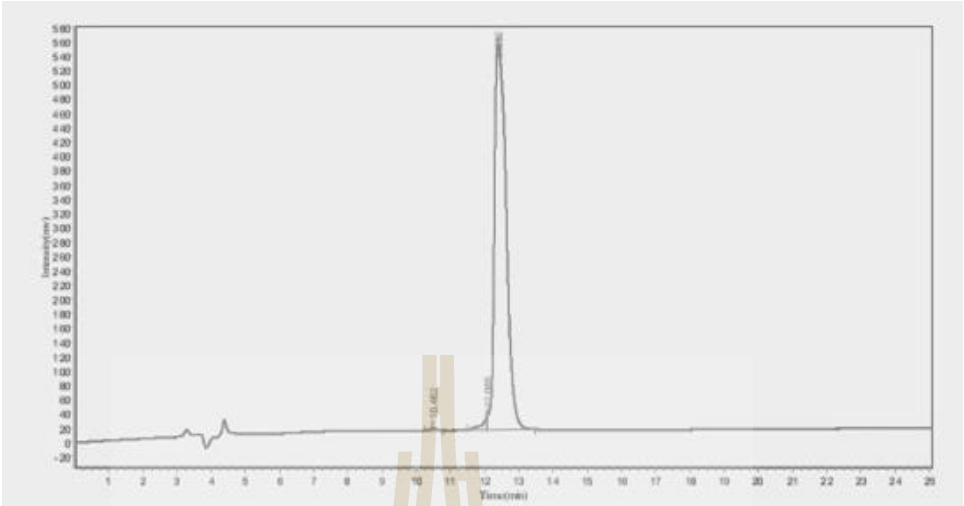
HPLC REPORT OF SYNTHESIZED PEPTIDES

Peptide: MLCSTTF



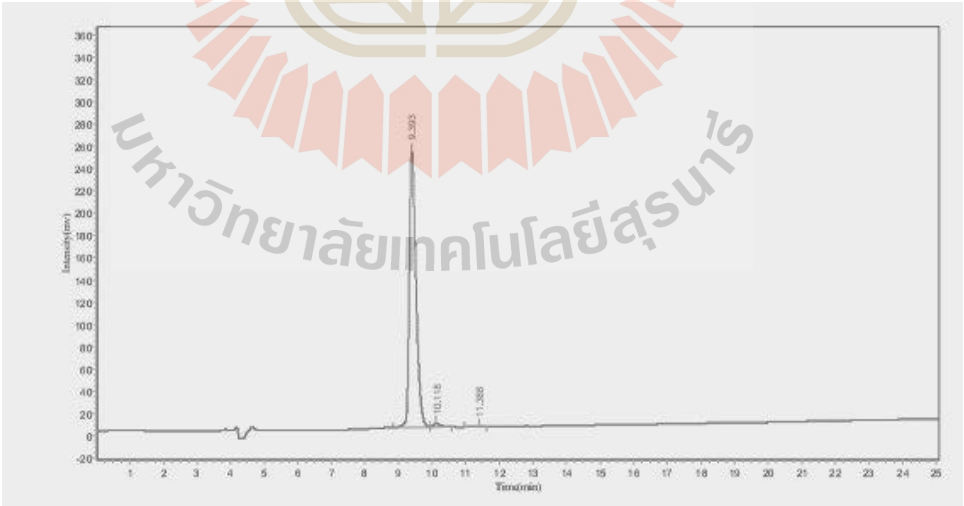
Rank	Time	Conc.	Area	Height
1	8.458	0.1823	17843	2763
2	9.486	0.2736	26787	4111
3	10.717	98.5598	9648354	901791
4	10.988	0.4353	42615	11294
5	11.249	0.5490	53739	6012
Total		100	9789338	925971

Peptide: ASPYAFGL



Peak No.	Ret Time	Height	Area	Conc..
1	10.462	3161.421	45046.406	0.3434
2	12.065	18615.221	187278.750	1.4275
3	12.392	537938.688	12886997.000	98.2291
Total				100.0000

Peptide: LASLFGNDP

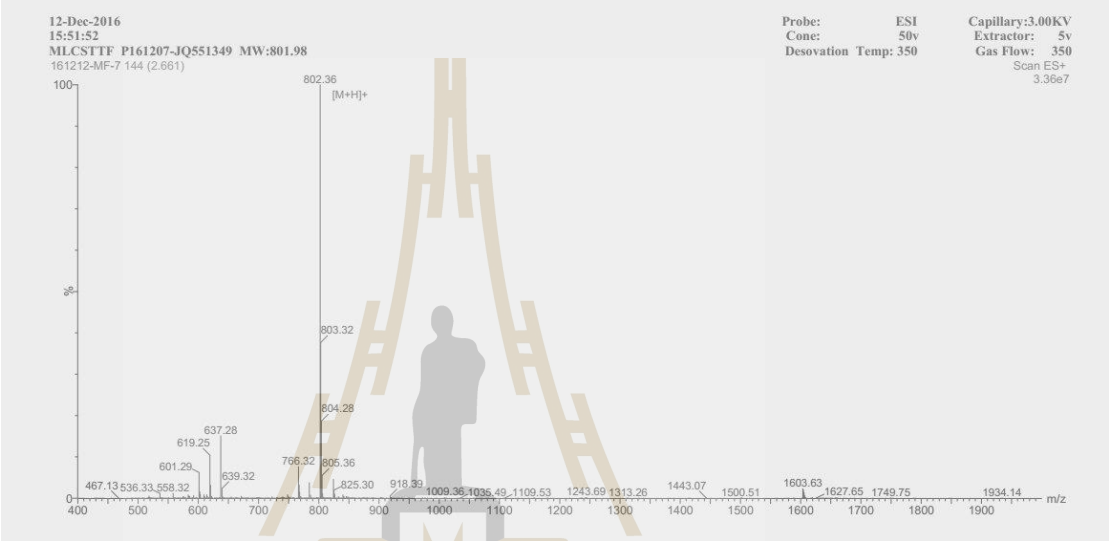


Peak No.	Ret Time	Height	Area	Conc..
1	9.393	247133.438	3407334.500	98.2104
2	10.118	3324.688	48037.695	1.3846
3	11.388	680.458	14050.402	0.4050
Total				100.0000

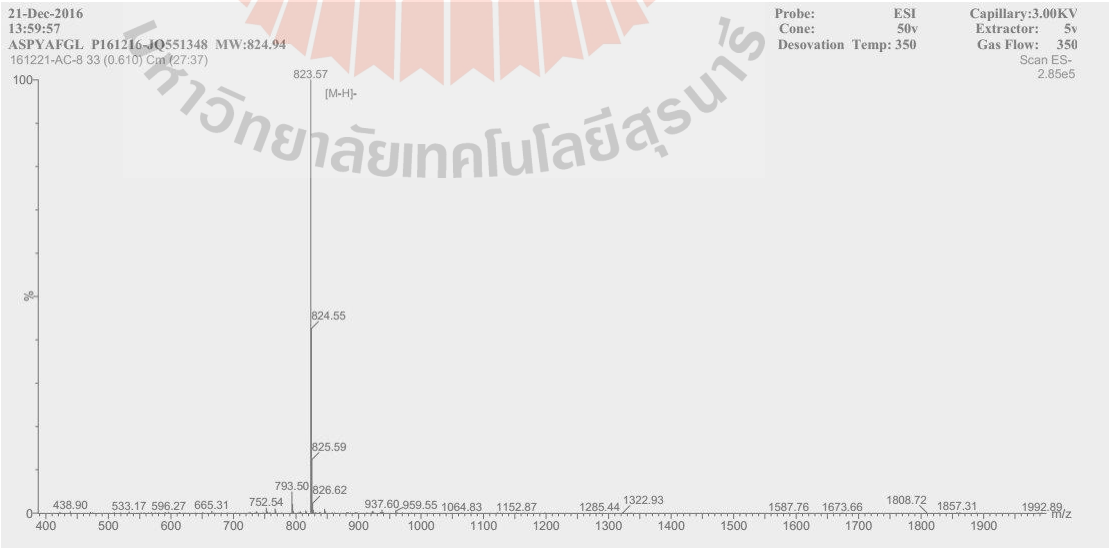
APPENDIX C

MS SPECTRUM OF SYNTHESIZED PEPTIDES

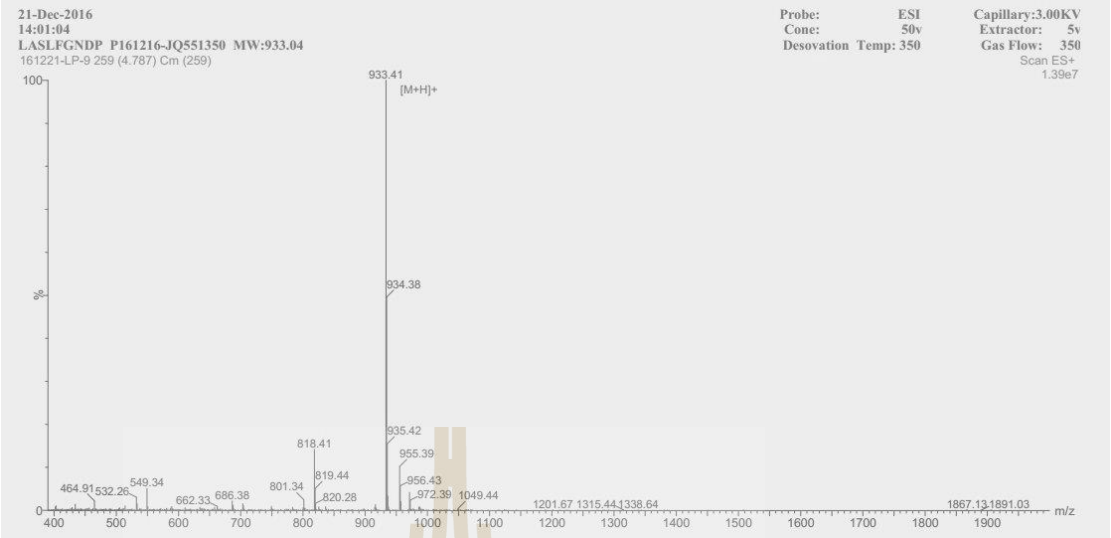
Peptide: MLCSTTF



Peptide: ASPYAFGL



Peptide: LASLFGNDP



APPENDIX D

PREDICTED ANTIHYPERTENSIVE ACTIVITY OF PEPTIDES WITH SIMILAR SEQUENCES

Peptide Sequence	SVM score	Prediction
AS**P**YAFGL	0.81	AHT
ASPYAFG	0.39	AHT
ASPYAF	0.27	AHT
ASPYA	0.16	AHT
AS**A**YAFGL	-0.72	Non-AHT
AS**C**YAFGL	-0.54	Non-AHT
AS**D**YAFGL	0.17	AHT
AS**E**YAFGL	-0.7	Non-AHT
AS**F**YAFGL	-0.47	Non-AHT
AS**G**YAFGL	0.54	AHT
AS**H**YAFGL	-0.59	Non-AHT
AS**I**YAFGL	-0.01	Non-AHT
AS**K**YAFGL	0.18	AHT
AS**L**YAFGL	-0.23	Non-AHT
AS**M**YAFGL	-0.58	Non-AHT
AS**N**YAFGL	-0.34	Non-AHT
AS**Q**YAFGL	-0.73	Non-AHT
AS**R**YAFGL	-0.05	Non-AHT
AS**S**YAFGL	-0.15	Non-AHT
AS**T**YAFGL	-0.49	Non-AHT
AS**V**YAFGL	-0.48	Non-AHT
AS**W**YAFGL	0.82	AHT
AS**Y**YAFGL	0.28	AHT

[a] Web server AHTpin: http://crdd.osdd.net/raghava/ahtpin/.

BIOGRAPHY

Pin Zhang was born on August 10th, 1984 in Henan province, P. R. China. In 2006, she attained the degree of Bachelor of Science (Food Science and Technology) from Henan Agricultural University. In 2009, she obtained her Master's degree in Agricultural Product Processing and Storage Engineering from Graduate School of Chinese Academy of Agricultural Sciences. In 2011, she started to study for her Doctoral degree under the supervision of Associate Professor Dr. Manote Sutheerawattananonda in School of Food Technology, Institute of Agriculture Technology, Suranaree University of Technology, Thailand. During her graduate study, she published her research work under the title of "Production and purification of glucosamine and angiotensin-I converting enzyme (ACE) inhibitory peptides from mushroom hydrolysates" in Journal of Functional Foods (Vol. 36, Pages, 72-83) in September 2017. Another manuscript entitled "Kinetic model for glucosamine production by hydrolysis of chitin from five mushrooms" is in preparation.