บทบาทของ *VIRGIBACILLUS* SP. SK37 และโปรติเนสต่อสารให้กลิ่นสำคัญในน้ำปลาภายใต้การลดปริมาณเกลือและเพปไทด์ต้านออกซิเดชัน

นางสาวนวพร ลาภส่งผล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2555

ROLE OF *VIRGIBACILLUS* SP. SK37 AND ITS PROTEINASE ON ODOR-ACTIVE COMPOUNDS OF FISH SAUCE UNDER REDUCED SALT CONTENT AND ANTIOXIDANT PEPTIDES

Nawaporn Lapsongphon

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 2012

ROLE OF *VIRGIBACILLUS* SP. SK37 AND ITS PROTEINASE ON ODOR-ACTIVE COMPOUNDS OF FISH SAUCE UNDER REDUCED SALT CONTENT AND ANTIOXIDANT PEPTIDES

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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นวพร ลาภส่งผล : บทบาทของ *VIRGIBACILLUS* SP. SK37 และโปรติเนสต่อสารให้กลิ่นสำคัญในน้ำปลาภายใต้การลดปริมาณเกลือ และเพปไทด์ต้านออกซิเดชัน (ROLE OF *VIRGIBACILLUS* SP. SK37 AND ITS PROTEINASE ON ODOR-ACTIVE COMPOUNDS OF FISH SAUCE UNDER REDUCED SALT CONTENT AND ANTIOXIDANT PEPTIDES) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.จิรวัฒน์ ยงสวัสดิกุล, 233 หน้า.

วัตถุประสงค์ของการศึกษานี้เพื่อใช้ประโยชน์ *Virgibacillus* sp. SK37 เป็นกล้าเชื้อหมักน้ำปลาภายใต้การเติมเกลือที่ลดลงจากการหมักน้ำปลาปกติ ศึกษาการย่อยของโปรตีนและสารให้กลิ่นสำคัญ รวมถึงศึกษาการผลิตโปรติเนสและประสิทธิภาพของโปรติเนสจาก *Virgibacillus* sp. SK37 ในการผลิตเพปไทด์ต้านออกซิเดชัน

การวิเคราะห์ชนิดและปริมาณของสารให้กลิ่นสำคัญในน้ำปลาไทยด้วยวิธี aroma extract dilution analysis (AEDA) และ static headspace dilution analysis (SHDA) ด้วย gas chromatography-olfactometry (GC-O) และ gas chromatography-mass spectrometry (GC-MS) วิเคราะห์ปริมาณของสารให้กลิ่นสำคัญด้วย stable isotope dilution assays (SIDA) และคำนวณค่า odor activity value (OAV) พบว่า สารให้กลิ่นสำคัญที่แสดงค่า OAV สูงสุด (มากกว่า 500) คือ มีเทนไธออล 2-เมธิลโพรพาแนล 3-เมธิลบิวทาแนล ไดเมธิลไตรซัลไฟด์ 3-เมธิลไทโอโพรพาแนล และกรดบิวทาโนอิค เมื่อทดลองโดยดึงสารเป็นกลุ่มออกจากโมเดลใช้ค่า R-index พบว่าสารระเหยในกลุ่มกรด แอลดีไฮด์ และซัลเฟอร์ มีผลต่อกลิ่นโดยรวมของน้ำปลา

จากการทดลองหมักน้ำปลาด้วยปลากะตักผสมด้วยเกลือที่ความเข้มข้น 10, 15, และ 20 เปอร์เซ็นต์ และเติมกล้าเชื้อ *Virgibacillus* sp. SK37 ประมาณ 5 log CFU/มิลลิลิตร พบว่าตัวอย่างปลาที่หมักด้วยเกลือ 10 เปอร์เซ็นต์ เกิดการเน่าเสียเมื่อหมักได้ 7 วัน ส่วนตัวอย่างที่หมักด้วยเกลือ 15 และ 20 เปอร์เซ็นต์ ยังคงพบการเจริญของกล้าเชื้อ *Virgibacillus* sp. SK37 ตลอดระยะเวลาการหมัก 3 เดือน อย่างไรก็ตามพบว่า *Virgibacillus* sp. SK37 ไม่สามารถช่วยย่อยโปรตีน ที่อัตราส่วนเกลือ 15 และ 20 เปอร์เซ็นต์ แต่การเติมกล้าเชื้อนี้ร่วมกับการลดปริมาณเกลือลงเหลือ 15-20 เปอร์เซ็นต์ มีส่วนช่วยในการเพิ่มกลิ่นมอลต์ (malty) และ/หรือ ช็อกโกแลตดำ (dark chocolate) ซึ่งพิจารณาจากค่า OAVs ของ 2-เมธิลโพรพาแนล 2-เมธิลบิวทาแนล และ 3-เมธิลบิวทาแนล ที่เพิ่มขึ้น

เมื่อศึกษาการใช้ของเสียจากอุตสาหกรรมอาหาร 5 ชนิด คือ กากถั่วเหลือง รำข้าว โปรตีนถั่วเขียว กากปลา และ กากยีสต์ พบว่ากากยีสต์ที่ความเข้มข้น 1 เปอร์เซ็นต์โดยน้ำหนักต่อปริมาตร สามารถทดแทนการใช้สารสกัดจากยีสต์ (yeast extract) สภาวะที่เหมาะสมในการผลิตโปรติเนส

คือโซเดียมคลอไรด์เข้มเข้น 2.5 เปอร์เซ็นต์ ค่าความเป็นกรดด่าง (pH) 7.5 บ่มที่อุณหภูมิ 40 องศาเซลเซียสเป็นเวลา 4 วัน ซึ่งมากกว่าการใช้สารสกัดจากยีสต์ทางการค้า 1.7 เท่า ผลการทดลองดังกล่าวแสดงถึงศักยภาพของกากยีสต์ในการผลิตโปรติเนสในระดับอุตสาหรรมโดยใช้อาหารเลี้ยงเชื้อในราคาไม่แพง โปรติเนสจากแบคมีเรียสายพันธุ์นี้มีศักยภาพเทียบกับนิวเทรสในการผลิตเพปไทด์ต้านออกซิเดชันจากโปรตีนถั่วเขียว การทำบริสุทธิ์ของเพปไทด์ถั่วเขียวที่ผ่านการย่อยด้วยโปรติเนสจาก *Virgibacillus* sp. SK37 ด้วยหลักการการแลกเปลี่ยนไอออน และการแยกตามขนาด การวิเคราะห์ลำดับของกรดอะมิโนด้วยเครื่อง LC-MS/MS พบว่า ตัวอย่างเพปไทด์ที่มีค่าจำเพาะในการต้านออกซิเดชันสูงประกอบไปด้วยเพปไทด์ 4 สาย ซึ่งแต่ละสายมีกรดอะมิโนอาร์จินีนที่ปลายซี (C-termini) นอกจากนี้เพปไทด์เหล่านี้มีความเสถียรต่อการต้านออกซิเดชันที่ pH 4-10 และอุณหภูมิ 25-121 องศาเซลเซียส

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2555 ลายมือชื่ออาจารย์ที่ปรึกษา_____

NAWAPORN LAPSONGPHON : ROLE OF *VIRGIBACILLUS* SP. SK37 AND ITS PROTEINASE ON ODOR-ACTIVE COMPOUNDS OF FISH SAUCE UNDER REDUCED SALT CONTENT AND ANTIOXIDANT PEPTIDES. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 233 PP.

VIRGIBACILLUS/PROTEINASE/ODOR-ACTIVE COMPOUND/FISH SAUCE/SPENT BREWERY YEAST/ANTIOXIDANT PEPTIDE

Objectives of this study were to utilize *Virgibacillus* sp. SK37 as a starter culture for fish sauce fermentation under reduced salt addition. Protein hydrolysis and odor-active compounds under starter culture inoculation were thoroughly investigated. In addition, production of *Virgibacillus* sp. SK37 proteinase and its efficacy as a processing aid of antioxidant peptides was elucidated.

Qualitative and quantitative analyses of odor-active compounds in Thai fish sauce samples were performed by aroma extract dilution analysis (AEDA), static headspace dilution analysis (SHDA), gas chromatography-olfactometry (GC-O), and gas chromatography-mass spectrometry (GC-MS). Odor-active compounds were quantified by stable isotope dilution assays (SIDA), and their odor activity values (OAVs) were calculated. Methanethiol, 2-methylpropanal, 3-methylbutanal, dimethyl trisulfide, 3-(methylthio)propanal, and butanoic acid showed the highest OAVs (>500). An Omission experiment using ranking R-index test revealed the importance of acid, aldehyde, and sulfur compounds to the overall odor of the complete model.

Virgibacillus sp. SK37 was inoculated with an approximate viable count of 5

log CFU/mL in anchovies with varied amounts of solar salt of 10, 15, and 20% of the total weight. Samples prepared using 10% salt underwent spoilage after 7 days of fermentation. The viable count of *Virgibacillus* sp. SK37 was found over 3 month period in the samples containing 15 and 20% salt. However, acceleration of protein hydrolysis was not pronounced in inoculated samples at both 15 and 20% salt. *Virgibacillus* sp. SK37, together with salt contents reduced to 15-20%, likely contributed to a stronger malty and/or dark chocolate note based on the higher OAVs of 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal.

Among the five food industrial wastes investigated, namely soybean pomace, rice bran, mungbean protein, fish sauce sludge, and spent brewery yeast sludge (Ys) for yeast extract replacement, *Virgibacillus* sp. SK37 proteinase was successfully produced from 1% (w/v) Ys. The highest levels of proteinase production obtained from the Ys medium containing 2.5% NaCl, pH 7.5, and incubated at 40°C for 4 days was about 1.7 times higher than the medium containing the commercial yeast extract, showing the potential of industrial scale of proteinase production using the inexpensive medium. Efficacy of this proteinase was comparable to Neutrase to produce mungbean meal hydolysate with antioxidant activities. *Virgibacillus* sp. SK37 proteinase-hydrolyzed mungbean peptides were purified using ultrafiltration, ion exchange, and gel filtration chromatography. The active fractions were characterized using LC-MS/MS, exhibiting the highest specific antioxidant activity which consisted of four peptides containing an arginine residue at their C-termini. These peptides were stable over a wide pH (4-10) and temperature (25-121°C) range.

School of Food Technology Student's Signature_____

Academic Year 2012 Advisor's Signature_____

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Nawaporn Lapsongphon

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LIST OF ABBREVIATIONS

ABTS	=	2,2′-Azinobis (3-ethyl-benzothiazoline-6-sulphonate)
$ABTS^{\bullet+}$	=	ABTS radical cation
AEDA	=	Aroma extract dilution analysis
AF	=	Acidic volatiles fraction
BHA	=	Butylated hydroxyanisole
BHT	=	Butylated hydroxytoluene
BSA	=	Bovine serum albumin
FRAP	=	Ferric-reducing antioxidant power
$^{\circ}C$	=	Degree celsius
CFU	=	Colony forming unit
C-terminal	=	Carboxy terminal
DEAE	=	Diethylaminoethyl
DH	=	Degree of hydrolysis
DHS	=	Dynamic headspace sampling
DSE	=	Direct solvent extraction
ESI	=	Electrospray ionization
FD	=	Flavor dilution
GC-O	=	Gas chromatography-olfactometry
h	=	Hour
M	=	Molar

LIST OF ABBREVIATIONS (Continued)

kDa	=	Kilodalton
MALDI	=	Matrix assisted laser desorption/ionization
MS	=	Mass spectrometry
MS/MS	=	Tandem mass spectrometry
MWCO	=	Molecular weight cut-off
m/z	=	Mass per charge ratio
Mw	=	Molecular weight
mg	=	Milligram
min	=	Minute
µg	=	Microgram
mL	=	Milliliter
mM	=	Millimolar
µL	=	Microliter
µm	=	Micrometer
nm	=	Nanometer
N-terminal	=	Amino terminal
NBF	=	Neutral/basic volatiles fraction
pI	=	Isoelectric point
PMF	=	Peptide mass fingerprint
%	=	Percent
SAFE	=	Solvent assisted flavor evaporation
SDE	=	Steam distillation extraction

LIST OF ABBREVIATIONS (Continued)

SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHDA	=	Static headspace dilution analysis
SHS	=	Static headspace sampling
SIDA	=	Stable isotope dilution assay
SPME	=	Solid phase micro extraction
TN	=	Total nitrogen content
U	=	Unit activity
w/v	=	Weight by volume
OAV	=	Odor activity value

CHAPTER I

INTRODUCTION

1.1 Introduction

Thai fish sauce or "Nam pla", a clear amber liquid having a unique aroma and taste, is commonly used as a condiment in Southeast Asia. Thai fish sauce is an indispensable seasoning in Thai cuisine and found in almost Thai dishes. Thus, this sauce imparts a unique character of Thai food. It was reported that fish sauce consumption in Thai population was estimated 17-20 mL/person/day (Gildberg and Thongthai, 2005). Thai fish sauce is exported globally and has been increasing annually. In 2011, the value of the exported Thai fish sauce was accounted to about 1.2 billion Baht which accounting for an increase of 5.34% from the previous year (Thailand Trading Report, MOC, http://www.ops3.moc.go.th/). Traditional Thai fish sauce production is a natural fermentation of anchovy (*Stolephorus* sp.) at high salt content of 28-30% (Yongsawatdigul, Rodtong, and Raksakulthai, 2007). The fermentation process mainly relies on fish protein hydrolysis by the action of fish endogenous and microbial proteinases. Due to high salt concentration, rendering a decrease in protein hydrolysis and leading to extremely long fermentation time of 12-18 months for complete flavor and aroma development. Thus, this extremely long fermentation time limits the growth of fish sauce industries.

There have been many attempts on the acceleration of fermentation time and/or flavor improvement in fish sauce fermentation, including reducing salt concentration

and/or the addition of enzymes (Beddows, Ismail, and Steinkrus, 1976; Fu, You, and Kim, 2008; Gildberg, Hermes, and Orejana, 1984), elevating fermentation temperature (Lopetcharat and Park, 2002), and addition of starter cultures (Akolkar, Duri, and Desai, 2010; Fukami, Funatsu, Kawasaki, and Watabe, 2004; Gildberg and Thongthai, 2001; Santo et al., 2005; Udomsil, Rodtong, Choi, Hua, and Yongsawatdigul, 2011; Yongsawatdigul, Rodtong, and Raksakulthai, 2007).

Several halophilic bacteria have been proposed to be a potential starter culture that can shorten fermentation time and improve flavor characteristics of fish sauce. In addition, the level of salt in the fermentation has a great impact on microbial population, rate of protein hydrolysis, and their metabolites during fermentation, resulting in different flavor compounds and fish sauce qualities. The reduced salt content can increase the activity of fish endogenous and microbial proteinases, resulting in an increased rate of protein hydrolysis and fermentation. However, low salt content during fish sauce fermentation may allow the growth of spoilage microorganisms. The use of starter cultures could inhibit spoilage microorganisms and render similar sensory and chemical characteristics to conventional fish sauce. There are reports describing the use of halophilic bacteria as a starter culture in reduced salt content during fish sauce fermentation. For example, *Tetragenococcus halophilus* and *Lactobacillus sakei* 2a were added in fish sauce made from sprat (*Sprattus sprattus*) at 20-23% (w/w) NaCl and in sardines (*Sardinella brasiliensis*) containing 6% NaCl, respectively (Gildberg and Thongthai, 2001; Santo et al., 2005).

The analyses of volatile compounds in fish sauce have been studied with different techniques of sample preparation and methods of analyses. Examples of different sample preparation for flavor analysis in fish sauce including, solvent extraction

(DSE) (McIver, Brooks, and Reineccius, 1982), steam distillation under reduced pressure (Sanceda, Kurata, Arakawa, 1986; Sanceda, Suzuki, and Kurata, 2003), simultaneous distillation and extraction (Peralta, Shimoda, and Osajima, 1996). These techniques usually contain a wide spectrum of volatile compounds. On the other hand, headspace analysis with Tenax trap (Fukami et al., 2002; Giri, Osaka, Okamoto, and Ohshima, 2010; Shimoda, Peralta, and Osajima, 1996; Wichaphon, Thongthai, Assavanig, and Lertsiri, 2012) and solid phase microextraction (SPME) (Pham, Schilling, Yoon, Kamadia, and Marshall, 2008) could be a good option for recovering highly volatile compounds lost during concentration step of DSE (Wampler, 1997). Thus, one single preparation technique is not able to accommodate compounds with a wide range of volatility. In addition, little work has been focused on odor active compounds that actually contribute to the aroma of Thai fish sauce.The key aroma compounds in Thai fish sauce have not yet been fully clarified.

Nawong (2006) isolated and screened proteinase-producing microorganisms from Thai fish sauce samples. *Virgibacillus* sp. SK37 showed the highest extracellular proteinase production among 165 strains studied. *Virgibacillus* sp. SK37 (GenBank accession number DQ910840) is gram positive, endospore-forming, rod-shaped, aerobic bacterium, and grows at a wide pH range of 4-11 (optimum 6.5-7.5), 20-45°C (optimum 30-40°C), and 0-25% NaCl (optimum 5-10%) (Nawong, 2006). The strain has been shown to produce Na^+-activated and Na^+-stable extracellular proteinases as well as possess cell-associated proteinases (Sinsuwan, Rodtong, and Yongsawatdigul (2007, 2012). Yongsawatdigul et al. (2007) reported that the use of *Virgibacillus* sp. SK37 as a starter culture in fish sauce made from Indian anchovy (*Stolephorus indicus*) at 25% solar salt fermented for 4 months resulted in the odor

quality perceived by the panelists comparable to the 12-mo-old commercial product ($P > 0.05$). However, high salt at fermentation (25-28%) would suppress growth and production of *Virgibacillus* sp. proteinases since its proteinase production has been reported to be optimal at 5% NaCl (Sinsuwan, Rodtong, and Yongsawatdigul, 2008). However, the effect of *Virgibacillus* sp. starter culture at varied salt contents on odor active compounds and fish sauce quality has not been systematically elucidated. Understanding such a relationship would lead to starter culture technological development of fish sauce fermentation.

Proteinases are one of the most important industrial enzymes accounting for nearly 60% of the total enzyme production, and are widely used in several industrial sectors, including peptide synthesis, detergent, food, pharmaceutical, chemical, and leather industry. Among the various proteinases, microbial proteinases play an important role in biotechnological processes accounting for approximately 59% of the total proteinase production because they can be genetically manipulated to generate new enzymes with altered properties that are desirable for various applications (Chu, 2007). The overall cost of enzyme production is another factor that should be taken into consideration. Agricultural byproducts have been used to replace yeast extract in order to reduce cost of fermentation medium. For example, Altaf, Naveena, Venkateshwar, Kumar and Reddy (2006) reported that wheat bran was successfully used to replace yeast extract for lactic acid production in solid state fermentation of *Lactobacillus amylophilus* GV6. However, proteinase production of moderately halophilic bacteria has rarely been reported. The utilization of byproduct as a substrate for the proteinase production should be sought. It will ultimately lead to the maximum utilization of byproduct for valuable enzyme production.

Different commercial proteinases have been used for the production of protein hydrolysates, such as pepsin, trypsin, chymotrypsin, papain, bromelain, and subtilisin. Besides commercial proteinases, other microbial proteinases used for the production of bioactive peptides have rarely been reported. Recently, proteinases from a different strain, *Virgibacillus* sp. SK33, hydrolyzed threadfin bream surimi wastes, yielding high antioxidant activity toward HepG2 cells (Wiriyaphan, Chitsomboon, and Yongsawatdigul, 2012). Three major extracellular subtilisin-like proteinases from *Virgibacillus* sp. SK37 with molecular weight of 19, 34, and 44 kDa showed optimum activity at pH 8 and 55-60°C and stability at a wide range of NaCl up to 30% NaCl (Phrommao, Rodtong, and Yongsawatdigul, 2010). These proteinases could hydrolyze protein at high NaCl concentration but properties of the resulting hydrolysates have not been investigated.

In Thailand, the quantity of mungbean supplied to glass noodle industry is estimated to be 200,000 tons/year and 27% of raw materials which is rich in protein are disposed as mungbean meal (Jantawat, Chinprahast, and Siripatrawan, 1998). After starch extraction for glass noodle production, the meal, consisting of 72-80% protein, is typically used as animal feed or discarded (Jantawat et al., 1998; Sonklin, Laohakunjit, and Kerdchoechuen, 2011). Since Chen, Muramoto, and Yamauchi (1995) isolated six antioxidant peptides from digested soybean β-conglycinin using proteinase from *Bacillus* sp., mungbean proteins could be a good source for antioxidant peptides. The efficient utilization of this byproduct as food should be sought. In order to maximum utilization of *Virgibacillus* sp. SK37 proteinases, its efficacy as a processing-aid of antioxidant mungbean peptides should be elucidated.

1.2 Research objectives

The objectives of this study were:

1. To identify, quantify, and characterize the key odor active compounds of two commercial Thai fish sauce samples.

2. To investigate the effect of *Virgibacillus* sp. SK37 together with the reduced salt content on fish sauce quality, particularly odor active compounds.

3. To investigate the suitable food industrial byproducts and conditions affecting proteinase production from *Virgibacillus* sp. SK37.

4. To evaluate, purify, and characterize active antioxidant mungbean peptides derived from *Virgibacillus* sp. SK37 proteinases.

1.3 Research hypotheses

The odor active compounds responsible for the characteristic of Thai fish sauce aroma can be accurately identified. The use of *Virgibacillus* sp. SK37 as a starter culture at reduced salt content during fermentation would improve flavor and chemical characteristics of fish sauce. In addition, food industrial byproducts can be used to replace yeast extract for proteinase production from *Virgibacillus* sp. SK37. The application of these proteinases could result in peptides with antioxidant activity as compared to commercial proteinases.

1.4 Scope of the study

Odor active compounds of two commercial Thai fish sauces were screened by aroma extract dilution analysis (AEDA) and static headspace dilution analysis (SHDA) using gas chromatography-olfactometry (GC-O) and quantified using stable

isotope dilution assays (SIDA). Protein hydrolysis and odor active compounds of fish sauce inoculated *Virgibacillus* sp. SK37 under reduced salt content was characterized. In addition, food industrial byproducts and conditions affecting proteinase production from *Virgibacillus* sp. SK37 were determined. Antioxidant peptides derived from *Virgibacillus* sp. SK37 proteinases were purified and characterized.

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CHAPTER II

LITERATURE REVIEWS

2.1 Fish sauce

Fish sauce is a clear brown liquid used as a condiment in Southeast Asia and worldwide. The sauce is known locally as Nuoc mam in Vietnam, Bakasang in Indonesia, Patis in Philippines, Budu in Malaysia, Yeesui in China, Shotturu in Japan, Tuk trey in Cambodia, Ngan-pya-ye in Burma, and Nam pla in Thailand (Lopetcharat, Choi, Park, and Daeschel, 200; Yoshida, 1998). Generally, fish sauces are prepared by mixing 1 to 3 parts of fresh anchovy (*Stolephorus* spp.), mackerel (*Ristrelliger* spp.), herring (*Clupea* spp.), or other small fish, depending on recipe and country of producers, with 1 part of solar salt (Wilaipan, 1990). Then, salt-mixed fish is transferred to a fermentation tank and requires about 12-18 months at ambient temperature to complete fermentation. The supernatant is firstly transferred from the fermentation tank to the ripening tank. After 2-12 weeks of ripening, first grade fish sauce is obtained whereas the residue is continually extracted up to 3-4 times for lower quality fish sauce (Lopetcharat et al., 2001; Sanceda, Suzuki, and Kurata, 2003). The conventional standard of fish sauce grading is based on total nitrogen. In Thailand, fish sauce with total nitrogen above 20 g/L (2.0%) is classified as grade 1 and as grade 2 if it contains total nitrogen between 15 and 20 g/L (1.5–2.0%) (Lopetcharat, Choi, Park, and Daeschel, 2001). If the total nitrogen content is below 15 g/L, it must be labelled as diluted or mixed product (Brillantes, 1999). Premium

grade are also claimed by some manufacturers, which is the product containing nitrogen content above 20 g/L and has been fermented for more than 18 months. However, the quality of fish sauce is also involved by color as well as taste and volatile compounds. Recently, Wichaphon, Thongthai, Assavanig, and Lertsiri (2012) attempted to find a correlation between total nitrogen contents (TN) and 7 volatile compounds, namely 2-butanol, *n*-propanol, acetic acid, propanoic acid, 2-methylpropanoic acid, butanoic acid, and 3-methylbutanoic acid. These authors found that samples with high amount of TN exhibited lesser all those volatile acids (cheesy odor). Thai Public Health Ministry classified fish sauce in Thailand into three types based on the production process including: (i) pure fish sauce obtained from fresh fish or fish residue fermented with salt or brine; (ii) hydrolyzed fish sauce derived from the fish hydrolysates or other kinds of animals, which are often treated with hydrochloric acid (HCl) or other hydrolyzing processes that are approved by the Thai Public Health Ministry; (iii) diluted fish sauce obtained from pure fish sauce or hydrolyzed fish sauce using approved additives or flavoring agents.

2.1.1 Microbiology of fish sauce fermentation

A wide variety of microorganisms have been found in raw ingredients for fish sauce production and various stages of fermentation. Bacteria involved in fish sauce can be classified into two major groups, such as bacteria that produce proteolytic enzymes contributed to hydrolysis of fish protein during fish sauce fermentation and flavor and aroma development (Lopetcharat et al., 2001). Since fish sauce contains high salt concentration of 25-30%, thus microorganisms found during fish sauce production are generally classified as halophiles (Thongthai, McGenity, Suntinanalert, and Grant, 1992). Halophilic bacteria found in fish sauce can be

categorized as halotolelant, slightly halophilic, moderately halophilic, and extremely halophilic bacteria. In the first two months of fermentation, halotolerant *Bacillus* species were found to be predominant and then a sharp increase of *Stapylococcus*, coryeform bacteria, and *Micrococcus* sp. became common in the third months (Chaiyanan, 2000). Moderately halophilic bacteria found during fish sauce fermentation include *Halobacillus thailandensis* (Chaiyanan, Maugel, Hug, Robb, and Colwall, 1999), *Tetragenococcus halophilus* and *T. muriaticus* (Thongsanit, Tanasupawat, Keeratipibul, and Jatikavanich, 2002), *B. subtilis* CN2 (Uchida et al., 2004), *B. vietnamensis*, (Noguchi et al., 2004), *B. subtilis* JM-3 (Kim and Kim, 2005), *Filobacillus* sp. RF2-5 (Hiraga et al., 2005), *Lentibacillus Salicampi* and *L. juripiscarius* (Namwong et al., 2005), *L. halophilus* (Tanasupawat et al., 2006), *Halobacillus* sp. SR5-3 (Namwong et al., 2006). For extremely halophilic archaea, *Halobacterium salinarum*, *Halococcus thailandensis* (Thongthai et al., 1992; Namwong, Tanasupawat, Visessanguan, Kudo, and Itoh, 2007), *Natrinema gari* sp. nov. (Tapinkae et al., 2008), *Chromohalobacter salexigens* JCM 8878, and *Halobacterium saccharolyticus* (Tanasupawat, Namwong, Kudo, and Itoh, 2009) have been isolated from fish sauce samples.

2.1.2 Microbial halophilic adaptation

Kushner (1978) firstly classified halophiles based on the level of salt requirement for growth. Among many definitions given by different authors, the most accepted categories of halophilic microorganisms according to their response to salt can be divided into slightly halophilic bacteria with optimal growth at 1-3% salt, moderately halophilic bacteria with optimal growth at 3-15% salt, and extremely halophilic bacteria with optimal growth above 15% salts. Bacteria grow optimally below 1% salt

as well as in the presence of relatively high salt concentrations (above 15% salt) are designated as halotolerant (Ventosa and Arahal, 2002; Ventosa, Nieto, and Oren, 1998).

Basically, there are two strategies used by halophilic microorganisms to maintain proper osmotic equilibrium across the membrane in their cytoplasm, such as "salt-in-cytoplasm strategy" and "organic-osmolyte strategy". In the "salt-in-cytoplasm strategy", cells accumulate high intracellular salt (potassium and sodium) concentration at equal molar concentrations to the medium. The second strategy is based on the biosynthesis and/or accumulation of organic osmotic solutes, called "organic-osmolyte strategy" (Kunte, Trüper, and Stan-Lotter, 2002; Oren, 2008). Most organic compatible solutes are uncharged or zwitterionic including amino acids or amino acid derivatives such as glutamine, glutamate, proline, ecotine, choline, betaine, and glycine betaine, as well as sugar and sugar alcohols (Galinski, 1995; Robert, 2005).

2.1.3 Application of starter culture in fish sauce fermentation

In traditional fish sauce fermentation, the rate of production depends only on the activity of enzymes in the fish and microflora. Thus, fermentation of fish sauce is considered to be a traditional process with long fermentation time. Many attempts have been made to accelerate the process by reducing salt concentration and/or the addition of enzymes (Beddows, Ismail, and Steinkrus, 1976; Fu, You, and Kim, 2008; Gildberg, Hermes, and Orejana, 1984), and elevating the temperature (Lopetcharat and Park, 2002). Salt reduction may allow the growth of spoilage microorganisms and affect the quality of finished products. The addition of halophilic bacteria as a starter culture has been used to inhibit spoilage microorganisms and/or accelerate

fermentation and/or flavor improvement for fish sauce fermentation. For example, the addition of a halophilic lactic acid bacteria (*Tetragenococcus halophilus*) in fish sauce made from sprat (*Sprattus sprattus*) resulted in an acceptable quality, but samples were rated as significantly inferior to the first grade commercial Thai fish sauce (Gildberg and Thongthai, 2001). Fish sauce samples treated with *Staphylococcus* strain R4Nu could reduce undesirable note of 2-ethylpyridine in fish sauce (Fukami, Funatsu, Kawasaki, and Watabe, 2004). The addition of *Lactobacillus sakei* 2a significantly inhibited the growth of spoilage microorganisms in the fermentation of sardines (*Sardinella brasiliensis*) (Santo et al., 2005). Yongsawatdigul, Rodtong, and Raksakulthai (2007) successfully applied three proteinase-producing strains of moderately halophilic bacteria, including *Virgibacillus* sp. SK33, *Virgibacillus* sp. SK37, and *Staphylococcus* sp. SK1-1-5 as starter cultures in fish sauce made from Indian anchovy (*Stolephorus indicus*) at 25% solar salt. The sample inoculated with *Virgibacillus* sp. SK37 obtained after 4 months fermentation appeared to have lower amount of distinctive volatile compounds and flavor intensity of fish sauce than the conventionally fermented sample. The use of four stains of *T. halophilus*, namely, *T. halophilus* MS33, *T. halophilus* MRC10-1-3, *T. halophilus* MRC10-5-10, and *T. halophilus* MRC10-7-8, as a starter culture for fish sauce fermentation likely eliminated the undesirable odor resulted from sulfur-containing compounds and to promote desirable odor characteristics of fish sauce (Udomsil, Rodtong, Choi, Hua, and Yongsawatdigul, 2011). In extremely halophilic archaebacterium, *Halobacterium* sp. SP1(1) has been used for acceleration of fish sauce fermentation. This strain provided higher protein and nitrogen contains as well as better flavor and aroma

aspects in the final product compared to the control containing microflora of *Halobacterium* sp. F1 and F2 (Akolkar, Duri, and Desai, 2010).

2.1.4 Flavor compounds in fish sauce

Volatile compounds of fish sauce have been identified in numerous studies and these compounds vary due to the origin, the production process as well as different sampling and analysis techniques. About 124-155 volatile compounds of fish sauce have already been identified including acids, nitrogen-containing compounds, sulfur-containing compounds, aldehydes, ketones, and aromatic hydrocarbons (Cha and Cadwallader, 1995; Peralta, Shimoda, and Osajima, 1996; Shimoda, peralta, and Osajima, 1996; Wichaphon et al., 2012). Dougan and Howard (1975) reported that three distinctive notes contributing to the odor of fish sauce were ammoniacal, cheesy, and meaty notes. The ammoniacal note is produced by ammonia, amines, and other basic nitrogen-containing compounds. The cheesy note is mainly from low molecular weight volatile fatty acids and methyl ketone. The meaty note is much more complicated, but it was believed that it could be produced by oxidation of precursors presenting in mature fish sauces.

All volatile components found in a typical chromatogram are not odor active compounds. Grosch (2000) reported that <5% of the volatile compounds identified in the food contribute to its aroma. Thus, a more comprehensive approach to evaluate individual compounds was performed by combining the dilution experiments in order to screen the potent compounds, quantification of the key aroma compounds exhibiting high flavor dilution (FD) factors followed by calculating their odor activity value (OAVs), and sensory evaluation of their reconstituted solution (Grosch, 2001).

Dilution technique has been used to identify the potent compounds in some fish

sauce samples. Fukami et al. (2002) used aroma extraction dilution analysis (AEDA) to screen high potent compounds.They reported that seven compounds with high FD factor (2-methylpropanal, 2-methylbutanal, 2-pentanone, 2-ethylpyridine, dimethyl trisulfide, 3-(methylthio)propanal, and 3-methylbutanoic acid) were principal contributors to the distinct odor of Thai fish sauce. Furthermore, contribution of four volatile compounds, including 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, and dimethyl trisulfide were investigated by sensory evaluation using eight descriptors for quantitative descriptive analysis (QDA). Each of these four volatiles cooperatively contributed to sweaty and rancid notes. Ethylpyridine and dimethyl trisulfide contributed to fishy and fecal notes. 2-Ethylpyridine, 2-methylpropanal, and 2-methylbutanal contributed to the meaty note. It was also indicated that dimethyl disulfide and butanoic acid contributed to unfavorable odor of fish sauce (Dougan and Howard, 1975; Shimoda, Peralta, and Osajima, 1996).

Pham, Schilling, Yoon, Kamadia, and Marshall (2008) characterized volatile compounds in 4 fish sauces (Korean and Thai fish sauces) using solid phase microextraction, gas chromatography-mass spectrometry, Osme, and gas chromatography olfactometry (SPME-Osme-GCO). Four distinct odors were detected in all samples. These 4 odors were identified as fishy (trimethylamine), pungent and dirty socks (combination of butanoic, pentanoic, hexanoic, and heptanoic acids), cooked rice and buttery popcorn (2,6-dimethyl pyrazine and/or 2-acetyl-1-pyrroline), and sweet/cotton candy (benzaldehyde).

Giri, Osaka, Okamoto, and Ohshima (2010) characterized the relative proportion (%) of the OAVs of the odor active compounds in Thai fish sauce samples. They found that butanoic acid showed the highest OAV value in the premium Thai fish

sauce (18 months ripened product), followed by 2-methylbutanoic acid, dimethyl trisulfide, 3-(methylthio)propanal, and acetic acid. For standard fish sauce (9 months ripened product), dimethyl trisulfide showed the highest OAV value, followed by 3-(methylthio)propanal, trimethylamine, butanoic, and 2-methylbutanoic acid. Sensory analysis also revealed that cheesy note of premium fish sauce showed significantly higher than that of standard fish sauce.

Wichaphon et al. (2012) identified 40 odor active compounds of 8 Thai fish sauce samples using dynamic headspace dilution analysis (DHDA) by combination scores. Eleven compounds, including trimethylamine, 2-butanol, *n*-propanol, dimethyl trisulfide, 1-octen-3-ol, acetic acid, 3-(methylthio)propanal, propanoic acid, 2-methylpropanoic acid, butanoic acid, and 3-methylbutanoic acid, were perceived in all samples and contributed to the aroma characteristic of Thai fish sauce. In addition, seventy-six volatile compounds were detected by DHS-GC-MS from 52 fish sauce samples. Twenty-one of them, including dimethyl sulfide, dimethyl disulfide, 2-propanone, 2-butanone, ethanol, *n*-propanol, 2-propanol, *n*-butanol, 2-butanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 3-pentanol, 1-penten-3-ol, *n*-pentanol, acetic acid, propanoic acid, butanoic acid, 2-methylpropanoic acid, 3-methylbutanoic acid, ethyl acetate, and phenol, were major compounds found on chromatograms.

2.2 Description of *Virgibacillus*

2.2.1 Morphology and physiology

The genus *Virgibacillus* was firstly reclassified from *Bacillus pantothenticus* on the basis of amplified DNA restriction analysis, fatty acid profiles, SDS-PAGE pattern of whole-cell protein and phenotypic characteristics (Heyrman et al., 2003).

This genus could be distinguished from members of *Bacillus* rRNA group 1 and from members of *Paenibacillus* and other aerobic endospore-forming bacteria by routine phenotypic tests. Two species of *Salibacillus* were also reclassified to *Virgibacillus* based on of genotypic and phenotypic characteristics (Heyrman et al., 2003). In addition, Yoon, Oh and Park (2004) also proposed to rename *Bacillus halodenitrificans* to *Virgibacillus halodenitrificans* based on 16S rRNA gene comparisons. Bacteria in the genus *Virgibacillus* are Gram-positive rods (0.3-0.7 x 2.0-0.6 μm), and single, pairs or long chains of cell arrangement. Their colonies are small, circular, low convex, and slightly transparent-to-opaque. They are aerobic, catalase-positive, motile, and spore forming. *Virgibacillus* bacteria grow at pH 6.0-10.0 (optimum at 7.5-8.0), and 10-55°C (optimum at 30-40°C). Members of this genus are moderately halophilic bacteria able to grow at 0-25% (w/v) NaCl with optimum of 5-10% NaCl (Phrommao, 2010). They currently consists of 27 recognized species. Table 2.1 shows some characteristics of some *Virgibacillus* spices isolated from saline environments.

2.2.2 Proteinases from *Virgibacillus*

This genus from various sources has been reported to produce a variety of extracellular hydrolytic enzymes. For example, *Virgibacillus marismortui* NB2-1 isolated from Pla-ra, fermented fish in Thailand, secreted five proteinases with molecular masses (MMs) ranging from 17 to 35 kDa. The enzymes were alkaline serine proteinases and showed optimum catalytic activity at pH 10, 50°C, and 5% NaCl (Chamroensaksri, Akaracharanya, Visessanguan, and Tanasupawat, 2008), alkaline serine proteinase from *Virgibacillus pantotheticus* MTCC 6729 isolated from fresh chicken meat samples. This stain was able to produce proteinase at 40°C at pH

9.0. The enzyme was thermostable alkaline by retaining its 100% and 85% stability at pH 10.0 and at 50°C, respectively (Gupta, Joseph, Mani, and Thomas, 2008), *Virgibacillus dokdonesis* VITP14 isolated from Kumta coast was found to produce halotolerant enzyme, and was a potential strain for producing novel proteinase for various industrial applications. The optimal condition for enzyme production was pH 7.0 and 40°C (Devi Rajeswari, Jayaraman, Rameshpathy, and Sridharan, 2012; Pooja and Jayaraman, 2009).

Sinsuwan, Rodtong, and Yongsawatdigul (2007, 2008a, 2008b, 2010a, 2010b) characterized *Virgibacillus* sp. SK33 and SK37 proteinases isolated from one-month-old Thai fish sauce. For *Virgibacillus* sp. SK33 proteinases, crude extracellular enzyme showed a subtilisin-like alkaline serine proteinase characteristic with optimal catalytic condition at 50°C and pH 8-11. Enzyme with MM of 19 and 32 kDa were purified and showed NaCl and $CaCl_2$-activated characteristics. In addition, the 32-kDa enzyme exhibited a high stability in various organic solvents at concentrations up to 25% (v/v) including dimethylsulfoxide, methanol, acetonitrile, and ethanol, suggesting the potential application in high ionic strength and aqueous-organic solvent systems. *Virgibacillus* sp. SK37 was found to possess both extracellular and cell-bound proteinases which were subtilisin-like serine proteinases with maximal activity at 65°C and pH 7-9.5. All enzymes were halotolerant showing NaCl-activated characteristic and stable up to 25% NaCl. When compared to extracellular proteinases from other moderately halophilic bacteria, the extracellular proteinase from *Virgibacillus* sp. SK37 exhibited activity at a higher NaCl concentration. Extracellular proteinases from *Pseudoalteromonas* sp. CP76 and *Salinivibrio* sp. AF-2004 showed maximum activity at 5.8% and 2.9%, respectively (karbalaei-Heidari et al., 2007;

Table 2.1 Some characteristics of *Virgibacillus* species isolated from saline environments.

Characteristic	Type culture strain[b]												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Habitat	Chigu saltern	salt lake	salt field	salt lake	Thai fermented fish	salt lake	saline lake	subsurface saline soil	marine solar saltern	permafrost core (Canadian high Arctic)	salt lake	sea water	salt lake
Spore shape[a]	S, E	E, S	E	E, S	E	E, S	E	E, S	S, O	E	E	E, S	E, S
Gram stain	+	+	+	+	+	+	+	+	v	+	+	+	+
Colony pigmentation	-	-	-	White	Red	Cream	Cream	-	-	Light pink	-	Cream	NA
Anaerobic growth	+	-	+	-	+	-	-	-	-	+	-	NA	-
Growth requirements													
NaCl range (optimum)(% w/v)	0-30 (5-10)	0-25 (10)	0-20 (5-10)	0.5-25 (7-10)	1-20 (5)	0-20 (5-7)	3-20 (10)	0-25 (9)	0-20 (8)	0-20 (5)	1-20 (5-10)	1-15 (4.7)	1-17 (5-10)
pH range (optimum)	5.0-9.0 (7.5)	6.0-10.0 (7.0)	5.5-9.0 (7.0)	5.5-10.0 (7.5)	7.0-8.0 (7.0)	6.5-9.5 (7.5-8.0)	6.0-10.0 (7.5)	6.0-9.0 (7.5)	7.0-8.0 (7.0)	4.4-9.1 (7.0)	6.0-10.5 (7.5-8.0)	6.0-10.0 (7.5)	4.0-9.0 (7.0)
temperature range (optimum) (°C)	15-50 (40)	10-50 (37)	10-45 (25)	10-50 (30-35)	15-40 (37)	8-52 (32-35)	15-40 (37)	10-50 (30)	4-40	0-30	10-55 (35-40)	10-45 (30)	15-45 (25-30)

Modified from: Kaewphuak (2011).

Note: NA = not available; w= weakly positive, v=variable; [a]E=Ellipsoidal, S=spherical, O=oval. [b]1=*V. chiguensis* (Wang et al., 2008), 2=*V. kekensis* (Chen et al., 2008), 3=*V. koreensis* (Lee et al., 2006), 4=*V. salaries* (Hua et al., 2008), 5=*V. siammensis* (Tanasupawat et al., 2010), 6=*V. xinjiangensis* (Jeon et al., 2009), 7=*V. salinus* (Carrasco et al., 2009), 8=*V.subterraneus* (Wang et al., 2010), 9=*V. byunsanensis* (Yoon et al., 2010), 10=*V. arcticus* (Niederberger et al., 2009), 11=*V. sediminis* (Chen et al., 2009), 12=*V. zhanjiangensis* (Peng et al., 2009), 13=*V. albus* (Zhang et al., 2012).

Sánchez-Porro et al., 2003). In addition, $CaCl_2$ could activate activity of enzyme from both extracellular and cell-bound fractions. Three major extracellular proteinases were partially-purified including 19, 34, and 44 kD and showed maximum activity at pH 8, 55-60°C, 25-30% NaCl, and 70-100 mM $CaCl_2$ (Phrommao, Rodtong, and Yongsawatdigul, 2010). Recently, Sinsuwan, Rodtong, and Yongsawatdigul (2012) showed that cell-bound proteinases from *Virgibacillus* sp. SK 37 with MM of 19, 20, 22, 32, 34, and 44 kDa showed proteolytic activity in either the absence or presence of 10 and 25% NaCl toward fish muscle, soy protein isolate, and casein substrates. The addition of whole cells of *Virgibacillus* sp. SK37 was able to accelerate proteolysis during fish sauce fermentation. These proteinases from both strains of SK33 and SK37 are likely to play an important role in protein hydrolysis of fish sauce fermentation. Besides commercial proteinases, other microbial proteinases used for the production of bioactive peptides have rarely been reported. Extracellular proteinase from *Virgibacillus* sp. SK37 could hydrolyze protein at high NaCl concentration but properties of the resulting hydrolysates have not been investigated. Therefore, antioxidant properties of the resulting hydrolysates produced by extracellular proteinases from *Virgibacillus* sp. SK37 should be investigated.

2.3 Overview of flavor analysis methodology

There are numerous methods for isolation and analysis of volatile flavor compounds from nonvolatile matrix. The method for sampling or isolation of volatiles should be selected by taking advantage of capability of chosen instrumental techniques and their compounds nature prior to analysis such as, volatility, polarity, stability and concentration of analytes, and sample matrix. Since the unique flavor of

fish sauce is one of the important quality parameters for consumer acceptability, volatile compounds in fish sauces have been identified by several researchers with different techniques of sample preparation and methods of analysis. Methods often employed in fish sauce flavor analysis are discussed below.

2.3.1 Isolation of volatile flavor compounds

2.3.1.1 Headspace sampling

2.3.1.1.1 Static headspace sampling (SHS)

SHS is a technique based on differences in volatility between flavor compounds and food matrix. The sample is placed in a sealed vial and letting volatile compounds above the sample come into equilibrium. Headspace vapor is then injected into injection port of the GC using gastight syringe or direct transfer with headspace sampler (Kim and Cadwallader, 2011). Volatile compounds isolated by SHS may closely be resemble the actual compounds coming from the sample that is detected by human nose (Genthner, 2010). There are many advantages of this technique, including simple preparation, high reproducibility, non-destructive analysis, low risk of artifacts, ability to analyze highly volatile low molecular weight compounds, and elimination of solvent peak and non-volatile contaminants (Kim and Cadwallader, 2011; Rouseff and Cadwallader, 2001; Snow and Slack, 2002). However, disadvantage of SHS is that it is not suitable for isolation of volatiles with high boiling points and poor sensitivity for trace level volatile analytes (Kolb and Ettre, 2006; Wampler, 1997).

2.3.1.1.2 Dynamic headspace sampling (DHS)

DHS or purge and trap analysis involves the passing of inert carrier gas such as nitrogen through the sample. The volatiles contained in the carrier

gas are then enriched by trapping onto adsorbent materials (generally porous polymers) or by cryogenic focusing. Then, volatile analytes are desorbed by heating the trap (thermal desorption), and the released volatiles are sent to the analytical GC column for analysis (Kim and Cadwallader, 2011). There are many types of solid sorbents, such as activated carbon, graphitized carbon blacks (Carbo-trap, Carbopack, Carbograph, Graphtrap, Graphon, Spheron), porous carbons (Carb, Hypercarb), carbon molecular sieves (Carbosieve, Carboxen, Purasieve, Spherocarb, Sortophase, Carbosphere, Saran Carbon, Ambersorb) and carbon-based porous polymers (Tenax, Chromosorb, Porapak, Hayesep, Amberlite resins) (Dewulf and Van Langenhove, 1999). Among these polymers, Tenax is the most widely used adsorbent material since it has a low affinity for water, exhibits high thermal stability (375°C), and can be subjected to repeated temperature cycling without deterioration (Etievant, 1996; Wardencki, 1998). However, the adsorbent material can be chosen according to the specificity of the target volatile analytes (Kim and Cadwallader, 2011). In general, DHS is usually used when the sample is a solid, while purge and trap is used when the sample is a liquid analyzed by bubbling the carrier gas through the liquid (Wampler, 1997). DHS offers many advantages as compared to static headspace, including analysis of just volatiles, automation, and easy sample preparation. In addition, DHS also offers increased sensitivity by permitting the analysis of volatiles present at the parts per billion (ppb) level, providing greater sensitive than SHS (Wampler, 1997). However, DHS is not suitable for isolating low volatile compounds. DHS could also lead to cross-contamination from sample to sample. Thus, the cleaning steps are needed when DHS is employed (Cadwallader and Macleod, 1998; Wanakhachornkrai and Lertsiri, 2003). DHS is one of the most popular techniques which have been

widely used for fish sauce flavor analysis (Fukami et al., 2002; Fukami, Funatsu, Kawasaki, and Watabe, 2004; Giri, Osaka, Okamoto, and Ohshima, 2010; Michihata, Yano, and Enomoto, 2002; Wichaphon et al., 2012).

2.3.1.1.3 Solid phase microextraction (SPME)

SPME provides many advantages over conventional sample preparation techniques. The SPME method is widely used for the extraction of volatiles due to its fast, simplicity and small sample volume (Blank, Milo, Lin, and Fey, 1999). Two sampling methods can be used to collect the compounds in the headspace above sample or directly immersed into the sample. After adsorption is completed, the compounds are thermally desorbed in a GC injector block for further analysis (Blank et al., 1999). This method is rapid, easy to automate for the extraction, and avoids the use of solvents for extraction. Extraction of volatile compounds based on their partitioning between the sample or sample headspace and a polymer-coated fibre (Kim and Cadwallader, 2011). There are three groups and seven commercial fiber types available: (i) non-polar (poly(dimethylsiloxane) (PDMS) at coating thickness of 100, 30, and 7μm sizes); (ii) polar (polyacrylate 85 μm, Carbowax/divinylbenzene (DVB) 65 μm, Carbowax/template resin); (iii) mixed polarity (PDMS/DVB 65 μm, PDMS/Carboxen 75 μm, DVB/Carboxen/PDMS) (Stashenko, and Martínez, 2007; Roberts, Pollien, and Milo, 2000). The use of SPME technique involves the risk of compound discrimination caused by the differences in polarities of the fibers and analytes. Volatility, polarity, and molecular weight of target analytes also need to be considered (McGorrin, 2007). Roberts, Pollien, and Milo (2000) reported that PDMS/DVB had the highest overall sensitivity whereas Carboxen/PDMS was the most sensitive to small molecules and acids. Generally,

sensitivity is better for nonpolar compounds which are easily detected at ppb levels, whereas polar compounds at the ppm level may be more difficult to detect. In addition, the concentration of analytes in the headspace is not proportional to its concentration bound to the fiber (Roberts et al., 2000). Several factors can cause biases in the quantitative determination of compounds. The use of stable isotopes as internal standards solves the above problems. The principles of this procedure are described below.

2.3.1.2 Solvent extraction and distillation extraction

2.3.1.2.1 Direct solvent extraction (DSE)

Direct solvent extraction is one of the simplest techniques for food flavor analysis. Flavor compound is extracted by the difference in polarity between the flavor compounds and food matrix. Most volatile compounds are considerably less polar than aqueous food matrix material. This technique is simple as putting a food sample into a vessel such as a separatory funnel, adding a solvent, and shaking (Kim and Cadwallader, 2011). The solvents commonly used for extraction are diethyl ether, diethyl ether/pentane mixtures, hydrocarbons, Freons, and methylene chloride (Parliment, 1997). After extraction, the solvent phase is collected, dried with anhydrous salt, and then concentrated prior to GC analysis (Kim and Cadwallader, 2011). Wanakhachornkrai and Lertsiri (2003) demonstrated that DSE detected a large number of semi-volatiles but the highly volatile compounds were lost during sample preparation and concentration. Moreover, the presence of solvent peak also could mask the analytes' peaks eluted at the beginning of the chromatography.

2.3.1.2.2 Steam distillation extraction (SDE)

Steam distillation is among the oldest techniques used to separate

volatile from non-volatile material. In 1964, Nickerson and Likens developed a versatile distillation unit for the simultaneous extraction of steam distillates by solvents. SDE is one of the most common steam distillation methods which widely used for fish sauce flavor analysis (Cha and Cadwallader, 1995; Kim et al., 2004; Peralta, Shimoda, and Osajima, 1996; Shih, Chen, Yu, Chang, and Wang, 2003). The sample (an aqueous solution or slurry of a solid material in water) and solvent are heated from flask A and B, respectively (Figure 2.1). Volatiles are then steam-distillated from the sample, and simultaneously the solvent is distilled. Both liquids are continuously recycled and the steam distillable-solvent soluble compounds are transferred from the aqueous phase to the solvent. Vapors condense together on the cold finger (C) where the extraction process occurs between both liquid films on the condenser surface. The solvent layer in area D was then collected in flask B (Figure 2.1) (Bouseta, and Collin, 1995; Chaintreau, 2001; Parliment, 1997). This method yields high recovery of stream-distillable volatiles with medium to high boiling points and ready to be injected into a GC system after concentration (Reineccius, 2006; Teranishi, Murphy, and Mon, 1977). However, disadvantages of SDE are poor recovery for polar compounds, such as hydroxyfuranones and phenols, and possible decomposition of volatiles or production of artifacts due to the presence of water and high extraction temperature (Reineccius, 1993; Werkhoff, Brennecke, Bretschneider, and Bertram, 2002). In particular, when sugars and free amino acids are present in the food sample, the Maillard or the Strecker reaction might lead to the formation of additional, non-genuine compounds (Schieberle, 1995). Thus, SDE is often operated under reduced pressure in order to minimize the formation of thermally-induced artifacts (Parliment, 1997).

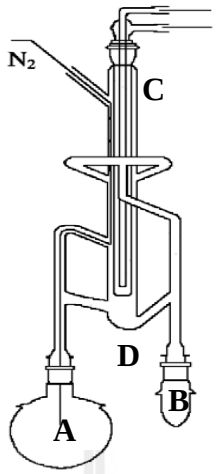

Figure 2.1 Scheme of the microextractor for simultaneous steam distillation-solvent extraction. A= Sample flask, B = Solvent flask, C= Cold-finger, D = Separation chamber.

Modified from: Bouseta and Collin (1995).

2.3.1.2.3 High vacuum distillation extraction

High vacuum distillation, which is one of the early classical techniques, has been applied to isolate low level (ppb to ppt) volatile components of food products containing high fat content. Volatile analytes are distilled from a sample for several hours under high vacuum (~10^{-5} Torr) and heat conditions (<60 $^{\circ}$C), with subsequent condensation of volatiles in a series of cold traps. The volatiles compounds are recovered from the condensed phase by solvent extraction (Kim and Cadwallader, 2011). This technique provides high yield of polar compounds and high recovey of authentic flavor extracts (Werkhoff et al., 2002). However, high boiling point aroma compound may partially condense inside the tubes before reaching the trap, sample containing high concentrations of saturated fat may plug up the stopcock

of the dropping funnel (Engle et al., 1999). In addition, highly volatile trace compounds may be lost during extraction and concentration. Engel, Bahr, and Schieberle (1999) developed a new technique called solvent assisted flavor evaporation (SAFE). This technique is based on high vacuum transfer (HVT) and gives more efficient isolation compared with classic high vacuum distillation methods. Engle et al. (1999) demonstrated that SAFE gave significantly higher percent yields for each high-boiling *n*-alkane (C10-C26) and gave higher yields when distilling polar compounds, such as vanillin, 3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolon) and 3-methylbutanoic acid as compared to HVT. Park, Lee, and Takeoka (2004) also proved that SAFE was able to higher recover the content of aromatic and aliphatic compounds as compared to steam distillation under reduced pressure coupled with continuous liquid-liquid extraction (DRP-LLE) and high-flow dynamic headspace sampling (DHS). In addition, SAFE can be applied to high-fat (50%) matrices, some foods without prior extraction (milk, beer, fruit pulps) (Engle et al., 1999) and to identify compounds present in low concentrations (Havemose, Justese, Bredie, and Nielsen, 2007). The use of this method is still limited in the study of fish sauce flavor. High recovery and sensitivity of volatile compounds makes SAFE an excellent choice in fish sauce flavor analysis.

2.3.2 Identification of volatile flavor compounds

Gas chromatography (GC), GC-olfactometry (GC-O), and GC-mass spectrometer (GC-MS) are standard instruments used in flavor analysis. For compound identification, Molyneux, and Schieberle (2007) recommended the following procedure to avoid false identifications including: (i) mass spectra and retention indices must be determined on at least two different GC columns that have stationary

phases of different polarities; (ii) the results must be compared to spectra and retention indices of reference compounds; (iii) for chiral compounds, the retention indices of both enantiomers must be determined by using the respective enantiomers; (iv) odor qualities or odor thresholds determined by GC-O may serve as appropriate tools to aid in compound identification; (v) if no data are available in the literature or a new structure is detected, a proposal for the structure can be derived from all mass spectrometric data, but the suggested structure must be synthesized and confirmed by nuclear magnetic resonance (NMR) measurements. If the synthesized reference agree in all mass spectrometric data, retention indices, and the bioactivity, NMR measurements can be omitted.

Retention index (RI) is a measure of relative retention time using normal (straight chain) alkanes as a standard reference. RI of each compound can be calculated from data obtained by programmed GC using the following formula derived by van den Dool and Kratz (1963):

$$RI = (100 \times L) + (100 \times N) \times [(RT_X - RT_L)/(RT_U - RT_L)]$$

Where L is carbon number of the lower alkane. N is the difference in carbon number of the two n-alkanes that bracket the compound. RT_X, RT_L, and RT_U are the retention time of unknown compound, the lower alkane, and the upper alkane, respectively.

2.3.2.1 Gas chromatography (GC)

GC is one of the chromatography used in analytical compounds that can be vaporized without decomposition. Thus, numerous volatiles compounds existing in the foods can be detected and separated. However, many of these volatile compounds are likely to have little or no impact on the actual aroma of the food. Thus, the odor-

active compounds from a complex mixture can be determined by GC in combination with olfactometric techniques (GC-O). In GC-O, volatile compounds are first separated by GC and then delivered to an oflactometer (sniffing port) where they are mixed with humidified air. The human nose “sniffers” continuously breathe (nasally) the air emitted from the olfactometer, and record the perceived odor descriptions and intensities of the detected odorants for discovering the odor-active compounds effluent from GC column (Kim and Cadwallader, 2011). Sniffing port connect in parallel to conventional detectors, such as flame-ionization detector (FID) or mass spectrometer (MS) (Plutowska and Wardencki, 2008).

Several techniques have been developed to evaluate GC-O data and to estimate the sensory contribution of single aroma compounds. Dilution techniques and time-intensity measurement are the two main GC-O methods (Blank, 1997). Two dilution techniques, such as aroma extract dilution analysis (AEDA) (Grosch, 1993) and combined hedonic aroma response measurement (Charm analysis) (Acree, 1993), have been developed and used to evaluate the intensities of odor active compounds in various food products. Both evaluate the odor activity of individual compounds by sniffing the GC effluent of a series of dilutions, usually as a series of 1:2 or 1:3 dilutions, of the original aroma extract. Both methods are based on the odor detection threshold. In AEDA, sniffer indicates whether or not an aroma can be perceived and describes the odor perceived. Each compound is expressed as the flavor dilution (FD) factor, which corresponds to the highest extract dilution value at which the odorant was detected. In Charm analysis, sniffer points out the beginning and end of each odor compound (duration of the smell) with a description of the odor. Times of the individual sniffs are combined and graphed to yield a chromatogram with peaks and

quantified peak areas (Charm values), which are used to quantify potency (Blank, 1997; van Ruth, 2001). Charm analysis differs from AEDA is that in Charm analysis the duration of perception is taken into consideration together with the final dilution (dilution value) in which a compound is detected, while only the maximum dilution value detected is noted in AEDA (Mistry, Reineccius, and Olson, 1997; van Ruth, 2001). AEDA and Charm analysis have been used to determine potent odorants in many different food products including fish/soy sauces (Cha and Cadwallader, 1998; Kanko, Kumazawa, and Nishimura, 2012; Steinhaus and Schieberle, 2007). For AEDA, the odor active compounds give different odor intensities at the same FD value (Pham et al., 2008). Recently, Wichaphon et al., (2012) assigned combination score which combined the FD factor and the odor intensity to identify odor active compounds of eight fish sauce samples. This score was assigned in order to discriminate the different intensity perceived on GC-O at each dilution.

In time-intensity measurement, this method was developed by McDaniel, Miranda-López, Watson, Micheals, and Libbey (1990). It is based on magnitude estimation of the odor intensity in only one injection. The subject rates the aroma intensity by using a computerized 16-point scale time-intensity device and indicates the corresponding aroma characteristics. The plot of the retention time versus odor intensity called Osmegram (Osme) (Blank, 1997). For example, the comparison of GC-MS chromatogram and Osme-GCO gram of fish sauce sample using SPME is shown in Figure 2.2. Other examples of using Osme for identification odor-active compounds are cashew apple nectar (Valim, Rouseff, and Lin, 2003) and freshly prepared essential oils by steam distillation (Kamath, Asha, Ravi, Narasimhan, and Rajalakshmi, 2001).

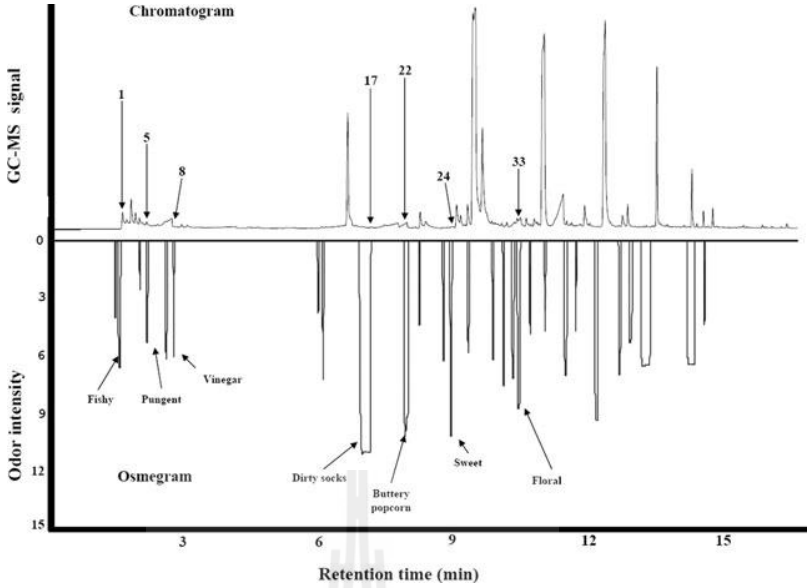

Figure 2.2 Comparison of SPME-GC-MS chromatogram (top) and SPME-Osme-GCO gram (bottom) of fish sauce sample.

From: Pham, Schilling, Yoon, Kamadia, and Marshall (2008).

2.3.2.2 Mass spectrometry (MS)

A technique widely used in flavor analysis is gas chromatography coupled with mass spectrometry (GC-MS). The method gives both qualitative (mass spectra) and quantitative (peak areas) information (Huston, 1997). Standard GC-MS used in flavor analysis is considered as fused silica, capillary column GC with bonded phase, providing high resolution, combined with fast scanning, high-sensitive MS operating in the electron impact ionization mode (Cadwallader and Macleod, 1998). Mass spectromegtry is an analytical technique which the sample is first ionized to create a population of ion fragments with distribution of mass/charge (m/z) ratios. Electron impact mass spectrometry (EI-MS) is the most common ionization technique used in flavor analysis (Kim and Cadwallader, 2011). The most commonly used mass

analyzers in food flavor analysis are quadrupole, ion trap, sector systems, and time-of-flight (Kaklamanos, Aprea, and Theodoridis, 2012). For fish sauce flavor analysis, EI-quadrupole MS is the most frequently used detector in literature.

Mass spectrometer requires charged and gaseous molecules for analysis. EI is one of an ionization method used in mass spectrometry, particularly for gases and volatile organic molecules. After the neutral analyte molecules of the sample are bombarded when subjected to high-energy electron bombardment at 70 eV generated by a filament (rhenium or tungsten wire), resulting in a positively charged ion. Typically, the sample molecules lose one electron (Kaklamanos et al., 2012). This process can either produce a molecular ion (same molecular weight and elemental composition of the original molecule) or fragment ion (a smaller piece of the original molecule) (Ashcroft, 1997). The results of charged ion fragments are then directed towards the mass analyzer and separated according to their mass/charge (m/z) ratio (Peppard, 1999). Separation process of ion by quadrupole mass analyzer relies on electric field generating by four metal rods (quadrupoles) (Leary and Schmidt, 1996). Each opposing rod pair is connected together electrically and a radio frequency (RF) voltage together with a direct current (DC) voltage is applied on the two rod pairs. These voltages produce an electric field that allow the selected m/z values (only ions of a certain m/z) to pass through the quadrupole and reach to the detector for a given ratio of voltages, while others are unstable trajectories and collide with the rods, thus losing their charge and being eliminated from the system (Kaklamanos et al., 2012). The m/z of particular ions is determined by correlating the field applied to the quadrupoles with the ions reaching the detector.

2.3.3 Quantification of volatile flavor compounds

Quantitative data for individual volatile compound contained in the complex mixtures are calculated based on comparisons between GC signals. The signal of a known quantity of standard (internal or external, or addition of the analyte itself) is used to determine the level of the analyte (IOFI, 2011). These approaches are described in some details below.

2.3.3.1 Internal standard method

This method is determined by addition of standard compound of known concentration into the sample and area of the peaks of interest compound compared with that of the standard. The advantage of this method is that the effect of the matrix can be eliminated. Response factors between each analyte and internal standard should be determined prior to the analysis the sample by injecting mixtures of known amounts of analyte and internal standard and measuring the peak ratios versus the amount ratios (Schieberle and Grosch, 1987). Compound used as the internal standard should not be present in the sample, not interfere/merge with any of the natural compounds of the mixtures, and has similar physicochemical properties to that of the analyte as possible (Kolb and Ettre, 2006). Therefore, the best internal standard would be the compound labeled with an appropriate isotope. The use of a stable isotope labeled internal standard for quantitative analysis or stable isotope dilution assay (SIDA) is a widely accepted technique. Due to physicochemical properties of the labeled compound is very close to the analyte, using stable isotopes for quantification can overcome the problem of extraction bias. The target compound (or unlabeled compound) can be quantified by adding a known amount of the stable isotope (or labeled compound) and number of deuterium (^{2}H or D) or carbon-13 (^{13}C) atoms as

an internal standard to the sample prior to extraction and GC-MS analysis. In combination with GC-MS, the unlabeled and labeled compound can easily be differentiated according to their different molecular mass (Schieberle and Grosch, 1987). The selected ions for relating the abundance of the unlabeled compound against the labeled compound requires careful consideration. The ideal for the selected ion will: (i) be present in only the spectrum of the unlabeled/labeled compound; (ii) not be present in the spectrum of another compound in the sample that coelutes with the target compound; (iii) be at least moderately abundant (Hausch, 2010). The peak areas of the selected ion of unlabeled/labeled compounds are then compared and used for determination of the target compound that present in the sample. The SIDA has been successfully applied to various food products for the quantification of desirable and also off-flavors as seen by its common use in recent years (Luisier, Buettner, lker, Rausis, and Frey, 2008; Ruisinger and Schieberle, 2012; Roland, Schneider, Le Guerneve, Razungles, and Cavelier, 2010; Stei nhaus and Schieberle, 2007). However, this approach is still limited in fish sauce flavor analysis. The most widely used approaches in the study of fish sauce flavor are relative abundance (%) and the ratios of peak area of any compound to that of internal standard (typically cyclohexanol, 2,4,6-trimethylpyridine, 2-methyl-3-heptanone).

2.3.3.2 External standard method

In the external standard method, the analysis of the analyte is compared with the analysis of the standard in a known concentration of pure solute. The standard is chromatographed separately from the sample. This method is based on two chromatographic conditions which must be carried out under identical conditions. Peak area and concentration are proportional. Thus, the concentration of the analyte

can be calculated from its peak area by comparing with the peak area of standard which known concentration (Kolb and Ettre, 2006). This method has a number of advantages. For example, standard can be identical to the analyte in the sample. This also means that the relative response factors between the standard and the analyte are no required to be determined (Scott, 1995). When chromatogram of the sample is too crowded or cannot find a place for internal standard, external standard is the preferred method for quantification. However, this method is the need to reproduce the matrix of the sample (Kolb and Ettre, 2006).

2.3.3.3 Standard addition method

This method is determined by analysis of original sample which is followed by analysis of the same sample to which know amounts of the analyte are added. Thus, the effect of the matrix can be eliminated. All measurements must be carried out under identical conditions. Peak area and amount of the analyte are propotional and thus no response factors are needed for calculation. This method is a universal procedure in the headspace measurement and has been recommended for quantitative headspace analysis (Kolb and Ettre, 2006). Some compounds in fish sauce were quantified using the standard addition method, such as trimethylamine, methyl mercaptan, 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, dimethyl trisulfide, 3-methylbutanol, and 2,6-dimethylpyrazine (Fukami et al., 2002; Fukami et al., 2004; Shimoda, Peralta, and Osajima, 1996).

2.4 Enzymatic protein hydrolysis

Enzymatic protein hydrolysis is the degradation of proteins into peptides and/or amino acids by proteinase. When proteins are hydrolyzed, amide (peptide) bonds are

cleaved, and, after addition of a water molecule, peptides and/or free amino acids are released. The newly formed peptides can be as new substrates for the enzyme (Adler-Nissen, 1993). Protein hydrolysate obtained by enzymatic hydrolysis of both plant and animal proteins have been reported to possess different biological activities which based on the initial protein source, enzyme used, and hydrolysis conditions.

2.4.1 Protein substrate

Various food proteins have been used as a source of substrate to produce protein hydrolysate with different biological and functional properties, including mill protein (whey and casein protein), fish protein, meat protein, and plant protein. Up to now, the main bioactive peptides described are from animal sources, especially milk proteins. In recent years, the antioxidant activities of enzymatic hydrolysates from plant-derived proteins, including soybean (Beermann, Euler, Herzberg, and Stahl, 2009; Moure, Domínguez, and Parajó, 2006; Zhang, Li, and Zhou, 2010), wheat (Zhu, Zhou, and Qian, 2006), canola (Cumby, Zhong, Naczk, and Shahidi, 2008), chickpea (Li, Jiang, Zhang, Mu, and Liu, 2008), alfalfa leaf (Xie, Haung, Xu, and Jin, 2008), sesame seed (Liu and Chiang, 2008), Zein (Zhu, Chen, Tang, and Xiong, 2008), curry leaves (Ningappa and Srinivas, 2008), flaxseed (Udenigwe, Lu, Han, Hou, and Aluko, 2009), rapeseed (Zhang, Wang, Xu, and Gao, 2009), buckwheat (Tang, Peng, Zhen, and Chen, 2009), hemp seed (Girgih, Udenigwe, and Aluko, 2011; Tang, Wang, and Yang, 2009), pea seed (Pownall, Udenigwe, and Aluko, 2010), peanut (Jamdar et al., 2010), and African yam bean (Ajibola, Fashakin, Fagbemi, and Aluko, 2011) have been evaluated using several *in vitro* antioxidant evaluation. The important difference between proteins is their primary amino acid sequence. For example, whey proteins contain hydrophobic and hydrophilic amino acids which are randomly distributed

over the peptide backbone whereas caseins contain distinct hydrophobic and hydrophilic domains. In addition, the three-dimentional structure of proteins also affect their sensibility towords proteolytic attack and the type of peptides formed during hydrolysis (Swaisgood, 1982).

Mungbean (*Vigna radiata* (L.) Wilczek) or green gram is an important food crop in South and Southeast Asia. Munbean seeds contain about 20-25% protein (Kavas and Nehir 1992). In Thailand, mungbean is commonly eaten as bean sprouts and the processed form. The most common products are vermicelli (or glass noodle) mungbean starch, different kinds of desserts and snacks. The quantity of mungbean seeds supplied to vermicelli industry is estimated to be 200,000 tons/year and 27% of these raw materials are usually discarded as feedstuff in Thailand (Jantawat, Chinprahast, and Siripatrawan, 1998). After starch extraction, the meal, consisting of 72-80% protein, is typically and used as animal feed or discarded (Jantawat et al., 1998; Sonklin, Laohakunjit, and Kerdchoechuen, 2011). It is estimated that mungbean byproduct are produced about 54,000 tons per year. The maximum utilization of these byproducts should be sought.

Major storage proteins of mungbean are shown in Table 2.2. The 8S globulin or vicilin type is the major storage protein in mungbean seed. Adachi, Bernardo, and Utsumi (2001) showed that 8S vicilin, 11S legumin, and basic 7S have high homology with soybean β-conglycinins, soybean glycinin, and soybean basic 7S, respectively. The 8S globulin consist of three isoforms (8Sα, 8Sα′, and 8Sβ) and were isolated, cloned, and characterized by Bernardo et al. (2004). They found that both the α and β subunits of soybean β-conglycinin (7S globulin) showed the highest homology of 58-61% with 8Sα′, 59-61% with 8Sα, and 66-68% with 8Sβ. (Bernardo

et al., 2004).

Table 2.2 Mungbean protein and their characteristics.

Globulin	% of the total globulin	Molecular weight (kDa)	
		Native	Subunit/Peptide
8S Globulin or vicilin	89	200	60, 48, 32, 26
11S Globulin or legumin	7.6	360	40, 24
Basic 7S	3.4	135	28, 16

From: Mendoza, Adachi, Bernardo, and Utsumi (2001).

Chen, Muramoto, and Yamauchi (1995) isolated six antioxidant peptides from digested β-conglycinin using proteinase S from *Bacillus* sp. These peptides were composed of 5-16 amino acid residues, including hydrophobic amino acids, valine or leucine, at the N-terminal positions, and proline, histidine, or tyrosine in the sequences. Some histidine-containing peptides can also act as a metal-ion chelator, a singlet oxygen quencher, and a hydroxy radical scavenger (Chen, Muramoto, Yamauchi, and Nokihara, 1996; Chen, Muramoto, Yamauchi, Fujimoto, and Nokihara, 1998). Beermann, Euler, Herzberg, and Stahl (2009) showed that peptides from soybean protein isolate prepared by tyrosine were shown to possess antioxidant capacities which might be linked to the presence of carboxy terminal tyrosine. There is a few research reported on the bioactive potential of mungbean protein hydrolysate. It has been reported that mung bean protein hydrolysates obtained with Alcalase exhibited angiotensin I-convertingyme (ACE) inhibitory activity *in vitro* (Li, Le, Liu, and Shi, 2005) and exerted antihypertensive effect in spontaneously hypertensive rats

(Li, Shi, Liu, and Le 2006). Three kinds of these peptides were isolated and their amino acid sequences were identified to be Lys-Asp-Tyr-Arg-Leu, Val-Thr-Pro-AlaLeu-Arg, and Lys-Leu-Pro-Ala-Gly-Thr-Leu-Phe (Li, Wan, Le, and Shi, 2006).

2.4.2 Proteinase

Commercial enzymes commonly used for protein hydrolysate varied in their principal enzyme activities, pH and temperature optimum. These proteinases can be classified based on their source (animal, plant, microbial), their mode of catalytic action (endopeptidase or exopeptidase), and their nature of active site residues involved in mechanism. Endopeptidase cleave the amide bond within the protein or peptide chain, whereas exopeptidase remove terminal amino acid either at the C-terminus (carboxypeptidases) or at the N-terminus (aminopeptidases). Based on the nature of the active site, proteinases can be classified as aspartic proteinases (*e.g.* cathepsins D and E, pepsin, chymosin, and renin), cysteine proteinases (*e.g.* cathepsins B, L, S, K, Q, papain, bromelain, and ficin), metalloproteinases (*e.g.* gelatinases A and B, collagenase, termoase, neutrase, and thermolysin), serine proteinases (*e.g.* plasmin, trypsin, chymotrypsin, subtillisin, alcalase, and elastase), and threonine proteinases (*e.g.* proteasome) (Alder-nissen, 1993, Beynon and Bond, 2001). These enzymes differ in their preferential specificity for the amino acid. For example, trypsin, chymotrypsin, and elastase are all serine proteinases. Trysin prefers either arginine (Arg) or lysine (Lys) residues at the carboxylic side of the amind bond whereas chymotrypsin prefers either phenylalanine (Phe), tryptophan (Trp), or tyrosine (Tyr) and elastase prefrers amino acids with small side chains such as alanine (Whitaker, 1994). The pH specificity of proteinases depends on the group of the nature of the active site; cysteine and metallo proteinases are active at neutral pH

whereas the serine and aspartic proteinases are active at alkaline and acidic pH, respectively (Alder-nissen, 1993). Table 2.3 shows some bacterial proteinases commercially used for protein hydrolysate production.

Table 2.3 Examples of commercial proteinase used for hydrolysis of food proteins.

Enzyme	Temperature range (°C)	pH range	Source	Specificity
Alcalase 2.4 L FG	55-70	6.5-8.5	*B. licheniformis*	Endopeptidase
Neutrase 0.5 L	45-55	5.5-7.5	*B.subtilis*	Endopeptidase
Protamex	35-60	5.5-7.5	-	Proteinase complex
Alkaline proteinase	60	9.0-10	*B. licheniformis*	Endopeptidase
Bromelain	50-60	3-9	Pineapple stem	Peptidase
Papain	65-80	5-7	*Carica papaya*	Peptidase
Corolase 7089	<60	5-7.5	*B. subtilis*	Endopeptidase
Validase TSP	45-55	6.5-8	*B. subtilis*	Endopeptidase
Corolase N	<60	5-7.5	*B. subtilis*	Endopeptidase
Corolase PN-L	<50	5-8	*A. sojae*	Endo-,exopeptidase
Corolase LAP	<70	6-9	*A. sojae*	Exopeptidase
Flavourzyme 100L	50	7.0	*A. oryzae*	Endo-,exopeptidase

Modified from: Gilmartin and Jervis (2002).

2.4.2.1 Halotolerant and halophilic proteinase

Halophilic microorganisms produce proteinases with high stability at high salt concentrations which can have novel applications (Margesin and Schiner, 2001). Haloneutrophilic bacteria produce extracellular halophilic proteinases with maximum activity at neutral pH. Several of these proteinases have been isolated and characterized (Kamekura and Seno, 1990; Kamekura, Seno, and Dyall-Smith, 1996;

Reddy, Jayalaksmi, and Sreeramulu, 2003; Schmitt, Rdest, and Goebel, 1990; Stepanov et al., 1992). Haloalkaphilic bacteria require both high salt concentration and high pH (10-11) for growth (Tindall, Ross, and Grant, 1984). Their extracellular enzymes have been purified and characterized. For example, the partially purified proteinase from haloalkaliphilic extracellular serine protease from *Halogeometricum borinquense* showed activity at pH on the range of 7.0-10.0 with optimal activity at 65°C and pH 10.0 (Vidyasagar, Prakash, Litchfield, and Sreeramulu, 2006), haloalkaline proteinase produced by *Salinivibrio* sp. strain AF-2004 exhibited its optimal activity at 65°C, pH 8.5, and 0-0.5 M NaCl with a high tolerance to salt concentrations of up to 4 M NaCl (Karbalaei-Heidari, Ziaee, Schaller, and Amoozegar, 2007). Halotolerant proteinases produced from halophilic or haloterant bacteria could retain their activity in either absence or presence of NaCl. At nearly saturated NaCl concentration, the enzyme activity can be either activated, or partially inhibited, or not affected by NaCl. For example, serine proteinase from thermotolerant *B. licheniformis* RKK-04 isolated from Thai fish sauce was halototerant showing 61 and 36% activity at 50 and 37°C, respectively, at a concentration of 30% NaCl, whereas the enzymes exhibited the highest activities in the absence of NaCl (Toyokawa et al., 2010). It has been shown that proteinase from halophilic archaea play an important role in the degradation of fish protein into amino acids (Thongthai, McGenity, Suntinanalert, and Grant, 1992). Thus, halotolerant/halophilic proteinases, which are active in the presence of salt, produced from microfloral halophilic bacteria are believed to play the major role during fermentation process.

Moderately halophilic bacteria constitute a very interesting group of

organisms with great potential used in biotechnology (Ventosa, Nieto, and Oren, 1998). Several extracellular proteinases from moderate halophiles from various sources have been isolated and characterized, such as *Salinivibrio costicola* 18AG isolated from salt spring (Lama, Romano, Calandrelli, Nicolaus, and Gambacorta, 2005), *Lentibacillus salicampi* isolated from salt field (Yoon, Kang, and Park, 2002), *Salinivibrio* sp. AF-2004 isolated from saline lake (Karbalaei-Heidari, Ziaee, Schaller, and Amoozegar, 2007). Both *Pseudoalteromonas* sp. CP76 and *Halobacillus karajensis* have been isolated from saline soil (Amoozegar, Malekzadeh, Malik, Schumann, and Spröer, 2003; Sánchez-Porro, Mellado, Bertoldo, Antranikian, and Ventosa, 2003).

2.4.2.2 Microbial proteinase production

Microbial proteinases are classified into various groups, dependent on whether they are active under acidic, neutral, or alkaline conditions. Alkaline proteinases are the most important group of microbial enzymes exploited commercially (Gupta, Beg, and Lorenz, 2002). Among the available microbial proteinases, alkaline proteinases produced by organisms belonging to the genus *Bacillus* are highly commercial valuable mainly used (eg. Alcalase, Novozyme 243 or subtilisin Carlsberg from *B. licheniformis*, subtilisin Novo or bacterial proteinase Nagase (BPN′) from *B. amyloliquefaciens*) (Gupta, Beg, and Lorenz, 2002; Rao, Tanksale, Ghatge, and Deshpande, 1998). The overall cost of enzyme production is the major obstacle against the successful application of any technology in the enzyme industry. Thus, there are several reports describing the use of agricultural byproducts for the production of microbial proteinases. For example, bug meal for alkaline proteinase production by *Bacillus* sp. AR009 (Gessesse, 1997), pigeon pea waste for a

thermostable alkaline proteinase production by *Bacillus* sp. JB-99 (Johnvesly, Manjunath, and Naik, 2002), green gram husk for alkaline protease production by *Bacillus* sp. (Prakasham, Rao, and Sarma, 2006), and proteinase production from *B. pantotheneticus* using molasses as a substrate (Shikha, Sharan, and Darmwal, 2007). Cost of raw materials for proteinase production is another factor that should be taken into consideration.

Composition of the culture medium was found to have great influence on extracellular proteinase production and it depends on microbial strain. In addition, physical factors, such as incubation time and temperature, inoculum concentration, and pH, significantly affect proteinase production (Gupta, Beg, Khan, and Chauhan, 2002). The nitrogen source is one of important parameters because it is metabolized to produce amino acids, nucleic acids, proteins, and cell wall components (Kumar and Takagi, 1999). Numerous studies reported that nitrogen sources have different effects on proteinase production of various strains of *Bacillus*. The best nitrogen source for both *B. cereus* 146 and *Bacillus* sp. K-30 was beef extract (Naidu and Devi, 2005; Shafee, Aris, Rahman, Basri and Salleh, 2005) while soybean meal and wheat bran were the best nitrogen source for proteinase production of *B. cereus* MCM B-326 and *B. pantotheneticus,* respectively (Nilegaonkar, Zambare, Kanekar, Dhakephalkar, and Sarnaik, 2007; Shikha, Sharan and Darmwal, 2007). Many of the extracellular proteinases produced by halophilic archaea are enable the degradation of proteins and peptides, likely to generate oligopeptide, dipeptide, and amino acid intermediates which feed into central metabolism for growth (De Castro, Maupin-Furlow, Gimenez, Herrera Seitz, and Sanchez, 2006). The mechanisms used by halophilic archaea to transport extracellular proteinases to the medium have been reported by using General

secretory (Sec) and twin-arginine-transport (Tat) pathways (Pohlschroder, Hartmann, Hand, Dilks, and Haddad, 2005; Ring and Eichler, 2004; Rose, Bruser, Kissinger, and Pohlschroder, 2002). In general, proteinase production was found to be repressed by rapidly metabolizable nitrogen sources, such as amino acids or ammonium ion concentrations in the medium. Proteinase production of *B. firmus* was repressed by excessive amount of yeast extract (Moon and Parulekar, 1991). However, proteinase production of *B. licheniformis* was not repressed by ammonium salts (Nehete, Shah, and Kothari, 1986).

2.4.3 Antioxidative peptides

Many food peptides derived by enzymatic hydrolysis with antioxidative properties in various oxidative reaction systems have been discovered. The antioxidant properties of these peptides include scavenging or quenching of reactive oxygen species (ROS)/free radicals and inhibition of ROS induced oxidation of biological macromolecules such as lipids, proteins, and DNA. Other mechanisms of antioxidant activity of peptides include transition metal chelating activity and ferric reducing power (Udenigwe and Aluko, 2012).

Some factors that may affect the antioxidant activity of food protein hydrolysates include specificity of proteinases used for hydrolysis, degree of hydrolysis (DH), and the structural properties of the resulting peptides, including molecular size, hydrophobicity, and amino acid composition (Udenigwe and Aluko, 2012). Many researches have been reported that the lowest molecular weight fraction using ultrafiltration membrane system showed more potent antioxidant peptides (Je, Park, and Kim, 2005; Rajapakse, et al., 2005; Ranathunga, Rajapakse, and Kim, 2006; Ren et al., 2008). Higher antioxidant activities of low molecular peptides are thought to be

because they can easily react with radicals. An increase in reducing power as DH increased has also been reported in Alcalase-hydrolysed porcine plasma protein (Liu, Kong, Xiong, and Xia, 2010) and canola protein hydrolysate (Cumby, Zhong, Y., Naczk, and Shahidi, 2008). Raghavan, Kristinsson, and Leeuwenburgh (2008) also reported that the reducing power of tilapia hydrolysates prepared from Flavourzyme increased with an increase in %DH, whereas reducing power of those prepared from *B. subtilis* proteinase did not vary with the extent of DH. These results also demonstrated that reducing power ability of protein hydrolysate vastly depends on the type of proteinase.

Regarding to amino acid composition, individual amino acid has been reported to possess different mechanisms of antioxidant activity. Aromatic amino acid (Tyr and Phe) have ability to donate protons easily to electron deficient radicals, while maintain their stability via resonance structures. Trp, Tyr, and His residue contained the indolic, phenolic, and imidazole group, respectively, which serve as hydrogen donors. In addition, Met and Cys have ability to donate their sulfur hydrogen (Ajibola, Fashakin, Fagbemi, and Aluko, 2011; Hernández-Ledesma, Dávalos, Bartolomé, and Amigo, 2005; Rajapakse, et al., 2005). The carboxylic and amino groups in branches of acidic (Asp and Glu) and basic (Arg, Lys, and His) amino acids have been reported to enhance metal chelation (Liu et al., 2010; Rajapakse, Mendis, Byun, and Kim, 2005; Saiga, Tanabe, and Nishimura, 2003). Moreover, the electron-dense aromatic rings of Tyr, Phe, and Trp residues of peptides can contribute to the chelating of pro-oxidant metal ions (Udenigwe and Aluko, 2012). Moreover, hydrophobic amino acids are important for enhancement of the antioxidant properties of peptides. The addition of hydrophobic amino acids, proline and leucine, to the N-

terminus of a dipeptide His-His resulted in enhanced antioxidative property of the peptides (Chen et al., 1998). The presence of hydrophonic amino acid has been reported to contribute to lipid peroxidation by increasing solubility of peptides and thereby facilitating better interaction with radical species (Rajapakse et al., 2005; Mendis, Rajapkse, and Kim, 2005).

2.4.4 Identification of protein hydrolysates

Mass spectrometry and tandem mass spectrometry (MS/MS) experiments are major tools used in protein/peptide identification. MS/MS is the technique for determining ion mass using mass spectrometer, which is coupled with several mass analyzers. Some techniques often employed in peptide identification are discussed below.

2.4.4.1 Ionization

Two ionization techniques, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are known as soft ionization techniques, allowing molecules to remain relatively intact during the ionization process. These techniques have been widely used for peptide identification. ESI has been developed for use as ionization method for biological macromolecules by Fenn, Mann, Meng, Wong, and Whitehouse (1989). This ionization technique is atmospheric pressure ionization (API). The ionization process of ESI is accomplished by forcing a liquid sample stream through a needle or capillary which produces a fine spray of highly charged droplets in the presence of a strong electric field. The droplet shrinks due to the solvent evaporation until it reaches the point that the surface tension can no longer sustain the charge. Then, the repulsive forces exceed the droplet surface tension to cause breakdown the droplets. The sequence of solvent evaporation and fission of

droplets is repeated until the small droplet containing only one solute molecule is obtained. These charged analyte molecules can be either singly or multiply charged. Then, ions free from solvent can enter the mass spectrometer via an intermediate vacuum region (Kicman, Parkin, and Iles, 2007).

For MALDI, the principle of this technique is based on depositing nonvolatile analyte mixed with a large excess of small organic material known as the matrix in an aqueous or organic solvent. Hillenkamp, Karas, Beavis, and Chait (1991**)** reported that α- cyano-4-hydroxycinnamic acid was suitable for analysis of peptide and proteins smaller than 10,000 Da, whereas sinapinic acid was used as a matrix for high mass proteins**.** A few microlitres is applied to a MALDI target plate which is positioned in the high vacuum source region of mass spectrometer and is irradiated with a pulsed laser beam (usually 337 nm). The matrix absorbs most of this energy in the form of heat, whereas the analytes such as proteins/peptide remain intact. A dense gas cloud is formed by sublimation of the matrix and rapid expansion of matrix and analyte into the gas phase. Then, desorption occurs by proton transfer between the photoexcited matrix and analyte, resulting in formation of charged analytes then introduction into the mass analyzer (Karas, Gluckmann, and Schafer, 2000; Kicman, Parkin, and Iles, 2007).

2.4.4.2 Mass analysis

ESI and MALDI interfaces are combined in various ways with different mass analyzers, including quadrupole (Q) mass analyzers, time-off-flight (TOF), quadrupole ion traps, and fourier transform ion cyclotron resonance (FTICR) mass spectrometry (Wysockia, Resingb, Zhanga, and Chenga, 2005). Molecular weight and amino acid sequence of various bioactive peptides are usually used a Q-TOF mass

spectrometer coupled with ESI ionization source. For examples for using of ESI-Q-TOF; antioxidative peptide from conger eel muscle protein derived by Alcalase (Ranathunga et al., 2006), tuna backbone protein hydrolyzed by pepsin (Je, Qian, Byun, and Kim, 2007), and bullfrog skin protein hydrolyzed by Alcalase (Qian, Jung, and Kim, 2008).

The generated ions from MALDI are also typically coupled with TOF mass analyzer. TOF mass analyzer is based on by measuring the time required for ions which are accelerated in equal energies and fly through the field-free drift tube (the analyzer). At, the fixed distance of the flight tube and accelating potential, the flight time of each ion is proportional to the square root of the mass-to-charge ratio (m/z). Thus, single charged ions with a larger mass will travel with a lower velocity down the tube compared to those with smaller mass (Kicman, Parkin, and Iles, 2007).

2.4.5 Protein/peptide identification

Protein identification using MS can be performed using peptide mass fingerprinting (PMF) and peptide sequencing using tandem mass spectoscopy. The basic of these approaches to identify proteins includes separation of proteins by gel electrophoresis or liquid chromatography (Thiede et al., 2005). Subsequently, the proteins are cleaved with sequence-specific endoproteinase (typically trypsin). Trypsin is normally used as a sequence-specific proteinase for generating the peptides. It cleaves peptide chains exclusively at the carboxyl side of the amino acids lysine or arginine (Olsen, Ong, and Mann, 2004).

2.4.5.1 Peptide mass fingerprinting (PMF)

The principle of this method is that absolute masses of peptides obtained from a specific proteolysis of a protein can be accurately measured, where a unique

mass fingerprint pattern of each individual protein is often obtained (Fabris et al., 1995). Mass profile of peptides is commonly performed using MALDI-TOF which shows higher sensitivity than ESI-MS without complication of multiple signals derived from each constituent (Jensen, Podtelejnikov, and Mann, 1996). Typically, spots from the gel are treated with a proteinase and each subsequent digest is spotted individually onto the MALDI plate. Then, sample/matrix crystals can be picked out and targeted by the laser beam as described above. This peptide fingerprint can be compared the masses of the peptides of the unknown protein to a predicted list of theoretical peptides masses derived from the choice of proteinase used and their corresponding masses for all proteins in a database. Mascot, MS-Fit, and Profound are the most frequently used as internet-accessible search programs for PMF (Thiede et al., 2005).

2.4.5.2 Peptide sequencing

PMF by MALDI-MS often fails to identify low molecular mass proteins and protein fragments due to the small number of detectable peptides (Thiede et al., 2005). Peptide sequencing involves the production of fragment ion spectra by tandem mass spectrometry (MS/MS). Most peptide sequencing experiments have been performed on ESI-triple quadrupoles (TQ). Masses of mixture peptides are analyzed in the first quadrupole and then one of these peptides are isolated and further fragmented by collision-induced dissociation (CID) by collision with inert gas such as argon and nitrogen in a collision cell (middle quadrupole). Then, the resulting fragments are subsequently analyzed in the third quadrupole in order to sequence the peptide. This usually results in the formation of two fragments, one containing the N-terminus and the other containing the C-terminus. N-Terminal fragments are known

as b-ions and are numbered starting from the N-terminus. C-Terminal fragments are known as y-ions and are numbered starting from the C-terminus (Figure 2.3).

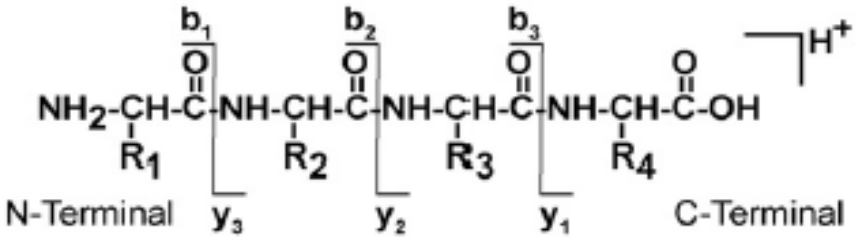

Figure 2.3 A diagram showing fragment ion nomenclature for N-terminal b-ions and C-terminal y-ions.

From: Kicman, Parkin, and Iles (2007).

The fragmentation pattern containing b- and y-series ions are matched with the patterns of fragment ions that are calculated from the database sequences using an algorithm. The sequence of peptides determined from fragmentation of peptides in MS/MS is called *de novo* peptide sequencing. Examples of existing database searching programs which are available on the worldwide web are SEQUEST, MASCOT, PeptideSearch, ExPASy (Aebersold and Goodlett 2001). Recently, peptide sequencing by MS/MS was also applied to identify antioxidative peptides from protein hydrolysates derived from egg white proteins using five different proteinase (Chen, Chi, Zhao, and Lv 2012). Two purified active peptides form reverse phase high performance liquid chromatography (RF-HPLC) separation were analyzed by LC-MS/MS in order to obtain mass profile and amino sequence. The MS/MS spectra were searched to characterize the amino acid sequence of those peptides using SEQUEST algorithm and performed on the Bioworks 3.2 software.

The fragment ions in these spectrum of the two antioxidant peptides were identified to be Tyr-Leu-Gly-Ala-Lys (551.54 Da) and Gly-Gly-Leu-Glu-Pro-Ile-Asn-Phe-Gln (974.55 Da).

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CHAPTER III

IDENTIFICATION AND QUANTIFICATION OF ODOR-ACTIVE COMPOUNDS IN COMMERCIAL THAI FISH SAUCE

3.1 Abstract

Qualitative and quantitative analyses of odor-active compounds in Thai fish sauce samples were performed using direct solvent extraction-solvent-assisted flavor evaporation (DSE-SAFE), aroma extract dilution analysis (AEDA), static headspace dilution analysis (SHDA), gas chromatography-olfactometry (GC-O), headspace-solid phase microextraction (H-SPME), and gas chromatography-mass spectrometry (GC-MS). For AEDA, butanoic acid, 3-methylbutanoic acid, 4-hydroxy-2-ethyl-5-methyl-3(2H)-furanone, and 2-phenylacetic acid showed the highest flavor dilution (FD) factor (log_3FD factor >6). For SHDA, thirteen compounds were detected, among them, methanethiol, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, dimethyl trisulfide, 3-(methylthio)propanal, and butanoic acid were the most predominant (FD factors >5). Twenty-odorants were quantified by stable isotope dilution assays (SIDA), and their odor activity values (OAVs) were calculated. Methanethiol, 2-methylpropanal, 3-methylbutanal, dimethyl trisulfide, 3-(methylthio)propanal, and butanoic acid showed the highest OAVs (>500). Omission experiment using ranking R-index test revealed the importance of acid, aldehyde, and sulfur compounds to the

overall odor of the complete model.

Keywords: fish sauce, aroma extract dilution analysis, odor-active compounds, odor activity value, omission experiment

3.2 Introduction

Thai fish sauce or *nam pla* is one of the important seasonings consumed widely in Southeast Asia, Asian people in Western countries, and the consumption is growing worldwide. Generally, Thai fish sauce is produced by mixing the cleaned anchovy (*Stolephourus* spp.) with solar salt at a ratio of 2:1 or 3:1, depending on the area of production (Lopetcharat, Choi, Park, and Daeschel, 2001). After 12-18 months of fermentation, liquid is drained off and ripened for another 2-12 weeks before filling (Saisithi, Kasemsarn, Liston, and Dollar, 1966). Flavor of fish sauce is one of the most important quality parameters of the product. All volatile compounds in fish sauces have been identified by several researchers with different techniques of sample preparation and methods of analyses. McIver, Brooks, and Reineccius (1982) investigated the volatile compounds of Thai fish sauce using solvent extraction, then separated into acidic, basic, and neutral fractions and identified 52 compounds in all fractions. Sanceda, Kurata, and Arakawa (1986) separated volatile compounds in fish sauce by steam distillation under reduced pressure and identified by gas chromatography-mass spectrometry (GC-MS). Headspace gas analysis with Tenax trap was also used to identify and quantify the potent odorants using standard addition method and 2,4,6-trimethylpyridine as an internal standard (Fukami et al., 2002; Giri, Osaka, Okamoto, and Ohshima, 2010). In addition, aroma-impact compounds in Thai fish sauce was characterized using solid phase micro extraction-GC-MS (SPME-GC-

MS) and SPME-Osme-GC-O, quantified using 1,3-dicholobenzene as an internal standard (Pham, Schilling, Yoon, Kamadia, and Marshall, 2008). The use of SPME technique alone involves the risk of compound discrimination caused by the differences in volatilities of the compounds and polarity of the fibers. To obtain volatile compounds of fish sauce, the isolation has to be completed by combination with solvent extraction. Several studies mentioned above have reported volatile profiles. Little work has been focused on the odor-active compounds of Thai fish sauce. Recently, Giri et al. (2010) investigated the relative proportion (%) of OAVs of all volatiles in premium Thai fish sauce. Butanoic acid showed the highest OAVs, followed by 2-methylbutanoic acid, dimethyl trisulfide, 3-(methylthio)propanal, acetic acid, and trimethylamine.

Quantification of volatile compounds in fish sauce, thus far, has been carried out in a semi-quantitative analysis using internal standards, which does not allow an accurate quantification. A stable isotope dilution assay (SIDA) is the method that provides the physicochemical properties of the isotopically labeled analogue as internal standard which are very close to those of the analyte. In combination with GC-MS, the analyte and internal standard can easily be differentiated according to their different molecular mass (Schieberle and Grosch, 1987). This technique gives high precision and accuracy in quantifying volatile compounds.

Although, odor-active compounds can be identified based on GC-O and the OAV concept, only synthetic blends of odorants (aroma models) are able to address the interaction between the odor-active compounds that generate the typical food aroma. This approach can be used to verify the results or reveal which of the odorants actually contribute to the aroma.

Therefore, objectives of this study were to screen the odor-active compounds of commercial Thai fish sauce by aroma extract dilution analysis (AEDA) and static headspace dilution analysis (SHDA) using gas chromatography-olfactometry (GC-O) and to quantify odor-active compounds using stable isotope dilution assays (SIDA). In addition, the impact of major compounds on overall odor of fish sauce was elucidated by omission studies in model systems.

3.3 Materials and methods

3.3.1 Fish sauce samples

Two commercial premium fish sauce samples were obtained from a supermarket in Thailand. Fish sauce A was a product from Rayong Fish Sauce Industry Co., Ltd. (Rayong, Thailand) and fish sauce B was from Marine Resources and Development Co., Ltd. (Chantaburi, Thailand). The contents of NaCl and total nitrogen (TN) were determined according to AOAC methods (1995). NaCl contents of fish sauce A and B were 23.99 and 24.85% (w/w), respectively. Total nitrogen contents of fish sauce A and B were 2.58 and 2.18% (w/w), respectively.

3.3.2 Chemicals

The following odorants were obtained from the commercial sources as follows: 2-acetyl-2-thiazoline, o-aminoacetophenone, 2,3-butanedione, butanoic acid, dimethyl sulfide, dimethyl trisulfide, 2-ethyl-3,5(or6)-dimethylpyrazine, 3-hydroxy-4,5-dimethyl-2(5H)-furanone, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, indole, methanethiol, 2- and 3-methylbutanal, 3-methylbutanoic acid, 2-methylpropanal, 2-methylpropanoic acid, 3-(methylthio)propanal, 3-(methylthio)propanol, (*E*)-2-nonenal, phenylacetaldehyde, 2-phenylacetic acid, 3-phenylpropanoic acid, propanoic acid,

skatole, and trimethylamine (Sigma-Aldrich, Inc., St. Louis, MO); acetic acid (Fisher Scientific, Pittsburgh, PA); β-damascenone (Firmenich Co., Geneva, Switzerland); (*Z*)-4-heptenal (Bedoukian Research Inc., Danbury, CT); 4-hydroxy-2-ethyl-5-methyl-3(2H)-furanone (TCI America, Portland, OR); 1-octen-3-one (Lancaster, Windham, NH). 2-Acetyl-1-pyrroline was synthesized using the procedure described by Fuganti, Gatti, and Serra (2007).

3.3.3 Isotropically labeled compounds

The following labeled compounds were obtained from the commercial sources: [$^{2}H_3$]-acetic acid, [$^{2}H_2$]-3-methylbutanal, [$^{2}H_9$]-trimethylamine (CDN, Pointe-Claire, Quebec); [$^{2}H_6$]-dimethyl sulfide, [$^{2}H_6$]-dimethyl trisulfide, [$^{2}H_2$]-2-methylpropanal (Sigma-Aldrich, Inc., St. Louis, MO)**;** [$^{2}H_5$]-propanoic acid (Cambridge Isotope Laboratories, MA, USA); [$^{13}C_2$]-2-phenylacetic acid (Isotec, Miamisburg, OH). The following labeled compounds were synthesized according to the literature: [$^{2}H_3$]-o-aminoacetophenone (Steinhaus and Schieberle, 2005), [$^{2}H_2$]-butanoic acid (Schieberle, Gassenmaier, Guth, Sen, and Grosch, 1993), [$^{2}H_5$]-3,6-dimethyl-2-ethylpyrazine (Steinhaus and Schieberle, 2005), [$^{13}C_2$]-4-hydroxy-2,5-dimethyl-3(2H)-furanone (Sen, Schieberle, and Grosch, 1991), [$^{2}H_3$]-methanethiol (Guth and Grosch, 1994), [$^{2}H_2$]-3-methylbutanoic acid (Steinhaus and Schieberle, 2005), [$^{2}H_2$]-2-methylpropanoic acid (Zimmermann, 2001), [$^{2}H_3$]-3-(methylthio)propanal (Sen and Grosch, 1991), [$^{2}H_3$]-3-(methylthio)propanol (Sen and Grosch, 1991), [$^{13}C_2$]-phenylacetaldehyde (Schuh and Schieberle, 2006), [$^{2}H_2$]-3-phenylpropanoic acid (Lin, Welti, Vera, Fay, and Blank, 1999), [$^{2}H_3$]-skatole (Steinhaus and Schieberle, 2005). Odorless deionized-distilled water was prepared by boiling glass-distilled water in an open flask until its volume was reduced by one-fourth of the original volume.

3.3.4 Sample preparation

3.3.4.1 Direct solvent extraction (DSE)

Fish sauce (20 g) was diluted and mixed well with 100 mL of odorless-distilled water, and spiked with 25 µL of internal standards (1.15 µg/µL of 2-ethyl butyric acid in methanol as acidic fraction internal standard, 1.32 µg/µL of 2-methyl-3-heptanone, and 1.13 µg/µL of 2, 4, 6-trimethyl pyridine in methanol as neutral/basic fraction internal standard). The pH was adjusted to ~2.0 using 2 M HCl and extracted three times with diethyl ether (100 mL total volume). After the third extraction, the pH of sample was raised to ~9.0 using 2 M NaOH and extracted three times with diethyl ether (100 mL total volume). The pooled solvent extract (200 mL) was evaporated to 50 mL using a Vigreux column in a 43ºC water bath.

3.3.4.2 Solvent-assisted flavor evaporation (SAFE)

Volatile compounds from fish sauce extracts were distilled using SAFE. The assembly used was similar to that described by Engel, Bahr, and Schieberle (1999). Distillation was carried out at 43ºC for 2 h under vacuum (~10^{-5} torr). To separate the acidic compounds from the neutral/basic compounds, the distillate was concentrated to 30 mL using a Vigreux column in a 43ºC water bath. Concentrated distill was washed three times with 0.1 M NaOH (3x20 mL), and the upper layer (diethyl ether) containing the neutral/basic volatiles fraction (NBF) was collected. The bottom layer (aqueous layer) was then acidified to pH ~2.0 with 4 M HCl, saturated with NaCl and extracted three times with diethyl ether (3x20 mL). The upper layer containing the acidic volatiles fraction (AF) was collected. Both fractions were washed twice with 15 mL of saturated NaCl solution, and then concentrated to 10 mL using Vigreux column, dried over 2 g of anhydrous Na_2SO_4, and further concentrated

to 900 μL of acidic fraction and 100 μL of neutral/basic fraction under nitrogen gas stream. Sample were prepared in triplicates and kept at -70ºC until analysis.

3.3.5 Aroma extract dilution analysis (AEDA)

AF and NBF were diluted in stepwise (1:3; 1 part aroma extract to 2 parts solvent) using diethyl ether as the solvent. Sniffing of dilution was continued until no odorant was detectable. Flavor dilution factor (FD factor) of each compound is the last dilution which was detectable. The FD factors were performed and averaged by two panelists which differing not more than two FD factors. Gas chromatography-olfactometry (GC-O) system consisted of a HP6890 GC (Agilent Technologies, Inc., Palo Alto, CA) equipped with a flame ionization detector (FID) and olfactory detection port (DATU Technology Transfer, Genava, NY). Column effluent was split 1:1 between the FID and the sniffing port using deactivated fused silica tubing (1 m x 0.25 mm i.d.; Restek, Bellefonate, PA). The GC oven temperature was programmed from 40 to 225ºC at a rate of 10ºC/min with an initial and final holding times of 5 and 20 min, respectively. The FID and sniffing port were maintained at a temperature of 250ºC. The sample (2 μL) was injected using a direct cool-on-column mode (+3ºC temperature tracking mode) into a polar column (Rtx-Wax, 15 m x 0.32 mm i.d.; 0.5 μm film; Restek) and a nonpolar column (Rtx-5 SLIMS, 15 m x 0.32 mm i.d.; 0.5 μm film; Restek).

3.3.6 Static headspace dilution analysis (SHDA)

GC-O was conducted on an HP6890 GC (Agilent Technologies, Inc.) equipped with flame ionization detector and olfactory detector port (ODP2, Gerstel). Sample (5 g) was placed in a 250-mL round bottom flask connecting with Wheaton connecting adapter and sealed with PTFE-lined septum. The flask was equilibrated at 40ºC for 30

min with agitation. Each headspace volume (25, 5, 1, 0.2, or 0.04 mL) was injected via a heated (50ºC) gas tight syringe into a CIS-4 cooled injection system (Gerstel) operating in the splitless mode [intial temperature -120ºC (0.10 min hold); ramp rate 12ºC/s; final temperature 260ºC (10 min hold)]. Seperations were performed on Rtx-Wax (15 m x 0.53 mm i.d.; 1 µm film; Restek) and Rtx-5 (15 m x 0.53 mm i.d.; 1 µm film; Restek). Column effluent was split 1:1 between the FID and ODP2 using deactivated fused silica tubing. The FID and ODP2 temperatures were maintained at 250ºC. The GC oven temperature was programmed from 35 to 225ºC at a rate of 6ºC/min with an initial and final holding times of 5 and 10 min, respectively.

3.3.7 Gas chromatography-mass spectrometry (GC-MS)

The GC-MS system consisted of an HP6890GC/5973 mass selective detector (MSD; Agilent Technologies, Inc.). Each aroma extract (1 µL) was injected into a CIS-4 cooled injection system (Gerstel) opering in the splitless-mode [intial temperature -50ºC (0.10 min hold); ramp rate 12ºC/s; final temperature 260ºC (10 min hold)]. Seperations were performed on a Stabilwax (30 m x 0.25 mm i.d.; 0.25 µm film; Restek) and Sac-5 (30 m x 0.25 mm i.d.; 0.25 µm film; Supleco, Bellefonate, PA). Helium was used as carrier gas at a constant rate of 1.0 mL/min. The GC oven temperature was programmed from 35 to 225ºC at a rate of 4ºC/min with initial and final holding times of 5 and 20 min, respectively. MSD conditions were as follows: capillary direct interface temperature, 280ºC; ionization energy, 70 eV; mass range, 35-350 amu; electron multiplier voltage (Autotune +200V); scan rate, 5.2 scans/s.

3.3.8 Headspace-solid phase microextraction (H-SPME)

For identification of highly volatile compounds, sample (1g) was placed in a 22-mL headspace vial and spiked with 5 µL of internal standards (1.15 µg/µL of 2-ethyl

butyric acid, 1.32 μg/μL of 2-methyl-3-heptanone, and 1.13 μg/μL of 2, 4, 6-trimethyl pyridine in methanol). The sample was sealed immediately with a PTFE-lined septum and pre-incubated at 40ºC for 10 min with agitation (500 rpm, 60 s on, 10 s off). Then, a SPME fiber (50/30 μm DVB/Carboxen/Polydimethylsiloxane fiber; Supelco, Bellefonte, PA) was exposed to the vial headspace for 20 min. Then, fiber was desorbed by splitless injection (injector temperature 260ºC; splitless time 4 min; vent flow 50 mL/min) into GC-MS system with the same settings as described above. The GC oven temperature was programmed from 35 to 225ºC at a rate of 6ºC/min with an initial and final holding times of 5 and 20 min, respectively.

3.3.9 Compound identification

Compounds were tentatively identified by GC-O, comparing their retention indices (RIs) and odor properties with referenced RIs and odor properties. Compounds were positively identified by GC-O, comparing their RIs and odor properties with referenced RIs and odor properties, and GC-MS. A homologous series of *n*-alkanes was used for determination of RIs according to the method of van den Dool and Kratz (1963).

3.3.10 Compound quantification

For methanethiol (no. 2), the concentration of [$^2H_3$]-methanethiol synthesized was determined by gas chromatography-flame photometric detector (GC-FPD). Headspace volume of methanethiol (50-300 μL) and [$^2H_3$]-methanethiol (5 mL) were drawn by a gas tight syringe and injected to 5 mL of methanol in a 22 mL headspace vials. Each sample (1 μL) was injected using hot split mode into Rtx-Wax column (15 m x 0.53 mm i.d.; 1 um film; Restek). The concentration of [$^2H_3$]-methanethiol was calculated by comparing its area with the area of methanethiol. Then, fish sauce

sample was spiked with headspace volume of [$^2H_3$]-methanethiol and quantified by H-SPME as described above.

Another highly volatile compounds (1, 3-6; Table 3.5) and intermediate/low volatility with low abundant compounds (7, 9-10, 14, and 16-21; Table 3.5) were quantified by H-SPME and DSE-SAFE as described above, respectively. Intermediate/low volatility with highly abundant compounds (8, 11-13, and 15; Table 3.5) were analyzed by DSE as described above, except that 5 g of sample was mixed with isotopically labeled compounds. Then, sample was diluted and mixed well with 25 mL of odorless-distilled water. The sample was extracted three times with diethyl ether (60 mL total volume). The pooled solvent extract (60 mL) was evaporated to 10 mL using a Vigreux column in a 43ºC water bath, dried over 2 g of anhydrous Na_2SO_4, and further concentrated to 200 µL under nitrogen gas stream.

The selected ions and response factor of the labeled and the aroma compounds no. 1-6 and 7-21 were analyzed by H-SPME-GC-MS and GC-MS, respectively, as described above. Response factor for each compound was determined by measuring the five levels of defined mixtures of the respective labeled and unlabeled compound. Concentration for each compound was calculated using response factor and area of the selected ions (Table 3.4), expressed as microgram per kilogram sample (ppb). Then, odor activity values (OAVs) were calculated by dividing the concentrations by the respective odor thresholds in water from literature.

3.3.11 Sensory analysis

3.3.11.1 Comparison of fish sauce and the complete fish sauce model

Aroma profiling was done by descriptive sensory analysis as previously described (Zhou, Wintersteen, and Cadwallader, 2002). One gram of fish sauce was

dissolved in the matrix (10 mL of 0.1 M phosphate buffer, pH 5.6, 25% NaCl) and presented to panelists in a 125-mL Nalgene PTFE wash bottles (Fisher, Pittsburgh, PA) with siphon tubes removed from the caps. Bottles were covered with aluminum foil to prevent any visual bias and labeled with random 3-digit codes. The 11–member panel (5 males, 6 females, 20-48 years old) were asked to identify and define descriptive terms of fish sauce sample and to determine appropriate aroma references. Standard references for “cheesy”, “potatoey”, “brothy/meaty”, “fishy/briny”, “malty”, and “sulfur” were presented at room temperature. Intensity of each standard was rated on 15-cm universal scales, with intensity ratings of 0 and 15 corresponding to none and very intense, respectively (Table 3.6). Intensity ratings of the standards were used as references for rating the intensities of the fish sauce sample and its model. Compound purities and the concentration of stock solution are provided in Table 3.7. The complete model mixture consisting of the 21 fish sauce odorants was prepared in the matrix based on the concentrations given in Table 3.5. The panelists were asked to evaluate the intensities of each odor note which represented in the sample and model. Final aroma profiles of samples were reported on the basis of discussion and consensus ratings.

3.3.11.2 Omission experiment

Omission models were prepared in the matrix same as the complete model, except that each time a different group of compound was omitted. Sensory difference testing of the omission model to the control was performed using the R-index measure by ranking (Lorjaroenphon, Cadwallader, Kim, and Lee, 2008). Panelists were asked to rank the omission models on how different they were from the complete model, from most similar to least similar (n = 19; 5 males, 14 females).

Degree of difference of each model against complete model was calculated using John Brown computations (O' Mahony, 1992).

3.4 Results and discussion

3.4.1 Identification of odor-active compounds

Odor-active compounds from two brands of premium Thai fish sauce (A and B) were isolated from the matrix through DSE, followed by high-vacuum distillation using SAFE. To avoid interferences during sniffing with GC-O, the distillate was further fractionated into the AF containing acidic volatile compounds and the NBF containing neutral and basic volatile compounds. Twenty-nine compounds with $\log_3$FD factor of ≥ 1 were detected by AEDA, consisting of 11 acidic volatile compounds (Table 3.1) and 18 neutral/basic volatile compounds (Table 3.2). The AEDA aroma profiles of the 2 fish sauce samples were similar. There were several compounds in AF showing high $\log_3$FD values, including butanoic acid (cheesy), 3-methyl-butanoic acid (cheesy, sweaty), 4-hydroxy-2-ethyl-5-methyl-3(2H)-furanone (burnt sugar), and 2-phenylacetic acid (rosy, plastic). These are regarded as the most odor-active ones based on high $\log_3$FD factor (> 6). The rest were also important due to their high $\log_3$FD values. In the NBF, 3-methylbutanal (malty, dark chocolate), 3-(methylthio)propanal (cooked potato), phenylacetaldehyde (rosy, plastic), and o-aminoacetophenone (musky, grape, stale) were the most odor-active compounds ($\log_3$FD factor > 2).

It has been reported that 2-methylpropanal, 2-methylbutanal, 2-pentanone, 2-ethylpyridine, dimethyl trisulfide, 3-(methylthio)propanal, and 3-methylbutanoic acid showed high FD factors and are principal contributors to the distinct odor of Thai fish

sauce (Giri et al., 2010). In this study, the importance of these compounds, except 2-pentanone and 2-ethylpyridine, was confirmed. Furthermore, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone, and 3-hydroxy-4,5-dimethyl-2(5H) furanone were identified for the first time as odor-active compounds in fish sauce.

4-Hydroxy-2,5-dimethyl-3(2H)-furanone has been found in many foods, including pineapples (Rodin, Himel, Silverstein, Leeper, and Gortner, 1965), strawberries (Douillard and Guichard, 1990), beef broth (Tonsbeek, Plancken, and van den Weerdhof, 1968), roasted almonds (Takei and Yamanishi, 1974), and soy sauce (Steinhaus and Schieberle, 2007). It is one of degradation products of the Amadori compound via 2,3-enolization, elongation by the Strecker aldehydes, and reduction of the resulting acetylformoin-type as intermediates (Blank and Fay, 1996). Its presence in fish sauce might be desirable because of its caramel/burnt sugar–like, sweet and fruity.

4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone and 3-hydroxy-4,5-dimethyl-2(5H)-furanone were tentatively identified and likely to be the important compounds due to their low odor threshold values of 1.15 and 0.001 (μg/L), respectively (Rychlik, Schieberle, and Grosch, 1998). However, these two compounds in this study were present in trace amount and were not detected by GC-MS. Sasaki, Nunomura, and Matsudo (1991) reported that 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone is produced by yeast during fermentation via pentose-phosphate cycle. In addition, this compound was also formed through Maillard reaction systems based on pentose (Blank and Fay, 1996). 3-Hydroxy-4,5-dimethyl-2(5H)-furanone is well known as a spicy/curry note and found in many food products. This compound is

Table 3.1 Odor-active (Log_3 FD factor ≥ 1) acidic volatile compounds in fish sauce A and B.

Compound	Odor[a]	RI[b]		Log_3 FD factor[c]	
		Wax	Rtx-5	A	B
Acetic acid[d]	Sour	1433	nd[e]	6	6
Propanoic acid[d]	Cheesy/fecal	1514	nd	4	5
2-Methylpropanoic acid[d]	Cheesy/Swiss cheese	1550	804	4	6
Butanoic acid[d]	Cheesy	1611	829	8	9
3-Methylbutanoic acid[d]	Cheesy/sweaty	1654	893	7	9
4-Hydroxy-2,5-dimethyl-3(2H)-furanone[d]	Burnt sugar	2005	1078	6	6
4-Hydroxy-2-ethyl-5-methyl-3(2H)-furanone[f]	Burnt sugar	2056	1149	7	6
4-Hydroxy-5-ethyl-2-methyl-3(2H)-furanone[f]	Burnt sugar	2077	nd	2	nd
3-Hydroxy-4,5-dimethyl-2(5H)-furanone[f]	Curry/spicy	2173	1115	3	5
2-Phenylacetic acid[d]	Rosy/plastic	2515	1272	8	8
3-Phenylpropanoic acid[d]	Rosy	2590	1366	2	1

Note: [a]Odor quality as perceived during GCO. [b]Retention indices were calculated from GCO data; Wax=Rtx-Wax. [c]Flavor dilution (FD) factor determined on Rtx-Wax column. [d]Compounds were positively identified by GCO, comparing their RIs and odor properties with referenced RIs and odor properties, and GC-MS. [e]nd=not detected. [f]Compounds were tentatively identified by GCO, comparing their RIs and odor properties with referenced RIs and odor properties.

Table 3.2 Odor-active (Log_3 FD factor ≥ 1) neutral-basic volatile compounds in fish sauce A and B.

Compound	Odor[a]	RI[b]		Log_3 FD factor[c]	
		Wax	Rtx-5	A	B
2-Methylpropanal[d]	Malty/dark chocolate	<900	nd[e]	0	2
2-Methylbutanal[f]	Malty/dark chocolate	904	nd	2	1
3-Methylbutanal[f]	Malty/dark chocolate	916	nd	3	3
(Z)-4-Heptenal[d]	Crabby/stale/fishy	1236	902	1	1
1-Octen-3-one[d]	Mushroom	1301	978	1	0
2-Acetyl-1-pyrroline[d]	Popcorn	1320	928	2	1
(Z)-1,5-Octadien-3-one[d]	Metallic	1380	988	1	0
3-(Methylthio)propanal[f]	Cooked potato	1452	908	6	4
3,6-Dimethyl-2-ethylpyrazine[f]	Roasted potato	1484	1068	2	2
(E)-2-Nonenal[d]	Stale/ hay	1537	1162	0	1
Phenylacetaldehyde[f]	Rosy/plastic	1640	1048	4	4
3-(Methylthio) propanol[f]	Potato	1712	985	0	1
2-Acetyl-2-thiazoline[d]	Popcorn	1760	1118	nd	1
(E)-β-Damascenone[d]	Floral/apple sauce	1826	1384	1	0
2-Phenylethanol[f]	Wine-like/rosy	1905	1122	1	1
o-Aminoacetophenone[f]	Musky/grape/stale	2202	1312	4	3
Indole[f]	Fecal	2428	1295	1	nd
Skatole[f]	Fecal	2480	1401	1	0

Note: [a]Odor quality as perceived during GCO. [b]Retention indices were calculated from GCO data; Wax, Rtx-Wax. [c]Flavor dilution (FD) factor determined on Rtx-Wax column. [d]Compounds were tentatively identified by GCO, comparing their RIs and odor properties with referenced RIs and odor properties. [e]nd=not detected. [f]Compounds were positively identified by GCO, comparing their RIs and odor properties, and GC-MS.

reported for the first time as high odor-active compound in fish sauce on the basis of its odor activity in air (GC-O). Its presence in fish sauce might be desirable because of its seasoning-like aroma. It has been described as a degradation product of threonine (Sulser, Depizzol, and Büchi, 1967). In addition, Kobayashi (1989) reported the formation of 3-hydroxy-4,5-dimethyl-2(5H)-furanone via aldol condensation of acetaldehyde and R-ketobutyric acid followed by lactonization in sake and wine.

Based on SHDA analysis of highly volatile compounds, 11 compounds were identified and 2 compounds remained unknown (Table 3.3). The highest FD factors (>5) were found in methanethiol (rotten, sulfurous, cabbage), 2-methylpropanal (malty, dark chocolate), 2-methylbutanal (malty, dark chocolate), 3-methylbutanal (malty, dark chocolate), dimethyl trisulfide (rotten, sulfurous, cabbage), 3-(methylthio)propanal (cooked potato), and butanoic acid (cheesy).

Methanethiol, acetaldehyde, and dimethyl sulfide were not detected by AEDA. Because of their high volatility, these compounds might be coeluted with solvent peak, lost during the extraction and concentration procedures. Therefore, the aroma contribution of very volatile compounds can only be estimated by directly analyzing the headspace above fish sauce with decreased volumes. In addition, 2-methyl-(3-methyldithio)furan was also not detected by DEAE, but tentatively identified by SHDA, comparing its RI and odor properties with referenced RI and odor properties. This sulfur-containing furan contributed to thiamin/meaty notes and played very important role in the overall flavor of fish sauce due to its very low odor threshold in water of 0.004 μg/L (Rychlik et al., 1998). This compound has been considered as an odor-active compound from a beef-like flavor produced by extrusion of enzyme-hydrolyzed vegetable protein (E-HVP) (Baek, Kim, Ahn, Nam, and Cadwallader,

2001) and yeast extract (Mahadevan and Farmer, 2006). 2-Methyl-(3-methyldithio)furan can be formed via the reaction of 2-methyl-3-furanthiol with methanethiol or Maillard reaction of ribose and cysteine (Mottram and Whitfield, 1995). This is the first report demonstrating that 2-methyl-(3-methyldithio)furan is one of the potent odorants in headspace of Thai fish sauce. Alcohols and esters were also were found in fish sauce samples but they showed very low FD factor (data no shown). Thus, these compounds were not considered as odor-active compounds and not taken to further quantification in this study.

3.4.2 Quantitative analysis of odor-active compounds

The concentrations and odor-activity values (OAVs) of 21 odorants in fish sauce are presented in Table 3.5. Precise quantitative measurements of the odorants were performed by the use of stables isotopomers of the analytes as internal standards. The highest abundant compounds of fish sauce A and B were acetic acid followed by butanoic acid, propanoic acid and 3-methylbutanoic acid. These four acids are major constituent of premium Thai fish sauce. In addition, pentanoic acid, hexanoic acid, 2-methylheaxanoic acid, heptanoic acid, and octanoic acid have been found in fish sauce (Guth and Grosh, 1994; Pham et al., 2008) but they are not considered as odor-active compounds in this study. Other high abundant compounds of both samples were 3-methylbutanal, 3-(methylthio)propanal, 2-methylpropanoic acid, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, 2-phenylacetic acid, and 3-phenylpropanoic acid. On the other hand, trimethylamine, 3,6-dimethyl-2-ethylpyrazine, *o*-aminoacetophenone, and skatole, were present in the lowest concentrations (< 10 μg/kg). Despite of their low amounts, they might have a significant impact on fish sauce flavor because of their low odor threshold values.

Table 3.3 Potent odorants in headspace of fish sauce by SHDA-GC-O.

Compound	Odor[a]	RI[b]		FD factor[c]			
				A		B	
		Wax	Rtx-5	Wax	Rtx-5	Wax	Rtx-5
Methanethiol[d]	Rotten/sulfurous/cabbage	652	431	25	25	25	25
Acetaldehyde[d]	Pungent/sweet	698	<400	5	1	5	nd[e]
Dimethyl sulfide[d]	Corn	714	525	1	5	nd	nd
2-Methylpropanal[d]	Malty/dark chocolate	804	561	25	25	25	25
2-Methylbutanal[d]	Malty/dark chocolate	909	658	25	1	25	5
3-Methylbutanal[d]	Malty/dark chocolate		648		25		25
2,3-Butanedione[d]	Buttery	972	602	nd	1	1	1
Unknown	Fishy/rancid/stale	1160	815	1	1	1	1
Dimethyl trisulfide[d]	Rotten/sulfurous/cabbage	1371	970	25	5	5	5
3-(Methylthio)propanal[d]	Potato	1446	906	25	5	25	5
Butanoic acid[d]	Cheesy	1616	nd	5	nd	25	nd
2-Methyl-(3-methyldithio)furan[f]	Thiamin/meaty	1661	1176	1	1	5	1
Unknown	Rotten/sulfurous/cabbage	1727	1218	1	5	5	5

Note: [a]Odor quality as perceived during GCO. [b]Retention indices were calculated from GCO data; Wax, Rtx-Wax. [c]FD factors of 1, 5, and 25 corresponding to headspace volumes of 25, 5, and 1 mL, respectively. [d]Compounds were positively identified by GCO, comparing their RIs and odor properties with referenced RIs and odor properties, and GC-MS. [e]nd=not detected. [f]Compounds were tentatively identified by GCO, comparing their RIs and odor properties with referenced RIs and odor properties.

Althought AEDA is a typical technique used to screen odor-active compound, it does not provide the information on the contribution of a single compound to the overall aroma. Since compounds are extracted out from the sample matrix with the solvents and volatilized during GC-O, these odorants are detected based on odor activity in air and interactions with matrix is inevitably neglected. OAV is better indicative for the odor-active compound as long as the accurate quantitative data and the odor detection thresholds are accurate.

In this study, the odor thresholds of the selected aroma compounds in water were used to calculate their OAVs due to the major phase of fish sauce is aqueous. 3-Methylbutanal had the highest OAV in both samples despite its low abundance. This is due to its low odor detection threshold. In addition, methanethiol, 2-methylpropanal, dimethyl trisulfide, 3-(methylthio)propanal, and butanoic acid showed OAVs > 500 and are also suggested as odor-active compounds of premium Thai fish sauce.

Giri et al. (2010) reported that the highest relative proportion (%) of OAVs in premium Thai fish sauce was butanoic acid, followed by 2-methylbutanoic acid, dimethyl trisulfide, 3-(methylthio)propanal, acetic acid, and trimethylamine. In this study, trimethylamine showed low OAV because of its very low concentration found in these 2 premium fish sauce samples. Shimoda, Peralta, and Osajima (1996) reported that volatility of trimethylamine increased with pH suggesting that the amount of trimethylamine might be affected by pH of fish sauce.

Valine and leucine are precursors for 2-methylpropanal and 3-methylbutanal, respectively, via amino acid catabolism pathway (Smit, Engels, and Smit, 2009). In addition, aldehydes can be formed via either an enzymatic including transamination

Table 3.4 Selected ion (m/z) and response factors used in the stable isotope dilution assay.

Compound	m/z	Labeled compound	m/z	Response factor
Trimethylamine	58	[$^{2}H_{9}$]-Trimethylamine	66	0.57
Methanethiol	48	[$^{2}H_{3}$]-Methanethiol	51	0.96
Dimethyl sulfide	62	[$^{2}H_{6}$]-Dimethyl sulfide	68	4.27
2-Methylpropanal	72	[$^{2}H_{2}$]-2-Methylpropanal	74	2.13
2-Methylbutanal	86	[$^{2}H_{2}$]-3-Methylbutanal	88	0.96
3-Methylbutanal	86	[$^{2}H_{2}$]-3-Methylbutanal	88	1.45
Dimethyl trisulfide	126	[$^{2}H_{6}$]-Dimethyl trisulfide	132	1.14
Acetic acid	60	[$^{2}H_{3}$]-Acetic acid	63	0.99
3-(Methylthio)propanal	104	[$^{2}H_{3}$]-3-(Methylthio)propanal	107	1.70
3,6-Dimethyl-2-ethylpyrazine	135	[$^{2}H_{5}$]-3,6-Dimethyl-2-ethylpyrazine	141	0.72
Propanoic acid	74	[$^{2}H_{5}$]-Propanoic acid	79	1.28
2-Methylpropanoic acid	73	[$^{2}H_{2}$]-2-Methylpropanoic acid	75	0.35
Butanoic acid	88	[$^{2}H_{2}$]-Butanoic acid	90	1.20
Phenylacetaldehyde	120	[$^{13}C_{2}$]-Phenylacetaldehyde	122	2.03
3-Methylbutanoic acid	87	[$^{2}H_{2}$]-3-Methylbutanoic acid	89	0.50
3-(Methylthio) propanol	106	[$^{2}H_{3}$]-3-(Methylthio) propanol	109	1.07
4-Hydroxy-2,5-dimethyl-3(2H)-furanone	128	[$^{13}C_{2}$]-4-Hydroxy-2,5-dimethyl-3(2H)-furanone	130	0.95
o-Aminoacetophenone	135	[$^{2}H_{3}$]-o-Aminoacetophenone	138	1.65
Skatole	130	[$^{2}H_{3}$]-Skatole	133	0.37
2-Phenylacetic acid	136	[$^{13}C_{2}$]-2-Phenylacetic acid	138	0.76
3-Phenylpropanoic acid	150	[$^{2}H_{2}$]-3-Phenylpropanoic acid	152	0.96

Note: Mass spectra of unlabeled and labeled compounds used for determining response factor are presented in Appendix.

Table 3.5 Concentrations and odor-activity values (OAVs) of compounds in fish sauce.

No.	Compound	Concentration[a] (µg/kg)		Odor threshold[b]	OAV[c]	
		A	B	(µg/kg)	A	B
1	Trimethylamine	0.84 (± 3.50)	1.28 (± 2.17)	0.47[d]	1.80	2.72
2	Methanethiol	58.19 (± 1.81)	57.05 (± 1.30)	0.02[e]	2910	2853
3	Dimethyl sulfide	40.15 (± 4.77)	10.14 (± 2.17)	0.3[f]	134	34
4	2-Methylpropanal	620 (± 2.44)	900 (± 2.52)	1[g]	620	900
5	2-Methylbutanal	347 (± 11.54)	852 (± 13.51)	3[h]	116	284
6	3-Methylbutanal	1291 (± 10.32)	1661 (± 13.98)	0.2[h]	6455	8305
7	Dimethyl trisulfide	17.51 (± 9.96)	25.64 (± 1.07)	0.01[h]	1751	2564
8	Acetic acid	4963025 (± 2.72)	4506166 (± 1.85)	22000[h]	226	205
9	3-(Methylthio)propanal	351(± 8.30)	1092 (± 14.77)	0.2[h]	1755	5460
10	3,6-Dimethyl-2-ethylpyrazine	1.26 (± 6.54)	5.48 (± 8.57)	8.6[h]	<1	<1
11	Propanoic acid	99869 (± 1.22)	561102 (± 3.22)	2000[h]	50	281
12	2-Methylpropanoic acid	5660 (± 1.61)	22352 (± 1.21)	50[h]	113	447
13	Butanoic acid	129336 (± 1.90)	307392 (± 1.07)	240[h]	539	1281
14	Phenylacetaldehyde	172 (± 5.01)	180 (± 3.17)	4[h]	43	45
15	3-Methylbutanoic acid	19572 (± 0.30)	94136 (± 2.64)	250[h]	78	377
16	3-(Methylthio)propanol	682 (± 5.62)	591 (± 2.92)	250[i]	2.73	2.36
17	4-Hydroxy-2,5-dimethyl-3(2H)-furanone	2456 (± 5.31)	2351 (± 3.90)	31[j]	79	76
18	o-Aminoacetophenone	4.01 (± 11.92)	2.87 (± 12.83)	0.2[h]	20	14
19	Skatole	0.47 (± 16.47)	0.44 (± 17.74)	3[k]	<1	<1
20	2-Phenylacetic acid	2675 (± 1.43)	20098 (± 1.62)	1000[l]	2.68	20
21	3-Phenylpropanoic acid	3344 (± 1.85)	1865 (± 2.71)	-[m]	-	-

Note: [a]Average concentration (±percent relative standard deviation) of data from triplicate samples. [b]Orthonasal odor threshold in water. [c]Odor-activity value=concentration divided by odor detection threshold. [d]Amoore (1977). [e]Guadagni, Buterry, and Okano (1963). [f]Buttery (1993). [g]Guadagni, Buttery, and Turnbaugh (1972). [h]Buttery and Ling (1998). [i]Fritsch and Schieberle (2005). [j]Buttery, Takeoka, Krammer, and Ling (1995). [k]Moss, Hawa, and Walker (1993). [l]Carunchia Whetstine, Cadwallader, and Drake (2005). [m]Not available.

and decarboxylation pathways or a nonenzymatic reaction including Strecker degradation (Ayad, Verheul, Engels, Wouters, and Smit, 2001; Smit, G., Smit, B. A., and Engels, 2005). Methionine is a precursor for 3-(methylthio)propanal, methanethiol, dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide (Bonnarme, Psoni, and Spinnler, 2000; Yvon and Rijnen, 2001). Butanoic acid is likely to form by atmospheric oxidation of fish lipids (Steinhaus and Schieberle, 2007), and it also can be derived from amino acids via bacterial activity (Beddows, Ardeshir, and Daud, 1980).

The remaining odorants in Table 3.5 with OAVs > 100 may also be important aroma contributors, including dimethyl sulfide, 2-methylbutanal, acetic acid, propanoic acid, 2-methylpropanoic acid, and 3-methylbutanoic acid. Two compounds, namely 3,6-dimethyl-2-ethylpyradzine, and skatole, should not contribute much to the aroma, because of low OVAs (<1).

3.4.3 Sensory attributes of fish sauce and its model

Descriptive sensory analysis was used to compare each of the complete model

(fish sauce A and B) against the actual fish sauce sample. Cheesy, malty, potatoey, brothy/meaty, fishy/briny, and sulfur were main attributes in both samples of fish sauce. Intensity of each standard reference was shown in Table 3.6.

Table 3.6 Attributes, references, and rating for the sensory descriptive analysis of fish sauce.

Attribute	Reference[a]	Rating[b]
Cheesy	0.1 g of parmesan cheese	9
Potatoey	10 g of mashed potato	8
Brothy/meaty	10 mL of a 1% yeast extract aqueous solution	5
Fishy/briny	10 mL of a 100 ppb trimethylamine aqueous solution	8
Malty	0.1 g of dark chocolate	8
Sulfur	2 g of boiled brussels sprouts	12

Note: [a]Samples were presented in125-mL PTFE bottles. [b]Aroma intensity values were rated on 15-cm universal scales, with intensity ratings of 0 and 15 corresponding to none and very, respectively.

According to odor properties obtained from GC-O analyses**,** low molecular weight volatile fatty acids should contribute to cheesy note. 2-Methylpropanal, 2-methylbutanal, and 3-methylbutanal contribute to malty note. Trimethylamine and 2-methyl-(3-methyldithio)furan contribute to fishy/briny and brothy/meaty note, respectively. Methanethiol, dimethyl sulfide, and dimethyl trisulfide might be a source of sulfur note, whereas 3-(methylthio)propanal and 3-(methylthio)propanol should be responsible for potatoey note. Dougan and Haward (1975) reported that three

distinctive notes contributed to the odor of fish sauce were ammoniacal, cheesy, and meaty notes. In addition, burnt, fishy, sweaty, fecal, rancid, and nutty notes were found as another distinctive notes for Thai fish sauce (Fukami et al., 2002; Giri et al., 2010).

Low molecular weight volatile fatty acids have been proposed to be responsible for the cheesy note of fish sauce, for example, acetic acid, butanoic acid, 2-methlylpropanoic acid, 2/3-methylbutanoic, pentanoic acid, heptanoic acid (Fukami et al., 2002; Giri et al., 2010; Pham et al., 2008). Fukami et al. (2002) found that 4 odor-active compounds contributing to the distinctive odor of Thai fish sauce were 2-methylpropanal, 2-methylbutanal, 2-ethylpyridine, and dimethyl trisulfide. All of these four compounds contributed to sweaty note. 2-Ethylpyridine and dimethyl trisulfide contributed to fishy note. 2-Methylpropanal together with 2-methylbutanal and 2-ethylpyridine has been reported to contribute to meaty odor, whereas 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal have been described as nutty odor in fish sauce (Giri et al., 2010).

Although all attributes in actual fish sauce were rated lower/higher than the models, the results revealed that all attributes of both samples were similar to their models (Figure 3.1, $P > 0.05$). These results demonstrate that selection of all odor-active compounds based on AEDA/SHDA and the quantitative experiments were highly accurate. Therefore, these complete models are good representative models of fish sauce for further omission experiments.

3.4.4 Omission studies

Omission experiments were conducted by omitting group of odorants having similar properties and performed by comparing the complete model against the

Table 3.7 Formulations of stock solutions used to prepare aroma models.

Compound	% Purity[a]	Amount of compound (mg) per 10 mL of stock solution	
		A Model	B Model
Group 1, Acids[b]			
Acetic acid	99.9	-[c]	-
Propanoic acid	99.4	-[d]	-
2-Methylpropanoic acid	99.5	12.9	44.8
Butanoic acid	99.8	263	617
3-Methylbutanoic acid	99.7	40.1	190
2-Phenylacetic acid	99.3	5.40	41.2
3-Phenylpropanoic acid	99.9	7.20	3.60
Group 2, Aldehydes[e]			
2-Methylpropanal	98.0	32.7	46.5
2-Methylbutanal	95.8	18.8	45.3
3-Methylbutanal	96.7	67.8	86.0
Phenylacetaldehyde	99.3	8.86	9.21
Group 3, Sulfur compounds[e]			
Methanethiol	99.5	0.0125	0.0125
Dimethyl sulfide	99.6	2.07	0.570
Dimethyl trisulfide	94.6	0.920	1.36
3-(Methylthio)propanal	97.4	19.4	56.9
3-(Methylthio)propanol	99.6	35.5	32.4
Group 4, Low OAV compounds[e]			
Trimethylamine	99.9	0.0430	0.0670
3,6-Dimethyl-2-ethylpyrazine	99.4	0.0660	0.277
4-Hydroxy-2,5-dimethyl-3(2H)-furanone	94.4	130	125
o-Aminoacetophenone	99.8	0.201	0.150
Skatole	99.6	0.0237	0.0241

Note: [a]Purity determined by GC analysis. [b]Stock solution was prepared in water. [c]Fisher Scientific (Pittsburgh, PA). [d]Sigma-Aldrich (St. Louis, MO). [e]Stock solution was prepared in methanol.

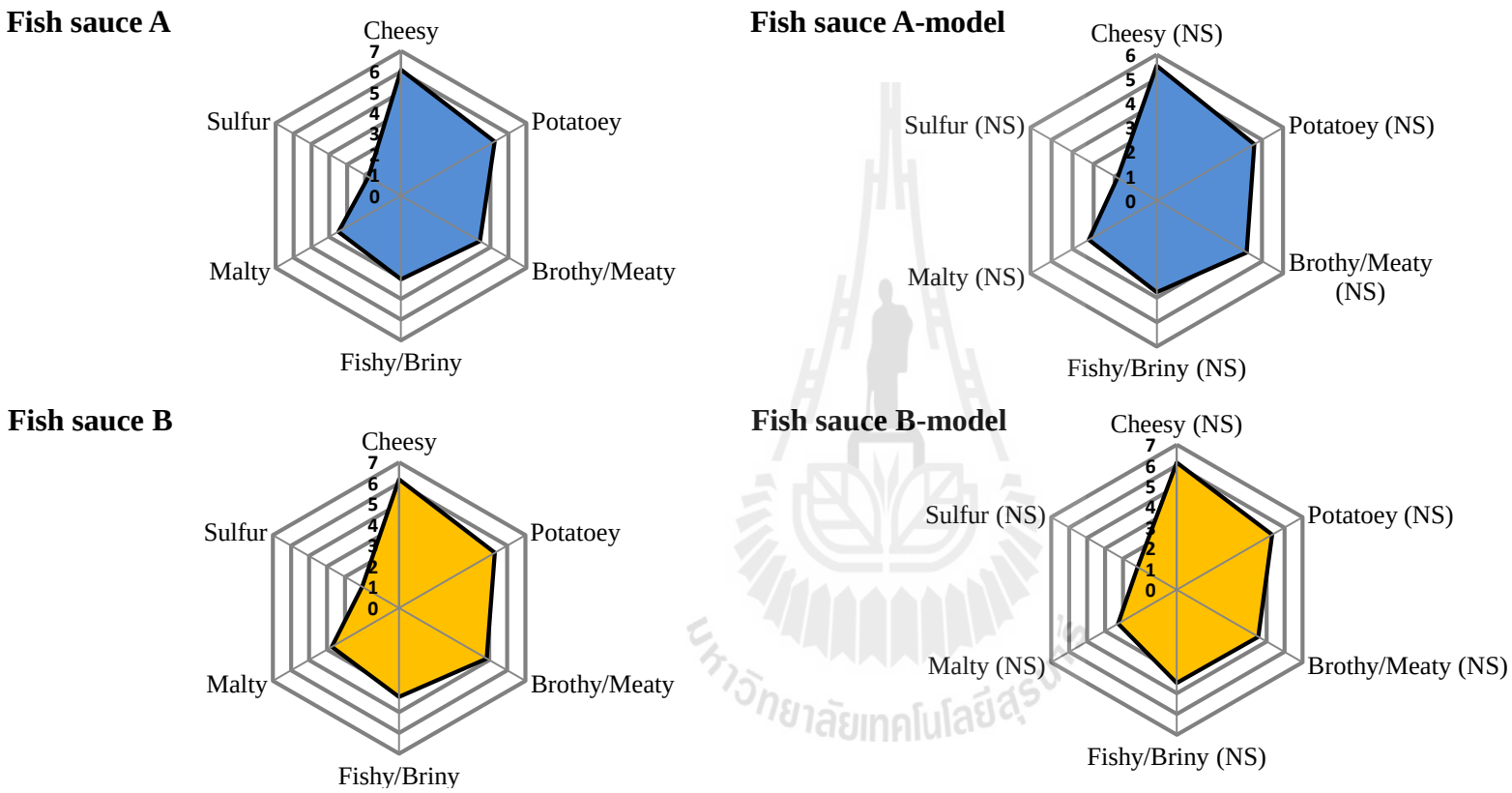

Figure 3.1 Sensory descriptive aroma profile comparison of fish sauce and the complete fish sauce model. NS= not significantly different between fish sauce and the complete fish sauce model (P > 0.05).

omission models. The impact of selected groups, including acids, aldehydes, sulfurous and low OAV compounds, on fish sauce flavor was performed using R-index. Results of omission tests showed that the omission of acids, aldehydes, and sulfurous compounds were significantly different ($\alpha = 0.05$) from the complete model (Table 3.8).

Table 3.8 R-index ranking test comparison of complete aroma models against omission model mixtures.

Comparison of complete model against	R-index (significance) [a]	
	Fish sauce A	Fish sauce B
Model with acids omitted	73.68 *	78.95 *
Model with aldehydes omitted	73.68 *	73.68 *
Model with sulfur compounds omitted	89.47 *	84.21 *
Model with low OAV compounds omitted	63.16	63.16

Note: [a]Degree of difference of each model against complete model (n = 19). *Significantly different from complete model at $\alpha = 0.05$ (critical value, expressed in percentage; R-index-50% for two-tailed test, $\alpha = 0.05$ and n = 19 is 20.97).

Omission of sulfur-containing compounds showed the highest degree of difference from the complete model (>80%). This could be because these compounds, including methanethiol, dimethyl trisulfide, and 3-(methylthio)propanal showed high OAVs. 2-Methylpropanal, 3-methylbutanal, and butanoic acid showing high OAVs play a significant role for overall aroma of the model omitted aldehydes and acids.

However, Lorjaroenphon et al. (2008) indicated that compounds with high OAVs do not necessarily have a profound effect on the overall aroma system. As expected, the omission of low OAV compounds had no effect on the model. This finding confirms that low OAV compounds, including trimethylamine, 3,6-dimethyl-2-ethylpyrazine, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, o-aminoacetophenone, and skatole, had no influence on the overall aroma of fish sauce.

3.5 Conclusions

The results of AEDA and SHDA from GC-O indicated that methanethiol, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, dimethyl trisulfide, 3-(methylthio)propanal, butanoic acid, 3-methylbutanoic acid, 4-hydroxy-2-ethyl-5-methyl-3(2H)-furanone, and 2-phenylacetic acid are odor-active compounds in Thai premium fish sauce on the basis of high flavor dilution factor. Quantitative data and calculated OAVs confirmed that these compounds were odor-active compounds based on high OAVs (>500), with the exception of 2-methylbutanal, 3-methylbutanoic acid, 4-hydroxy-2-ethyl-5-methyl-3(2H)-furanone, and 2-phenylacetic acid due to their low concentrations and high odor thresholds. Quantitative data were further confirmed by descriptive analysis of the model mixtures. Omission experiments demonstrated that the sulfur-containing compounds showed the highest influence on the overall aroma of fish sauce, followed by acid and aldehyde compounds.

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CHAPTER IV

CHARACTERIZATION OF PROTEIN HYDROLYSIS AND ODOR-ACTIVE COMPOUNDS OF FISH SAUCE INOCULATED *VIRGIBACILLUS* SP. SK37 UNDER REDUCED SALT CONTENT

4.1 Abstract

The effect of *Virgibacillus* sp. SK37 together with the reduction of salt content on fish sauce quality, particularly odor-active compounds, was elucidated. *Virgibacillus* sp. SK37 was inoculated with approximate cell count of 5 log CFU/mL in samples with varied amounts of solar salt of 10, 15, and 20% of total weight. Eighteen selected odorants were quantified by stable isotope dilution assays (SIDA), and their odor activity values (OAVs) were calculated. Samples prepared using 10% salt underwent spoilage after 7 days of fermentation. The viable count of *Virgibacillus* sp. SK37 was observed throughout 3-months in the samples containing 15 and 20% salt, but had no influence on accelerating protein hydrolysis of finished product. *Virgibacillus* sp. SK37 under reduced salt addition of 15-20% appeared to increase the content of 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, acetic acid, and 2-methylpropanoic acid. However, only aldehyde compounds were found to have an effect on the overall aroma of fish sauce based on high OAV values, suggesting that samples inoculated with *Virgibacillus* sp. SK37 under reduced salt content of 15-20**%**

contributed to stronger malty and dark chocolate note.

Keywords: *Virgibacillus* sp. SK37, fish sauce, salt reduction, odor-active compounds, odor activity value

4.2 Introduction

Thai fish sauce or *nam pla* is one of the important seasonings consumed widely in Southeast Asia and has been increasingly recognized worldwide. Generally, Thai fish sauce is produced by mixing the cleaned anchovy (*Stolephorus* spp.) with solar salt at a ratio of 2:1 or 3:1, depending on the area of production (Lopetcharat, Choi, Park, and Daeschel, 2001). After 12-18 months of fermentation, liquid is drained off and ripened for another 2-12 weeks before filling (Saisithi, Kasemsarn, Liston, and Dollar, 1966). Due to high salt concentration, proteolysis takes place slowly, leading to extremely long fermentation time of 12-18 months. Several moderately halophilic bacteria have been proposed to be potential starter cultures that could shorten fermentation time and improve aroma characteristics of fish sauce. Fukami, Funatsu, Kawasaki, and Watabe (2004) reported that fish sauce inoculated with *Staphylococcus xylosus* contained lower amounts of compounds contributing to undesirable fecal note, which were dimethyl disulfide, 2-ethylpyridine, dimethyl trisulfide, and butanoic acid than the control (without inoculation). Sensory evaluation showed that fishy, sweaty, fecal, and rancid notes were significantly reduced by this stain. *Tetragenococcus halophilus* has also been shown to reduce dimethyl disulfide as well as improves amino acid content, particularly glutamic acid (Udomsil, Rodtong, Choi, Hua, and Yongsawatdigul, 2011).

Recently, a moderately halophilic bacterium, namely *Virgibacillus* sp. SK37 (GenBank/NCBI no. DQ910840) isolated from 1-month-old Thai fish sauce mashes has been proven to reduce fermentation time by 50% (Yongsawatdigul, Rodtong, and Raksakulthai, 2007). The strain has been shown to produce Na^+-activated and Na^+-stable extracellular proteinases as well as possess cell-associated proteinases (Sinsuwan, Rodtong, and Yongsawatdigul, 2007; 2012), rendering an increase in protein hydrolysis during high salt fermentation. However, bacterial counts of this stain drastically decreased at fermentation containing 25% solar salt. Proteinase production of *Virgibacillus* sp. SK33 isolated from fish sauce has been reported to be optimal at 5% NaCl (Sinsuwan, Rodtong, and Yongsawatdigul, 2008). High salt at fermentation (25-28%) would suppress growth and production of *Virgibacillus* proteinases.

The level of salt in the fermentation has a great impact on microbial population and their metabolites as well as the extent of proteolysis. Most studies of fish sauce starter cultures have been carried out under Na^+-saturated condition at a ratio of fish and salt of 3:1. The growth of moderately halophilic starter cultures is typically limited as these organisms have optimal growth at 3-15% NaCl (Ventosa, Nieto, and Oren, 1998). The reduced salt content would extend survival of the inoculated cultures. In addition, the reduced salt content would also increase activities of fish endogenous and microbial proteinases, rendering an increase in protein hydrolysis. Formation of volatile compounds which are derived from peptides and/or amino acid precursors or directly from microorganisms is expected to vary with salt content. However, the effect of *Virgibacillus* starter culture at varied salt contents on volatile compounds and fish sauce quality has not been systematically elucidated.

Understanding such a relationship would lead to starter culture technological development of fish sauce fermentation.

Extraction and quantification of volatile compounds in fish sauce is typically carried out by purge and trap or solid phase microextraction (SPME) using regular internal standard (Fukami et al., 2002; 2004; Giri, Osaka, Okamoto, and Ohshima, 2010; Peralta, Shimoda, and Osajima, 1996; Pham, Schilling, Yoon, Kamadia, and Marshall, 2008; Udomsil et al., 2011; Wichaphon, Thongthai, Assavanig, and Lertsiri, 2012). According to different physico-chemical properties of various compounds, quantitative determination of odor-active compounds in fish sauce requires selective and sensitive analytical methods. It has been reported that the use of isotopically labeled analogue as internal standard for quantification provides high accurate data due to physico-chemical properties of both the internal standard and analyte are very similar (Schieberle, and Grosch, 1987). The different sampling techniques offer a number of individual advantages but also suffer specific limitations (Sides, Robards, and Helliwell, 2000). Combination the precision of stable isotope dilution assay (SIDA) with appropriate sampling technique of each compound extraction should be taken into account. The objectives of this study were to investigate the effect of *Virgibacillus* sp. SK37 together with the reduced salt content on fish sauce quality, particularly odor-active compounds.

4.3 Materials and methods

4.3.1 Starter culture preparation

Starter culture was prepared by inoculating a loopfull of *Virgibacillus* sp. SK37 into a 100 mL of modified halophilic medium or Y-medium (Sinsuwan et al., 2008)

(1% yeast extract, 0.3% trisodium citrate, 0.2% KCl, 2.5% $MgSO_4.7H_2O$) containing 15% NaCl, pH 7 at 35°C. *Virgibacillus* sp. SK37 was cultured for 3 days with a shaking speed of 100 rpm (New Brunswick Scientific Co. Inc., Edison, NJ) to attain an approximate cell count of 7 log CFU/mL. The cultured broth was centrifuged and cell pellets were collected and washed with sterile 15% NaCl solution three times and resuspended in 100 mL of 15% NaCl solution before adding to the fermentation.

4.3.2 Fish sauce fermentation

Indian anchovy (*Stolephorus* spp.) were caught off the Gulf of Thailand and kept in ice during transported to Suranaree university laboratory. Solar salt was collected from a fish sauce factory. One kilogram of anchovy was mixed with varied amounts of solar salt of 10, 15, and 20% of total weight. Cell suspensions of *Virgibacillus* sp. SK37 (approximately 6 Log CFU/mL) were added at 10% (v/w). The mixtures were packed in a glass jar (9 cm diameter x 17 cm height). The controls without starter culture at each salt content were also prepared by adding sterile 15% NaCl at 10% (v/w). The sample representing the conventional process was also prepared by mixing anchovies with solar salt of 25%. All samples were incubated in a 35°C incubator for 90 days. Changes of microbial population, oligopeptide and α-amino content were monitored at 0, 7, 14, 30, 45, 60, 75, and 90 days of fermentation. Amino acid profiles, sodium (Na) content, volatile compounds, and other physico-chemical properties, namely total nitrogen, ammonical nitrogen content, degree of browning, and pH, were determined in the finished products after 3-month fermentation**.**

4.3.3 Microbiological analyses

Fish sauce mashes (10 g) were taken aseptically from the fermentation jar at

each time interval. Total plate count of all samples were performed on plate count agar (PCA) (without NaCl) incubated at 35°C for 2-3 days under aerobic condition. Halophilic bacteria were enumerated using the spread plate technique on the modified JCM 168 agar medium containing 15% NaCl and incubated at 35°C for 4-5 days under aerobic condition.

4.3.4 Chemical analyses

4.3.4.1 Oligopeptide content

Fish sauce mashes (3 g) were added to 27 mL of 5% (w/v) cold trichloroacetic acid (TCA) solution, then the mixture was homogenized using an IKA homogenizer (IKA Works Asia, Bhd, Malaysia) and centrifuged at 10,000×*g* for 15 min at 4°C. The supernatant was analyzed for oligopeptide content using Lowry's assay method (Lowry, Rosebrough, Farr, and Randall, 1951) with tyrosine as a standard. Oligopeptide content was expressed as μmol tyrosine/g of fish sauce mash.

4.3.4.2 α-Amino content

The α-amino content was performed using trinitrobenzenesulfonic acid (TNBS) with leucine as a standard (Field, 1971). Fifty μL of the filtered fish sauce was mixed with 0.5 mL of 0.2125 M phosphate buffer, pH 8.2 and 0.5 mL of 0.05% TNBS reagent. The mixture was incubated at 50°C for 1 h in a water bath. After incubation, the reaction was stopped by the addition of 1 mL of 0.1 N HCl and left at room temperature for 30 min. Absorbance was measured at 420 nm using a spectrophotometer. α-Amino content was expressed as mM of leucine.

4.3.4.3 Amino acid profiles

Total amino acid contents of samples fermented for 3 months were determined following AOAC method (AOAC, 2000). Samples were hydrolyzed with

6 N HCl at 110°C for 24 h using autoclave. Hydrolyzed samples were dried by rotary evaporation under vacuum. The residue was then dissolved in sodium citrate buffer (pH 2.2). Samples for tryptophan determination were hydrolyzed under vacuum with 4.25 M NaOH at 110°C for 20 h and neutralized by HCl to pH 4.25 before analysis. Samples for cystine and methionine determination were oxidized to cysteic acid and methionine sulfone, respectively, with performic acid for 16 h in an ice bath. Consequently, sodium metabisulfite was added to decompose the performic acid before hydrolysis with HCl. Free amino acids were determined by directly diluting all samples with sodium citrate buffer. All samples were derivatized with propyl chloroformate. Quantitative analysis of amino acids was achieved by gas chromatography–mass spectrometry (GC-MS) system equipped with an Agilent 6890NGC/5973 mass selective detector (MSD; Agilent Technologies, Inc., Darmstadt, Germany). Seperations were performed on ZB-AAA column (10 m x 0.25 mm i.d.; 0.25 μm film, Phenomenex, Torrance, USA). The amino acid content was expressed as mg per 100 g of fish sauce.

4.3.4.4 Sodium (Na) content

All samples (50 mL) were digested with 10 mL of concentrated HNO_3 for about 3 h. After cooling, the digested sample was adjusted to 100 mL with deionized water. Na content was determined by air-acetylene flame atomization technique using atomic absorption spectroscopy (AAS) (Model AAnalyst 100, Perkin Elmer Corp., Norwalk, CT).

4.3.4.5 Biogenic amines content

One mL of sample was used to determine biogenic amines using high performance liquid chromatography (HPLC). The sample was added with 100 μL of 1

μg/mL diaminoheptane as an internal standard, 200 μL of 0.2 N NaOH, and 300 μL of saturated sodium bicarbonate. Two milliliters of 10 mg/mL dansyl chloride was added. The mixture solution was incubated at 40°C for 45 min. Subsequently, the sample was added with 300 μL of 30% ammonia solution to eliminate dansyl chloride residue. The mixture solution was adjusted to 5 mL using acetonitrile and centrifuged at 2,500×g for 5 min. Supernatant was collected and filtered through a 0.45-μm membrane filter (Agilent Technologies Inc., Palo Alto, CA., U.S.A.). Dansyl-derivatized biogenic amines were separated using a mobile phase consisting of the mixture of acetonitrile and 0.02 M acetic acid (1:9) as a solvent A. The mixture of solvent B was prepared from 0.02 M acetic acid, acetonitrile and methanol (1:4.5:4.5) at a flow rate of 1 mL/min. A Hypersil BDS column C18 (3 μm, 100 × 4 mm, Agilent Technologies Inc., Palo Alto, CA., U.S.A.) equipped with HPLC (HP 1100, Agilent Technologies Inc., Palo Alto, CA., U.S.A.) was used to analyze. Fifty percent of solvent B was run isocratically for 5 min. Subsequently, the gradient elution was started and ended at 90% solvent B in 25 min. The column was equilibrated with 50% solvent B for 10 min before the next injection. Diode array detector was set at 254 nm and 550 nm as a reference wavelength (Yongsawatdigul, Choi, and Udomporn, 2004).

4.3.4.6 Other chemical parameters

Fish sauce samples fermented for 3 months were analyzed for total nitrogen and ammonical nitrogen (AOAC, 1995). Degree of browning was monitored by diluting sample with distilled water at a ratio of 1 to 4 and measuring absorbance at 440 nm.

4.3.5 Odor-active compound quantification

Odor-active compound which considered as odor-active compounds in

commercial Thai fish sauce were selected for quantification using SIDA as described in section 3.3.10. Mass spectra of unlabeled and labeled compounds used for determining response factor are presented in Appendix.

4.3.6 Sensory evaluation

The panel consisted of 9 panalists who work at Rayong province, Thailand. The panelists were asked to give acceptance scores for 4 attributes: color, odor, flavor and overall acceptance, using the 7-point hedonic scale. Five fish sauce samples were presented to each panelist in a random ordor. Samples were not added sugar or any other additives. Ten mL of samples contained in a 15-mL glass cup with approximately 2-cm headspace was served to each panelist. Before sensory evaluation, the sample cups were covered with lids and left at room temperature (approximately 28°C) for 30 min. The panelists compared odor by opening the lid of the cup and sniffing. Flavor preference were assesses by tasting approximately 0.5 ml of fish sauce samples using a plastic spoon. The panelists were asked to use drinking water and plain cracker for rinsing their mouth before tasting the next sample.

4.3.7 Statistical analyses

The fermentation experiment was prepared in duplicate. All chemical analysis was carried in duplicate and mean values were presented. Analysis of variance (ANOVA) was carried out using SPSS program (SPSS version 14, Windows version). Differences among mean values were established using Duncan Multiple Range Test (DMRT) at $P < 0.05$.

4.4 Results and discussion

4.4.1 Microbiological changes

The initial bacterial counts on PCA of all samples were approximately 5 log CFU/mL, whereas inoculated samples showed higher count than 5 log CFU/mL on JCM 168 agar (Figure 4.1). This result demonstrated that *Virgibacillus* sp. SK37 grew in JCM 168 agar better than in PCA. Samples prepared using 10% salt underwent spoilage after 7 days with total viable counts of 6-7 log CFU/mL on both PCA and JCM 168 agar. The use of *Virgibacillus* sp. SK37 as a starter culture at this level of salt could not prevent microbial spoilage. Thus, these samples were not further investigated.

The viable counts on JCM agar of samples inoculated starter cultures were 4.4-4.8 log CFU/mL at day 7 and remained approximately 5.0 log CFU/mL throughout the course of fermentation. While the controls without starter cultures showed a drastic decrease of microbial population in the first 15 days of fermentation and relatively low bacterial counts of 2.0-2.5 CFU/mL during 3-months period. These results demonstrated that *Virgibacillus* sp. SK37 grew equally well in the fermentation containing 15 and 20% salt. Higher survival of *Virgibacillus* sp. SK37 was observed in this study as compared to those previously reported in the fermentation containing 25% NaCl, which was about 3 log CFU/mL at 3 months (Yongsawatdigul et al., 2007). This finding suggests that reducing salt content to 15-20% could extend viable counts of the inoculated culture.

4.4.2 Chemical changes

4.4.2.1 Oligopeptide content

Oligopeptide content of all fish sauce samples increased during

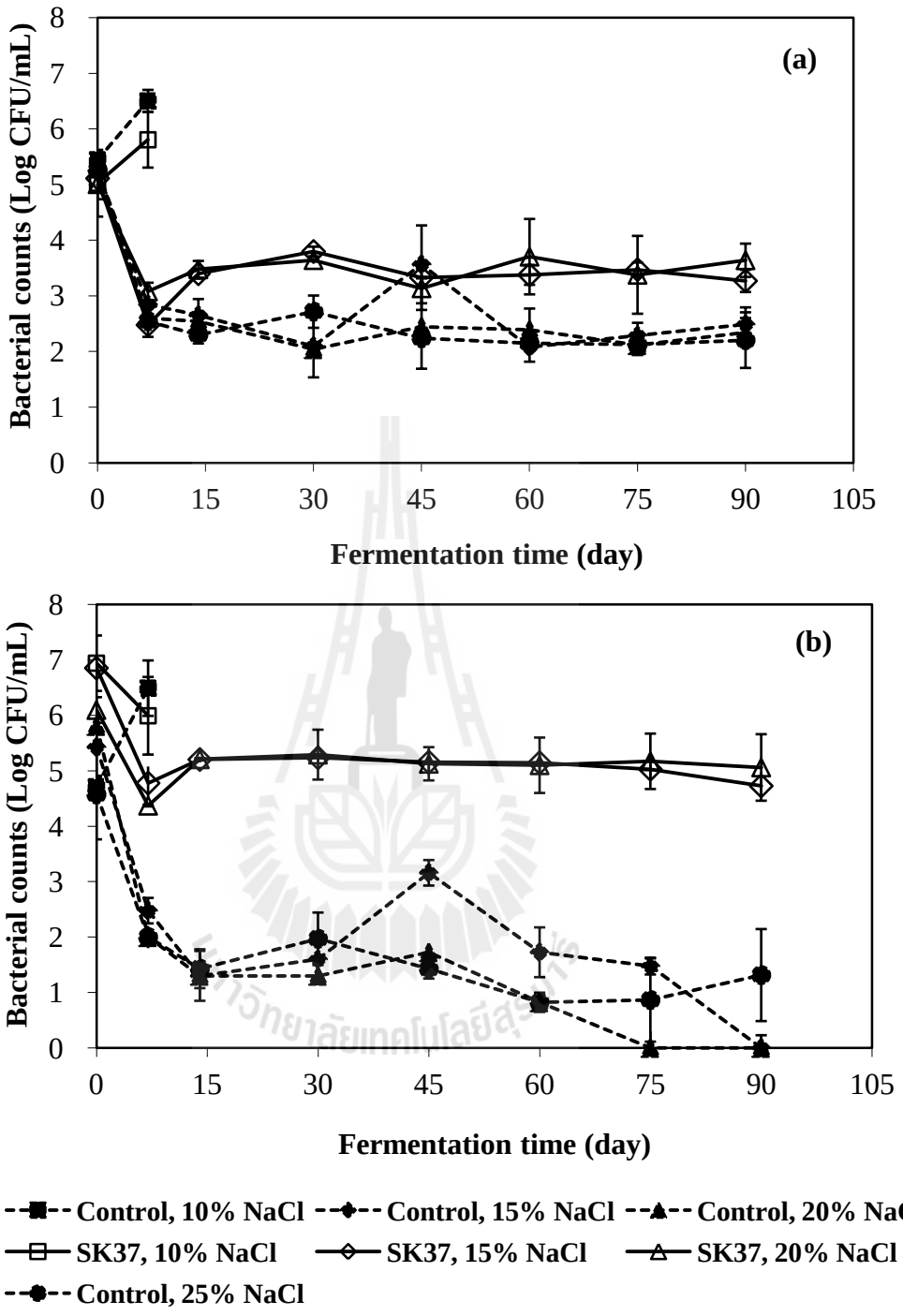

Figure 4.1 Changes in bacterial counts of fish sauce samples inoculated with *Virgibacillus* sp. SK37 and incubated at 35°C for 3 months on PCA without NaCl (a) and JCM 168 agar medium containing 15% NaCl (b).

fermentation (Figure 4.2). Oligopeptide content of samples fermented at 10% salt rapidly increased to the highest level of 21-22 μmol/g sample at day 7. Growth of spoilage microorganisms contributed to extensive proteolysis. In addition, lowering salt content is likely to increase the activity of fish/microflora proteinases. Samples prepared at 15% salt addition with starter culture showed the highest oligopeptide content up to 45 days of fermentation ($P < 0.05$). At day 60th, oligopeptide content of the inoculated samples was comparable to the control ($P > 0.05$). These results demonstrated that addition of *Virgibacillus* sp. SK37 increased protein hydrolysis at the early stage of fermentation.

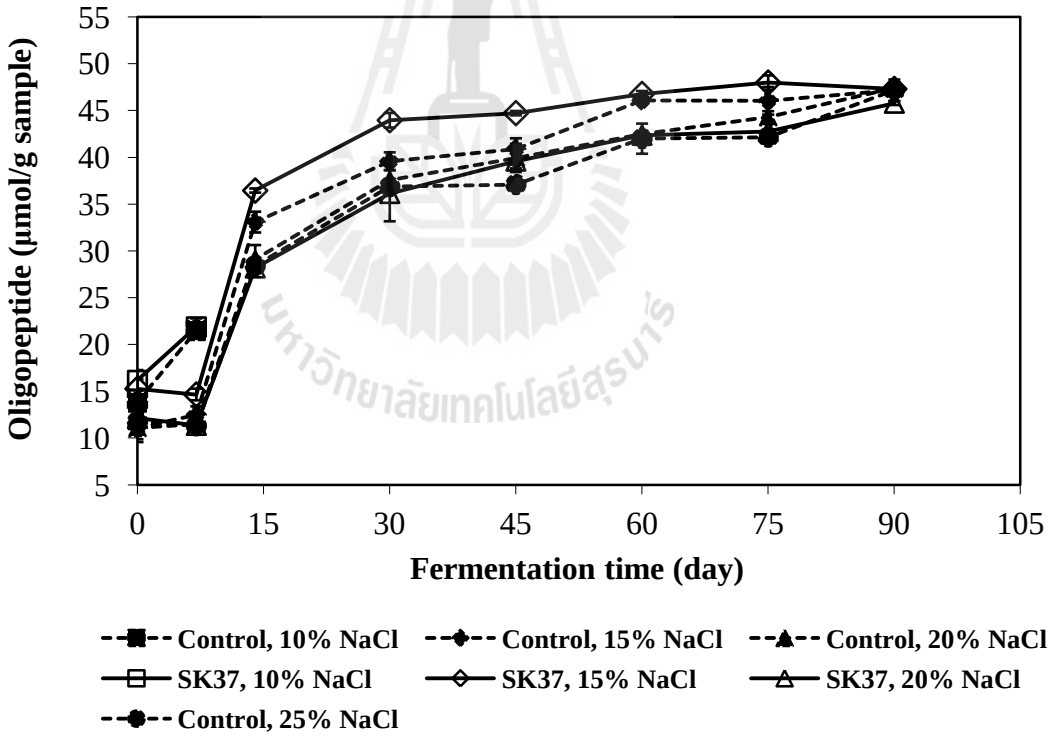

Figure 4.2 Changes in oligopeptides of fish sauce samples inoculated with *Virgibacillus* sp. SK37 and incubated at 35°C for 3 months.

This could imply that proteolytic activity of *Virgibacillus* sp. SK37 was rather limited at the later stage of fermentation. Proteinase is a primary metabolite which is synthesized parallel to microbial growth (Yang and Lee, 2001). As fermentation progressed, counts of *Virgibacillus* sp. SK37 did not continually increased, but rather stable, leading to limited proteinase secretion. Oligopeptide contents of fish sauce inoculated with *Virgibacillus* sp. SK37 at addition of 20% salt were comparable to its respective controls during the course of fermentation ($P > 0.05$). This indicated that addition of starter culture at 20% salt had no benefit on accelerating protein hydrolysis. Salt stress has been reported to decrease expression of the *aprE* gene encoding proteinase of *B. subtilis* (Kunst and Rapoport, 1995). For this reason, production of *Virgibacillus* sp. SK37 proteinases could be limited at high salt environment.

4.4.2.2 α-Amino content

The α-amino content of all fish sauce samples increased during fermentation in the similar pattern to oligopeptide content (Table 4.1). An increase in α-amino content of the control samples was resulted from proteolytic activity of microflora and fish endogenous enzymes. The fish sauce samples inoculated with *Virgibacillus* sp. SK37 starter culture together with the reduced salt content of 15% showed the highest α-amino content at day 30 ($P < 0.05$), corresponding to the highest oligopeptide content (Figure 4.2). However, when fermentation was progressed to 90 days, α-amino content of samples with added starter culture was comparable to their respective controls ($P > 0.05$). This confirmed that addition of *Virgibacillus* sp. SK37 increased protein hydrolysis only at the early stage of fermentation, but not at the later stage. When higher amount of salt was added at 20%, α-amino content of inoculated

samples was comparable to its control during the course of fermentation ($P > 0.05$). It was observed that the controls (without starter culture) with reduced salt content at 15 and 20% showed higher α-amino content than the sample prepared at 25% salt addition at 90 days of fermentation ($P < 0.05$).

4.4.2.3 Other chemical parameters

The total nitrogen content of all inoculated samples was lower than that of their respective controls ($P < 0.05$, Table 4.2). At 3 months of fermentation, the total nitrogen of all fish sauce samples was higher than 2%, which is classified as first grade fish sauce (Lopetcharat et al., 2001). The ammoniacal nitrogen of all samples was comparable ($P > 0.05$). A high number of inoculated cultures (5 Log CFU/g) during fermentation might lead to an increase in N-source utilization as C-sources were very limited in the system. As a result, total nitrogen of inoculated samples were lower. This was in agreement with a lower browning index of inoculated samples as main substrates, N-compounds, for Maillard browning were relatively lower than respective controls. It should be mentioned that browning index of controls with salt reduction to 15 and 20% was greater than the traditionally fermented sample containing 25% NaCl. Histamine was the predominant biogenic amine found in all samples. Reduction of salt content to 15 or 20% without starter culture resulted in histamine content of 23.9-24.5 mg/100 mL, which was comparable to 22.5 mg/100 mL of histamine found in 25%-NaCl sample ($P > 0.05$). Inoculation of *Virgibacillus* sp. SK37 at 15 or 20% NaCl decreased histamine levels to 13.0-15.6 mg/100 mL, which were lower than those of their respective controls without inoculation ($P < 0.05$). Histamine level of all samples was lower than the allowable limit of 40 mg/100 mL, imposed by Codex (2011). This study confirmed the histamine reduction ability

of *Virgibacillus* sp. SK37 as previously reported (Yongsawatdigul et al., 2007).

Although the content of salt addition was varied in the fermentation, Na content of the liquid drained from the mash showed similar Na content of 9.4-10.3 g/100 mL which was equivalent to 24.0-26.1% NaCl. Samples prepared at 15% salt showed lower salt content than those prepared at 25% salt ($P < 0.05$) but comparable with those prepared at 20% salt addition ($P > 0.05$). During salting of fish, fish are surrounded by granular salt, which is initially dissolved by the surface moisture of the fish. Then, water is removed from the fish and salt ions are penetrated into the fish during osmotic dehydration (Hernandez-Herrero, Roig-Sagues, Lopez-Sabater, Rodriguez-Jerez, and Mora-Ventura, 2002). It has been reported that rate of salt penetration into the muscle varies with the fat content, freshness of the fish, surface/volume ratio of the flesh, and temperature (Narayanaswamy, Narashimha Rao, and Govidan, 1980). Moisture content of fresh anchovies used in this study was about 65%. Limited water flux from the fish resulted in almost saturated salt content in the drained liquid. Although addition salt was reduced in the fermentation yielding the greater extent of proteolysis, the salt content of the finished product did not deviate from the standard of fish sauce which is set to be not less than 20% (Codex, 2011).

4.4.3 Amino acid profiles

Total and free amino acids profiles are presented in Table 4.3. Total amino acid content was in the range of 8,087 to 9,578 mg/100 g, while sum of free amino acid content of ranged from 10,670 to 11,383 mg/100 g. Total amino acid contents of all samples were lower than free amino acid content. This might be because some amino acids could be oxidized during hydrolysis for total amino acid determination. As compared to the traditional salt addition (25%), reduction salt content to 15 and 20%

Table 4.1 α-Amino content of fish sauce samples inoculated with *Virgibacillus* sp. SK37 and fermented at 35°C for 3 months.

Fermentation time (day)	α-Amino content (mM) [a]				
	15% NaCl		20% NaCl		25% NaCl
	Control	SK37 [b]	Control	SK37	Control
30	710.65 ± 12.10 c	841.05 ± 26.20 a	719.14 ± 15.09 bc	754.63 ± 12.20 b	730.71 ± 14.23 bc
45	865.52 ± 22.43 ab	899.92 ± 8.03 a	844.23 ± 10.32 bc	810.65 ± 34.73 c	827.03 ± 6.92 bc
60	874.22 ± 4.70 ab	901.93 ± 5.91 a	875.90 ± 9.50 ab	838.96 ± 19.45 b	848.19 ± 23.90 ab
75	946.62 ± 10.90 a	958.60 ± 3.60 a	915.83 ± 35.10 ab	878.19 ± 10.98 b	917.54 ± 6.10 ab
90	975.25 ± 2.30 a	938.70 ± 23.50 abc	962.79 ± 38.80 ab	911.30 ± 10.33 bc	895.51 ± 14.23 c

Note: [a] Different letters within a row indicate significant differences ($P < 0.05$). [b] SK37 = *Virgibacillus* sp. SK37.

Table 4.2 Physico-chemical properties of fish sauce samples inoculated with *Virgibacillus* sp. SK37 and fermented at 35°C for 3 months.

Physico-chemical properties	Fish sauce sample [a]				
	15% NaCl		20% NaCl		25% NaCl
	Control	SK37 [b]	Control	SK37	Control
Total nitrogen (%)	2.26 ± 0.01 a	2.06 ± 0.03 c	2.20 ± 0.02 ab	2.01 ± 0.03 c	2.14 ± 0.04 b
Ammonical nitrogen (%)	0.12 ± 0.004	0.13 ± 0.008	0.11 ± 0.003	0.13 ± 0.006	0.11 ± 0.002
Color (Abs@440 nm)	0.446 ± 0.003 a	0.338 ± 0.001 c	0.419 ± 0.002 b	0.326 ± 0.001 d	0.319 ± 0.001 e
Na (g/100 mL)	9.57 ± 0.20 b	9.44 ± 0.08 b	9.95 ± 0.21 ab	9.93 ± 0.31 ab	10.27 ± 0.11 a
pH	5.46 ± 0.01 b	5.50 ± 0.01 a	5.45 ± 0.01 b	5.45 ± 0.01 b	5.43 ± 0.01 c

Note: [a] Different letters within a row indicate significant differences ($P < 0.05$). [b] SK37 = *Virgibacillus* sp. SK37.

increased total amount of total and free amino acids, indicating the higher degree of protein hydrolysis. At each sat ratio, *Virgibacillus* sp. SK37 is unlikely to increase total and free amino acids corresponding to the results of oligopeptide and α-amino contents.

The taste and flavor precursor of fish sauce is derived from some free amino acids (Chayovan, Rao, Liuzza, and Khan, 1983; Lee, Kim, Jean, Kim, and Kim, 1982). Thus, free amino acid was focused. It was observed that salt reduction yielded fish sauce with higher amount of free alanine, aspartic acid, glutamic acid, methionine, phenylalanine, and serine content. Lysine, histidine, glutamic, and aspartic acid were predominant amino acids in all samples.

Free glutamic acid content of inoculated samples was comparable to their respective controls. Glutamic acid and alanine were reported to be the taste-active components of fish sauce along with threonine, tyrosine, histidine, valine, proline, cystine, and methionine (Park, Watanabe, Endoh, Watanabe, and Abe, 2002).

Inoculated samples at both levels of salt addition showed higher free isoleucine and leucine contents than their respective controls. Branched-chain amino acids (leucine, isoleucine, and valine) are reported as precursors for major volatile compounds in fish sauce, including aldehydes (3-methylbutanal, 2-methylbutanal, and 2-methylpropanal), alcohols (3-methylbutanol, 2-methylbutanol, and 2-methylpropanol), and acids (3-methylbutanoic acid, 2-methylbutanoic acid, and 2-methylpropanoic acid) (Ardo, 2006).

Free phenylalanine content of inoculated samples appeared to be lower than the controls at both levels of salt addition. The addition of *T. halophilus* starter culture increased higher free phenylalanine content (Udomsil et al., 2011). This amino acid

Table 4.3 Amino acid contents of fish sauce samples inoculated with *Virgibacillus* sp. SK37 and fermented at 35°C for 3 months.

Amino acid	Amino acid content (mg/100 g)									
	Control (15% NaCl)		SK37 (15% NaCl)		Control (20% NaCl)		SK37 (20% NaCl)		Control (25% NaCl)	
	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free
Alanine	306	493	288	452	287	502	265	449	275	446
Arginine	51	95	59	84	54	101	62	102	44	91
Aspartic acid	706	766	677	731	692	885	612	642	642	675
Cysteine	229	289	278	280	166	225	192	247	172	216
Glutamic acid	1145	1323	1144	1335	1054	1248	1026	1255	1053	1085
Glycine	227	307	223	296	201	221	207	318	205	300
Histidine	1221	1236	1305	1400	1176	1235	1177	1308	1312	1480
Hydroxylysine	167	168	16	23	152	165	14	35	14	17
Hydroxyproline	19	50	19	49	19	31	7	14	8	29
Isoleucine	354	421	391	474	334	377	269	481	341	462
Leucine	422	553	444	578	391	448	405	608	419	632
Lysine	1975	2359	2076	2330	2021	2308	1712	2383	1953	2389
Methionine	243	294	237	288	219	270	222	273	227	266
Phenylalanine	346	651	351	359	300	563	305	397	313	345
Proline	273	317	249	361	242	245	227	338	250	321
Serine	433	516	420	459	433	603	307	449	406	438
Threonine	562	635	546	666	548	458	330	460	527	556
Tryptophan	264	205	269	295	230	232	226	266	215	247
Tyrosine	147	219	116	227	135	198	109	210	105	255
Valine	488	486	463	474	460	503	413	435	435	488
Total	**9578**	**11383**	**9571**	**11161**	**9114**	**10818**	**8087**	**10670**	**8916**	**10738**

has been reported as a precursor for phenylacetaldehyde, 2-phenylethanol, 2-phenylacetic acid, and benzaldehyde via amino acid catabolism by aminotransferase (Ardo, 2006; Groot and Bont, 1998). The role of benzaldehyde on fish sauce flavor has not been reported but has been identified in some fermented foods (Wittanalai, Raksriyatham, and Deming, 2011). 2-Phenylethanol has been regarded as the odor-active compound in soy sauce and also showed higher flavor dilution (FD) factor than phenylacetaldehyde and 2-phenylacetic acid (Steinhaus and Schieberle, 2007). Free phenylalanine was not judged to be as a taste-active component of fish sauce (Park et al., 2002). Thus, the reduced content of phenylalanine by *Virgibacillus* sp. SK37 might not affect the taste of fish sauce.

Inoculated samples showed lower free serine content than their respective controls. Serine is converted to pyruvate in the gluconeogenesis pathway by L-serine dehydrogenase which is encoded by two genes, s*dhA* and *sdhB*, in *Bacillus subtilis* (Wu, Mao, Olman, and Xu, 2007). These genes might also be present in *Virgibacillus* sp. SK37, resulting in decrease of this compound in inoculated samples.

Addition of *Virgibacillus* sp. SK37 as a starter culture did not increase free methionine content at both salt addition levels when compared to its control. Methionine has been reported to contribute to umami and overall taste of fish sauce (Park et al., 2002). In addition, methionine is a precursor for methanethiol, 3-(methylthio)propanal, 3-(methylthio)propanol, 3-(methylthio)propanoic acid, dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide ((Ardo, 2006; Bonnarme, Psoni, and Spinnler, 2000; Yvon and Rijnen, 2001). All of these sulfur-containing compounds have been shown as important volatile compounds in fish sauce (Fukami et al., 2002; Giri et al., 2010; Peralta et al., 1996; Wichaphon et al., 2012). However,

some of these compounds are responsible for a fecal note that is undesirable for the product.

4.4.4 Odor-active compounds

The concentrations of selected odor-active compounds were quantified using stables isotope of the analytes as internal standards (Table 4.4). The highest abundant compounds of all fish sauce samples were acetic acid followed by butanoic acid, 3-methylbutanoic acid, and propanoic acid. Wichaphon et al. (2012) showed that organic acids, including acetic acid, propanoic acid, 2-methylpropanoic acid, butanoic acid, and 3-methylbutanoic acid, contributed to the volatile profiles of the first and second grade fish sauce samples.

Inoculated samples appeared to have higher content of volatile acids, particularly acetic acid and 2-methylpropanoic acid ($P < 0.05$), as compared to its respective controls. A reduction of salt to 15-20% did not affect these volatile acids ($P > 0.05$). Acetic acid and 2-methylpropanoic acid have been reported to contribute to sour and cheesy/sweaty note, respectively (Wichaphon et al., 2012). Acetic acid can be formed via oxidation of ethanol and acetaldehyde. Bulthuis, Rommens, Koningstein, Stouthamer, and Verseveld (1991) reported that ethanol and acetaldehyde were among volatile compounds formed via glycolysis and pentose phosphate pathway during the anaerobic growth of *B. licheniformis* in batch culture. 2-Methylpropanoic acid is formed by oxidation of 3-methylpropanal, which is derived from valine via transamination and decarboxylation pathways (Yvon and Rijnen, 2001). Shimoda, Peralta, and Osajima (1996) suggested that volatile fatty acids, especially 2-methylpropanoic acid, could be major contributors to the cheesy and stinging odor of fish sauce, based on their quantitative and odor threshold values.

Inoculation with *Virgibacillus* sp. SK37 increased butanoic acid and 3-methylbutanoic acid contents at salt addition of 15% ($P < 0.05$) but not 20% ($P > 0.05$). 3-Methylbutanoic acid is derived from leucine via transamination and decarboxylation pathways (Yvon and Rijnen, 2001). Butanol and 2-methylbutanol could be further converted to butanal and 2-methylbutanal, respectively, by the action of a broad-specificity NADPH-dependent alcohol dehydrogenase, which has been found in the genome of *Virgibacillus* sp. SK37 (Phrommao, 2010). The oxidation of butanal and 3-methylbutanal also yields butanoic acid and 3-methylbutanoic acid, respectively. It was observed that an increase in 3-methylbutanoic acid content is likely to be related to a high leucine content in inoculated samples.

Free phenylalanine content of inoculated samples appeared to be lower than their respective controls, which led to a reduced precursor for phenylacetaldehyde formation. In addition, 2-phenylacetic acid, 3-phenylpropanoic acid, and 4-hydroxy-2,5-dimethyl-3(2H)-furanone of inoculated samples showed lower contents than their respective controls ($P < 0.05$). Phenylacetaldehyde and 2-phenylacetic acid are responsible for honey-like/hot chocolate and sweet note, respectively (Steinhaus and Schieberle, 2007). Phenylacetaldehyde can be formed via Strecker degradation of phenylalanine (Hofmann and Schieberle, 2000). 4-Hydroxy-2,5-dimethyl-3(2H)-furanone has been found in different kinds of food. However, the role of this compound on fish sauce flavor has not been reported. It is one of degradation products of the Amadori compound via 2,3-enolization, elongation by the Strecker aldehydes, and reduction of the resulting acetylformoin-type as intermediates and responsible for caramel-like odor (Blank and Fay, 1996; Yvon and Rijnen, 2001). This compound was regarded as one of the odor-active compounds in soy sauce and is

Table 4.4 Concentration of odor-active compounds in fish sauce samples inoculated with *Virgibacillus* sp. SK37 at 35°C for 3 months.

No.	Compound	Concentration[a] (µg/kg)				
		Control (15% NaCl)	SK37 (15% NaCl)	Control (20% NaCl)	SK37 (20% NaCl)	Control (25% NaCl)
1	Trimethylamine	1.38 b	1.65 a	1.27 c	1.31 bc	1.29 bc
2	Methanethiol	41 a	40 a	36 ab	37 ab	31 b
3	Dimethyl sulfide	17	15	16	15	14
4	2-Methylpropanal	340 b	355 ab	286 c	383 a	299 c
5	2-Methylbutanal	209 e	539 a	396 c	463 b	331 d
6	3-Methylbutanal	373 c	823 a	566 b	849 a	628 b
7	Dimethyl trisulfide	5.03	4.78	4.76	4.71	4.5
8	Acetic acid	482452 c	735923 a	484265 c	549590 b	514783 bc
9	3-(Methylthio)propanal	810 a	714 ab	598 b	651 b	609 b
10	Propanoic acid	3409 b	3835 ab	3913 ab	4193 a	3730 ab
11	2-Methylpropanoic acid	1391 b	2347 a	1468 b	2184 a	1453 b
12	Butanoic acid	12461 bc	25905 a	13575 bc	14252 b	11392 c
13	Phenylacetaldehyde	79 a	35 c	51 b	27 c	57 b
14	3-Methylbutanoic acid	5333 b	8405 a	5906 b	6613 b	6171 b
15	3-(Methylthio)propanol	103 b	307 a	81 bc	71 c	57 c
16	4-Hydroxy-2,5-dimethyl-3(2H)-furanone	1061 a	831 bc	920 b	730 cd	689 d
17	2-Phenylacetic acid	1605 a	1200 b	1619 a	1083 b	1613 a
18	3-Phenylpropanoic acid	41 a	34 bc	35 ab	28 c	27 c

Note: [a] Average of four determinations (n = 4). Different letters within a row indicate significant differences ($P < 0.05$).

Table 4.5 Odor-activity value (OAV) of compounds in fish sauce samples inoculated with *Virgibacillus* sp. SK37 at 35°C for 3 months.

No.	Compound	Odor threshold[a] (μg/kg)	OAV[b]				
			Control (15% NaCl)	SK37 (15% NaCl)	Control (20% NaCl)	SK37 (20% NaCl)	Control (25% NaCl)
1	Trimethylamine	0.47	2.90 b	3.58 a	2.71 c	2.89 bc	2.75 bc
2	Methanethiol	0.02	2050 a	2000 a	1800 ab	1850 ab	1550 b
3	Dimethyl sulfide	0.3	57	50	53	50	47
4	2-Methylpropanal	1	340 b	355 ab	286 c	383 a	299 c
5	2-Methylbutanal	3	70 e	180 a	132 c	154 b	110 d
6	3-Methylbutanal	0.2	1865 c	4115 a	2830 b	4245 a	3140 b
7	Dimethyl trisulfide	0.01	503	478	476	471	450
8	Acetic acid	22000	22 c	33 a	22 c	25 b	23 bc
9	3-(Methylthio)propanal	0.2	4050 a	3570 ab	2990 b	3255 b	3045 b
10	Propanoic acid	2000	1.70 b	1.92 ab	1.96 ab	2.10 a	1.87 ab
11	2-Methylpropanoic acid	50	28 b	47 a	29 b	44 a	29 b
12	Butanoic acid	240	52 bc	108 a	57 bc	59 b	47 c
13	Phenylacetaldehyde	4	20 a	9 c	13 b	7 c	14 b
14	3-Methylbutanoic acid	250	21 b	34 a	24 b	26 b	25 b
15	3-(Methylthio)propanol	250	0.41 b	1.23 a	0.32 bc	0.28 c	0.23 c
16	4-Hydroxy-2,5-dimethyl-3(2H)-furanone	31	34 a	27 bc	30 b	24 cd	22 d
17	2-Phenylacetic acid	1000	1.61 a	1.20 b	1.62 a	1.08 b	1.61 a
18	3-Phenylpropanoic acid	NA[c]	-	-	-	-	-

Note: [a] Orthonasal odor threshold in water. [b] Odor-activity value=concentration divided by odor detection threshold. [c] Not available.

responsible for caramel-like odor (Steinhaus and Schieberle, 2007). It could be speculated that this compound could contribute to the flavor of fish sauce as it has a low threshold value of 31 μg/kg (Buttery, Takeoka, Krammer, and Ling, 1995).

2-Methylpropanal, 2-methylbutanal, and 3-methylbutanal contents of inoculated samples were higher than the controls at both levels of salt addition ($P < 0.05$). These compounds were derived from valine, isoleucine, and leucine via amino acid catabolism pathway, respectively (Ardo, 2006; Smit, Engels, and Smit, 2009). They have been reported to contribute to malty, nutty, almond, and meaty odor of fish sauce (Fukami et al., 2002; Giri et al., 2010; Peralta et al., 1996; Steinhaus and Schieberle, 2007). Giri et al. (2010) reported that 2-methylpropanal and 2-methylbutanal showed high FD factors and are principal contributors to the distinct odor of Thai fish sauce. In addition, Steinhaus and Schieberle (2007) reported that 2-methylbutanal and 3-methylbutanal are among the most potent odorants in soy sauce based on high OAVs. Thus, addition of *Virgibacillus* sp. SK37 in conjunction with salt reduction to 15 and 20% rendered fish sauce with more intense desirable volatile aldehydes.

Virgibacillus sp. SK37 and salt reduction had no effect on volatile sulfur compounds, including methanethiol, 3-(methylthio)propanal, dimethyl sulfide, and dimethyl trisulfide ($P > 0.05$). Methionine plays a crucial role in the metabolism of sulfur-containing compounds. L-Methioninase has been reported in various microorganisms, including *B. subtilis* (El-Sayed, 2010). Demethiolation of methionine by L-methioninase results in methanethiol, α-ketobutyrate, and ammonia (El-Sayed, 2010). Methanethiol can be further oxidized to dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide (Bonnarme et al., 2000; Smit et al., 2009; Yvon and Rijnen, 2001). These compounds are responsible for cooked cabbage, onion, fishy, sulfury

(Giri et al., 2010) and believed as distinctive fish sauce odor (Fukami et al., 2002; Peralta et al., 1996). 3-(Methylthio)propanal is also converted from methionine via aminotransferase to 4-methylthio-2-oxobutyric acid which can be further decarboxylated via α-ketoacid decarboxylase. Reduction and oxidation of 3-(methylthio)propanal yield 3-(methylthio)propanol and 3-(methylthio)propanoic acid, respectively (Varlet and Fernandez, 2010). 3-(Methylthio)propanol content of inoculated samples at 15% salt addition was higher than the control ($P < 0.05$). Its presence in fish sauce might be desirable because of its meaty, nutty, and cooked potato-like notes (Steinhaus and Schieberle, 2007; Wichaphon et al., 2012).

The odor-activity values (OAVs) of 17 odorants in the samples are presented in Table 4.5. Six compounds, such as methanethiol, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, dimethyl trisulfide, and 3-(methylthio)propanal showed the highest OVAs, implying that these compounds have high influence on the overall aroma of fish sauce samples because of their high concentrations/low odor threshold values. The rest showed low OAV-value, indicating a less important role for overall aroma in fish sauce. It should be noted that high abundant acid compounds, including acetic acid, propanoic acid, butanoic acid, and 3-methylbutanoic acid showed low OAVs.

3-(Methylthio)propanal had the highest OAV in fish sauce samples without inoculation, whereas 3-methylbutanal showed the highest OAV in the inoculated samples. This result implied that inoculation of *Virgibacillus* sp. SK37 along with salt reduction to 15 and 20% rendered fish sauce with more intense malty, nutty, almond, and meaty odor. As compared to the typical salt addition of 25%, the reduced salt content to 15% increased methanethiol in the finished product, which was likely to cause undesirable note. OAV of 2-methylpropanal, 2-methylbutanal, and 3-

methylbutanal significantly increased in fish sauce inoculated *Virgibacillus* sp. SK37 together with reduced salt addition ($P < 0.05$). Relation of aldehydes, particularly 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal, to sensory characteristics of inoculated fish sauce deserves further investigation. Salt content and *Virgibacillus* sp. SK37 had no influence on OAV of dimethyl trisulfide.

4.4.5 Sensory evaluation

Sensory evaluation based on color, odor, taste, and overall acceptance of all fish sauce samples was shown in Table 4.6.

Table 4.6 Hedonic score of color, odor, taste, and overall acceptance of fish sauce samples

Fish sauce samples		Attributes			
		Color	**Odor**	**Taste**	**Overall acceptance**
15% NaCl	Control	5.56 ± 0.73 a	4.33 ± 1.50 ab	4.44 ± 0.73	4.89 ± 0.78
	SK37	4.67 ± 1.22 ab	5.33 ± 0.87 a	4.44 ± 1.24	4.89 ± 1.05
20% NaCl	Control	5.22 ± 0.67 ab	4.78 ± 0.67 ab	4.11 ± 1.27	4.22 ± 0.97
	SK37	4.56 ± 1.13 b	4.33 ± 0.71 ab	4.00 ± 1.00	4.00 ± 0.71
25% NaCl	Control	5.11 ± 0.78 ab	4.00 ± 1.22 b	4.11 ± 1.05	4.22 ± 0.83

Note: Acceptance score: 7 = extremely like; 4 = neither like nor dislike; 1 = extremely dislike. Different letters within a column indicate significant difference ($P < 0.05$).

The color and odor preference of fish sauce samples inoculated with starter cultures

were comparable to its control ($P > 0.05$), although the profile of odor-active compounds were different. Taste and overall acceptance of samples with added starter culture was comparable to their respective controls ($P > 0.05$).

4.5 Conclusions

The reduction of salt content to 15-20% increased the rate of protein hydrolysis, resulting in increases in α-amino content, total nitrogen, browning index, pH, and free amino acids in the finished product, as compared to 25%-salt treatment. The addition of *Virgibacillus* sp. SK37 at 15% salt content increased protein hydrolysis only at the early stage of fermentation, whereas it did not accelerate protein hydrolysis at 20% salt content at any time during fermentation. Total nitrogen and amino contents of inoculated samples at reduced salt content were lower than those of their respective controls, leading to lower degree of browning. This is the first report demonstrating that *Virgibacillus* sp. SK37 at 15-20% salt content increases key aroma active compounds, namely 2-methylbutanal and 3-methylbutanal. Therefore, flavor of fish sauce could be controlled through application of *Virgibacillus* sp. SK37 starter culture.

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CHAPTER V

SPENT BREWERY YEAST SLUDGE AS A SINGLE NITROGEN SOURCE FOR FIBRINOLYTIC ENZYME PRODUCTION OF *VIRGIBACILLUS* SP. SK37

5.1 Abstract

Among the five food industrial wastes including soybean pomace (Sp), rice bran (Rb), mungbean protein (Mp), spent brewery yeast sludge (Ys), and fish sauce sludge (Fs), Ys was the best nitrogen source for proteinases production from *Virgibacillus* sp. SK37 isolated from fish sauce fermentation. Ys at 1% (w/v) successfully replaced yeast extract in the medium. The highest level of proteinase production was obtained in the medium containing 2.5% NaCl, pH 7.5 and incubated at 40°C for 4 days, which was about 1.7 times higher than medium containing the commercial yeast extract. At least 10 proteinases with molecular weight (Mw) of 19, 24, 28, 32, 42, 44, 60, 61, 63, and 64 kDa were detected in zymograms using casein, gelatin, and fibrin as a substrate. *Virgibacillus* sp. SK37 proteinases and plasmin completely hydrolyzed fibrinogen within 50 min with Aα-chain being the first target and followed by Bβ- and γ-chains. Degradation products produced by *Virgibacillus* sp. SK37 proteinases consisted of the fragments with Mw of 39, 41, and 42 kDa, while peptides with mass of 41, 42, and 44 kDa were observed by plasmin digestion. Therefore, *Virgibacillus* sp. SK37 proteinases could be a potential ingredient for a

functional food development.

Keywords: *Virgibacillus* sp. SK37, spent brewery yeast sludge, fibrinolytic enzyme

5.2 Introduction

Fibrin is the major protein component of blood clots, which is formed from fibrinogen by thrombin (EC 3.4.21.5). There are more than 20 enzymes in the body that assist in blood clotting, while there is only one that can break down the clot known as plasmin (Ali and Ibrahim, 2008). When fibrin is not hydrolyzed due to some disorders, thromboses can occur which is one of the main causes of cardiovascular diseases. Zymography has been used to identify proteinase activity in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This method is based on SDS-PAGE copolymerization with the selected protein substrate, such as gelatin, casein, and fibrin. Activities in zymograms are visualized as clear zones (where the protein substrates are digested) after electrophoretic separation (Garcia-Carreno, Dimes, and Haard, 1993; Lantz and Ciborowski, 1994). *Bacillus* from traditional fermented foods is an important group of microorganisms that have been found to produce the fibrinolytic enzymes and identified as effective sources of thrombolytic agents. In 1987, *B. natto* producing Nattokinase was firstly isolated from a traditional Japanese fermented soybean called natto and studied extensively (Fujita et al., 1993; Sumi, Hamada, Tsushima, Mihara, and Muraki, 1987). Subsequently, *Bacillus* sp. from different fermented foods was discovered to produce fibrinolytic enzymes. For example, *Bacillus* sp. CK 11-4 from the Korean fermented-soybean sauce called chungkook-jang (Kim et al., 1996), *Bacillus* sp. KA38 from the Korean salty fermented fish called jeot-gal (Kim et al., 1997), *B. subtilis* DC33 from Chinese

traditional fermented-soybean call *Douchi* (Wang et al., 2006), and *Bacillus* sp. nov. SK006 from Asian traditional fermented shrimp paste (Hua, Jiang, Mine, and Mu, 2008).

Extracellular proteinase from *Virgibacillus* sp. SK37 could be activated by NaCl and retained its activity at 20-25% NaCl (Sinsuwan, Rodtong, and Yongsawatdigul, 2007). Thus far, fibrinolytic activity of *Virgibacillus* sp. SK37 has not been fully realized. In addition, production of *Virgibacillus* sp. SK37 proteinases was based only on the culture medium containing yeast extract, casamino acid, and peptone as nitrogen sauce. This would definitely limit the large scale production of the enzyme.

Composition of the culture medium and physical factors significantly affect microbial growth and proteinase production (Gupta, Beg, Khan, and Chauhan, 2002). Nitrogen source is one of the important parameters because it is metabolized to produce amino acids, nucleic acids, proteins, and cell wall components (Kole, Draper, and Gerson, 1988). Several reports describe the use of food byproducts for the production of microbial proteinases. For example, bug meal, pigeon pea, green gram husk, and molasses were used for the production of alkaline proteinases from *Bacillus* sp. AR009, *Bacillus* sp. JB-99, *Bacillus* sp., and *B. pantotheneticus*, respectively (Gessesse, 1997; Jhonvesly, Manjunath, and Naik, 2002; Prakasham, Rao, and Sarma, 2006; Shikha Sharan and Darmwal, 2007).

Food industrial byproducts might have a potential to be used for biomass and enzyme production. Soybean pomace is a byproduct from the soy sauce industry. Its major components are carbohydrates and proteins. Because of high sodium chloride content, soybean pomace is normally discarded as a waste, resulting in serious

environmental problems. Soybean pomace was found to be a good substitute for yeast extract for the production of pullulan by *Aureobasidium pullulans* HP-2001 (Seo et al., 2004). Rice bran is a byproduct of the rice milling. When mungbean is used for starch and vermicelli production, a large quantity of valuable protein is produced and discarded as feedstuff. Yeast sludge is a byproduct from the brewing industry and mainly used for animal feed. It has rich nutritional compositions and amino acids. Fish sauce sludge is a byproduct from the fish sauce industry. It is mainly used as low value fertilizer. The utilization of these byproducts as an alternative nitrogen source of *Virgibacillus* sp. SK37 should be sought. This would lead to the maximum utilization of these byproducts for valuable enzyme production. Therefore, the objective of this study was to investigate the suitable food industrial byproducts and conditions affecting proteinase production from *Virgibacillus* sp. SK37. In addition, fibrinolytic activity of these proteinases was also investigated.

5.3 Materials and Methods

5.3.1 Chemicals

Succinyl (Suc)-Ala-Ala-Pro-Phe-AMC was purchased from Bachem A.G. (Bubendorf, Switzerland). Human fibrinogen, thrombin, plasmin and casein were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

5.3.2 Food industrial byproducts

Soybean pomace (Sp) from soy sauce production; rice bran (Rb) from the rice milling process; mungbean protein (Mp) from vermicelli production; spent brewery yeast sludge (Ys) from the brewing process, and fish sauce sludge (Fs) from the fish

sauce industry were used as a nitrogen source for developing the economical culture medium. Total nitrogen and NaCl content of all byproducts were determined according to AOAC (1995).

5.3.3 Medium

Medium for cell growth and proteinase production was the modified halophilic medium or Y-medium (Ym) containing (%): yeast extract, 1.0; trisodium citrate, 0.3; KCl, 0.2; $MgSO_4.7H_2O$, 2.5; NaCl, 5 (Sinsuwan, Rodtong, and Yongsawatdigul, 2008). The modified medium was prepared by substituting 1% yeast extract with byproducts. Each byproduct was mixed with reverse osmosis water at 1% (w/v) and boiled for 10 min. The mixture was centrifuged at 8000×*g* for 20 min. The supernatant was added with 0.3% trisodium citrate, 0.2% KCl, and 2.5% $MgSO_4.7H_2O$. After the pH and NaCl concentration was adjusted to 7.0 and 5% (w/v), respectively, the medium was sterilized at 121°C for 15 min and used for cell culture.

5.3.4 Screening of suitable medium

Pure culture of *Virgibacillus* sp. SK37 was prepared by streaking on the Ym agar. Starter culture was prepared by inoculating a loopfull of *Virgibacillus* sp. SK37 into a 50 mL of Ym, pH 7 at 35°C and shaking at 100 rpm. Cells were cultured until the optical density (OD) at 600 nm reached 0.3 (approximately 10^7 CFU/mL), which took about 3 days. The inoculum (5 mL) was transferred to Ym or the modified media (45 mL) containing only Sp, Rb, Mp, Ys, or Fs in a 250-mL Erlenmeyer flask and incubated at 35°C for 6 days with a shaking speed of 100 rpm.

Both bacterial growth and proteinase activity were monitored at 0, 2, 4, and 6 days of incubation. Bacterial enumeration was performed using a spread plate

technique on Ym agar and incubated at 35°C for 2 days. Each cultured medium was collected and centrifuged at 8000×*g*, 4°C for 30 min. Proteinase activity of the supernatant was determined by the modified method of Sinsuwan et al. (2007) as described below.

5.3.5 Effect of medium composition and temperature on proteinase production

Starter culture was prepared as described above. The Ys was selected because it gave the highest proteinase activity. Inoculum (5 mL) was transferred to the modified medium containing Ys as a nitrogen source (45 mL) in a 250-mL Erlenmeyer flask at different amounts (0.5, 1.0, 3.0, and 5.0% w/v). Then, the optimum concentration of Ys determined from this study was used to evaluate the effect of incubation temperature (30, 35, 40, and 45°C), pH (6.0, 6.5, 7.0, 7.5, and 8.0) and NaCl concentration (0, 2.5, 5.0, 7.5, and 10% w/v).

At each studied condition, a sample was incubated for 5 days with a shaking speed of 100 rpm. Bacterial growth was monitored at 0 and 4 days of incubation using a spread plate technique on Ym agar and incubated at 35°C for 2 days. Each cultured medium was collected and centrifuged at 8,000×*g*, 4°C for 30 min. Proteinase activity of the supernatant was determined every day as described below. The optimal condition was selected based on the highest proteinase activity.

5.3.6 Proteinase activity assay

The assay was determined according to the method of Sinsuwan et al. (2007) using Suc-Ala-Ala-Pro-Phe-AMC as a substrate with some modifications. Reaction mixture (1 mL total volume) containing 50 µL enzyme solution, 1 µM of Suc-Ala-Ala-Pro-Phe-AMC in Mcllvain buffer (0.2 M trisodium phosphate, 0.1 M trisodium

citrate), and pH 8.0 was incubated at 60°C for 5 min. The reaction was stopped by adding 1.5 mL of stopping reagent (30% butanol, 35% methanol, and 35% deionized water). Fluorescence intensity was measured at excitation wavelength of 380 nm and emission wavelength of 460 nm (RF-1501, Shimadzu Co., Kyoto, Japan). One unit activity (U) was defined as 1 nmol of AMC released per 1 mL of enzyme.

5.3.7 Production of proteinases from *Virgibacillus* sp. SK37

Starter culture (200 mL) was prepared as described above and transferred to the modified medium (1,800 mL) containing 1% Ys, 2.5% NaCl, and pH 7.5. The culture was incubated at 40°C for 4 days with a shaking speed of 100 rpm. This was the optimum condition for proteinase production of *Virgibacillus* sp. SK37 obtained from the above study. Cells were harvested using tangential flow filtration equipped with a 0.45-μm membrane cassette installed in LV centramate™ holder (Pall Co., New York, USA). Two liters of the cell-free supernatant was further subjected to ultrafiltration (UF) membrane cassette with molecular weight cut-off (MWCO) 30 kDa (Pall Co., New York, USA). Then, the retentate (100 mL) was dialyzed against two liters of 50 mM Tris-HCl pH 8.0. The dialyzed proteinase was stored at 4°C.

5.3.8 Zymographic assay

Casein, gelatin, and fibrin zymograms were carried out according to the method of Kim, Choi, and Lee (1998). Casein, gelatin, or human fibrinogen (0.12%, w/v) were initially dissolved in a 20 mM sodium phosphate buffer (pH 7.4). In the case of fibrin gel, 100 μL of human thrombin (10 NIH unit/mL) was added in the human fibrinogen solution to make a soft fibrin. Casein, gelatin, and fibrin were separately co-polymerized with 12% (w/v) acrylamide, 0.32% (w/v) bisacrylamide, and 0.375 M Tris-HCl (pH 8.8) for a running gel preparation. Stacking gel was prepared to contain

5% (w/v) acrylamide, 0.11% (w/v) bisacrylamide, and 330 mM Tris-HCl (pH 6.8). *Virgibacillus* sp. SK37 proteinase was loaded into gel at approximately 0.5 U per well. Electrophoresis was run at a constant current of 12 mA at 4°C (Laemmli, 1970). After the electrophoresis was completed, the gel was incubated in 50 mM Tris-HCl (pH 7.4) which contained 2.5% Triton X-100 for 30 min at room temperature. Then, gel was washed twice with distilled water to remove Triton X-100. Proteolytic reaction was carried out in a buffer containing 30 mM Tris-HCl, pH 7.4, 200 mM NaCl, 10 mM $CaCl_2$, and 0.02% Brij-35, incubated at 37°C for 30 min. Gel was stained with 0.1% Coomassie brilliant blue R-250, 40% methanol and 10% acetic acid for 1 h and destained in 30% ethanol and 10% acetic acid. Clear zone indicated the presence of proteinase.

5.3.9 Degradation of fibrinogen

The reaction mixture (60 µL) containing 50 µL of human fibrinogen [0.12% (w/v) in 20 mM sodium phosphate buffer (pH 7.4)] and 5 µL of deionized water was pre-incubated at 37°C for 15 min. Proteinase activity of *Virgibacillus* sp. SK37 proteinase was determined by addition of 5 µL of crude proteinase (88.2 U/mL). At various time intervals (0, 10, 20, 30, 40, 50, and 60 min), the mixture was taken and mixed with an equal volume of a treatment buffer (0.125 mM of Tris-HCl pH 6.8, 20% glycerol, 10% β-mercaptoethanol, and 0.1% bromophenol blue) and heated at 90°C for 5 min. Degradation products of fibrinogen were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gel. Degradation pattern of fibrinogen by *Virgibacillus* sp. SK37 proteinase was compared to that by plasmin, which was carried out at the same manner at 150 µg/mL.

5.3.10 Statistical analyses

All experiments were done in duplicate and mean values were presented. Analysis of variance (ANOVA) was carried out using SPSS program (SPSS version 13, Windows version). Differences among mean values were established using Duncan Multiple Range Test (DMRT) at $P < 0.05$. The effect of each condition on proteinase production and bacterial growth was analyzed using completely randomized design (CRD).

5.4 Results and discussion

5.4.1 Screening of food industrial byproducts for proteinase production

Mungbean protein (Mp) showed the highest total nitrogen content and rice bran (Rb) contained the lowest (Table 5.1). Proteinase production of *Virgibacillus* sp. SK37 in Y-medium (Ym) was the highest as compared to media substituted by byproducts (Figure 5.1). Yield of proteinase drastically increased at day 2 of cultivation and slowly increased afterward (Figure 5.1a). Among five byproducts studied, brewer yeast sludge (Ys) yielded the highest proteinase production at day 4 and 6 ($P < 0.05$). Kumar and Tagaki (1999) reported that extracellular proteinase production of microorganisms varied with specific nitrogen source. The best nitrogen source for both *B. cereus* 146 and *Bacillus* sp. K-30 was beef extract (Naidu and Devi, 2005; Shafee, Aris, Rahman, Basri, and Salleh, 2005) while soybean meal and wheat bran were the best nitrogen source for proteinase production of *B. cereus* MCM B-326 and *B. pantotheneticus*, respectively (Nilegaonkar, Zambare, Kanekar, Dhakephalkar, and Sarnaik, 2007; Shikha Sharan and Darmwal, 2007).

Table 5.1 Total nitrogen and NaCl content of commercial yeast extract and various food industrial byproducts.

Samples	Total nitrogen (% w/w)	NaCl (% w/w)
Yeast extract	10.79 ± 0.05	-
Mungbean protein (Mp)	12.29 ± 0.09	0.52 ± 0.16
Fish sauce sludge (Fs)	2.22 ± 0.14	74.67 ± 0.74
Rice bran (Rb)	2.16 ± 0.01	0.48 ± 0.11
Yeast sludge (Ys)	7.79 ± 0.01	0.46 ± 0.13
Soybean pomace (Sp)	3.47 ± 0.14	19.82 ± 0.80

Rice bran provided the highest biomass within 2 days, while Ym and other byproducts required 4 days to reach the maximum biomass (Figure 5.1b). Cell growth in all culture media was similar at day 4 ($P > 0.05$). Proteinase production of *Virgibacillus* sp. SK37 in the medium containing soybean pomace (Sp) or Mp was the lowest during the course of cultivation ($P < 0.05$). Despite the maximum biomass obtained from the Rb medium at day 2, proteinase activity of Rb was lower than that in Ys and Ym ($P < 0.05$). This result indicated that proteinase production does not entirely depend on biomass. Medium composition plays a vital role on proteinase production. Proteinase production in Ym increased rapidly and reached the maximum activity at day 2 (7.51 ± 0.63 U/mL), while proteinase production in Ys gradually increased and reached the maximum activity at day 4 (6.60 ± 0.53 U/mL). Although total nitrogen of Mp was higher than Ys (Table 5.1), lower proteinase activity was found in Mp. Mp contains mungbean proteins, which are mainly intact proteins. In contrast, brewer yeast, *Sarccharomyces cerevisiae*, in Ys is rich in amino acids,

peptides, vitamins and minerals. Small nutrients in Ys appeared to be more effective in stimulating proteinase production of *Virgibacillus* sp. SK37 than large protein molecules contained in Mp. Large protein like casein was also found to repress proteinase production of other strains of *Virgibacillus* sp. (Sinsuwan et al., 2008). Addition of yeast extract to the medium has been reported to enhance proteinase production of various Bacillus species (Boominadhan and Rajakumar, 2009; Hadj-Ali et al., 2007). Proteinase gene transcription was observed in the medium containing yeast extract (Maunsell, Adams, and O'Gara, 2006). Recently, Ys has been applied as a substitute of a yeast extract in the culture medium for production of various enzymes (do Nascimento, Junior, and Coelho, 2011; Panagiotou, Granouillet, and Olsson, 2006; Xiros and Christakopoulos, 2012). As Ys are considered as a complex medium constituting of various compounds, the single compound responsible for protein expression at the molecular level has not been identified. This study indicated that Ys was the suitable inexpensive material that could be used as a main component for stimulating proteinase production of *Virgibacillus* sp. SK37. Therefore, Ys was chosen for further optimization.

It should be noted that content of carbon source is not considered in this study. It has been demonstrated that nitrogen is more essential than carbon for extracellular proteinase synthesis (Mckellar and Cholette, 1984). Carbon source is of important for biomass, and less important for proteinase production of bacteria. Boethling (1975) also reported that rate of proteinase secretion in the presence of yeast extract alone was significantly higher than that observed when both yeast extract and carbon source were present.

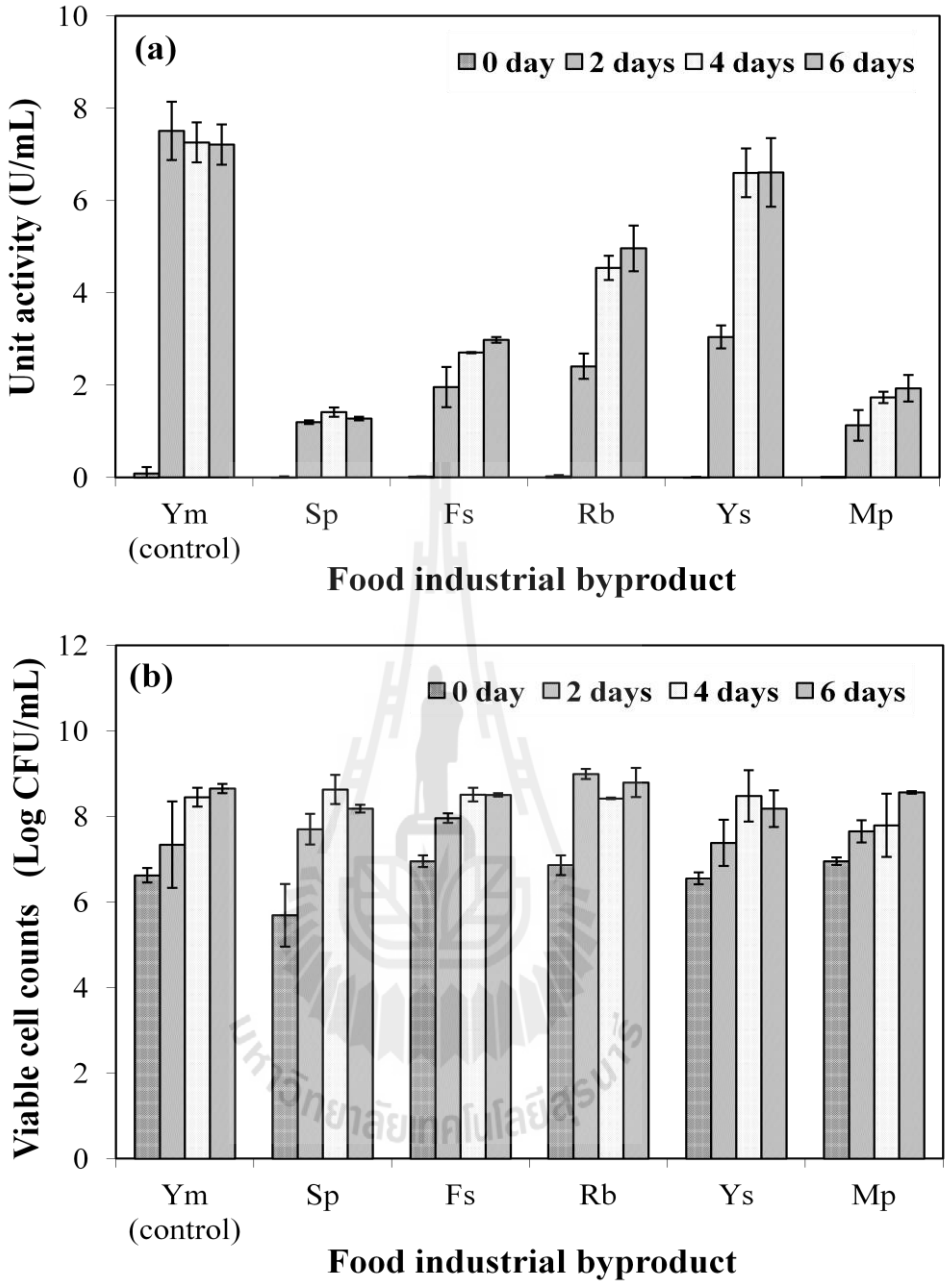

Figure 5.1 Effect of food industrial byproducts on proteinase production (a) and viable cell counts (b) of *Virgibacillus* sp. SK37. Media containing 5% NaCl, pH 7.0 and incubated at 35°C for 6 days. Ym, modified Y-medium containing either yeast extract; Sp, soybean pomace; Rb, rice bran; Mp, mungbean protein; Ys, yeast sludge; Fs, fish sauce sludge at 1% (w/v).

5.4.2 Effect of yeast sludge content and conditions on proteinas production

5.4.2.1 Yeast sludge concentration

The highest proteinase production of 6.72 ± 0.44 U/mL was achieved at day 3 in the medium containing 1% Ys (Figure 5.2a). At high concentration of 3-5% Ys, proteinase production was not observed within the first 3 days, but gradually increased in day 4 and reached comparable activity to that of 1% at day 5 ($P > 0.05$). The onset of proteinase production of *Virgibacillus* sp. SK37 in the medium containing 0.5% Ys was observed at the earliest stage in the first day. Proteinase activity was low throughout the course of cultivation at 0.5% Ys. This was perhaps because of the limited nitrogen source for its metabolism. The higher content of Ys in the medium appeared to prolong the lag period of proteinase production. Moon and Parulekar (1991) reported that, in general, proteinase production was found to be repressed by rapidly metabolizable nitrogen sources, such as amino acids or ammonium ion concentrations in the medium. Several studies have demonstrated that excessive nitrogen concentration showed a repression in proteinase production of various strains. Proteinase production of *B. firmus* was repressed by an excessive amount of yeast extract. However, proteinase production of *B. licheniformis* was not repressed by ammonium salts. Yeast extract at >0.03% and peptone at >0.2% repressed the proteinase production of *B. firmus* and *Bacillus* sp. SMIA-2, respectively (do Nascimento and Martins, 2004; Moon and Parulekar, 1991; Nehete, Shah, and Kothari, 1986). Proteinase production of alkaliphilic actinomycete was repressed at either >1.5% peptone or >1% yeast extract (Mehta, Thumar, and Singh, 2006). The proteinase production of *Virgibacillus* sp. SK37 was also repressed at concentration of Ys>1% (w/v). Proteinase production at 1% (w/v) Ys was the highest

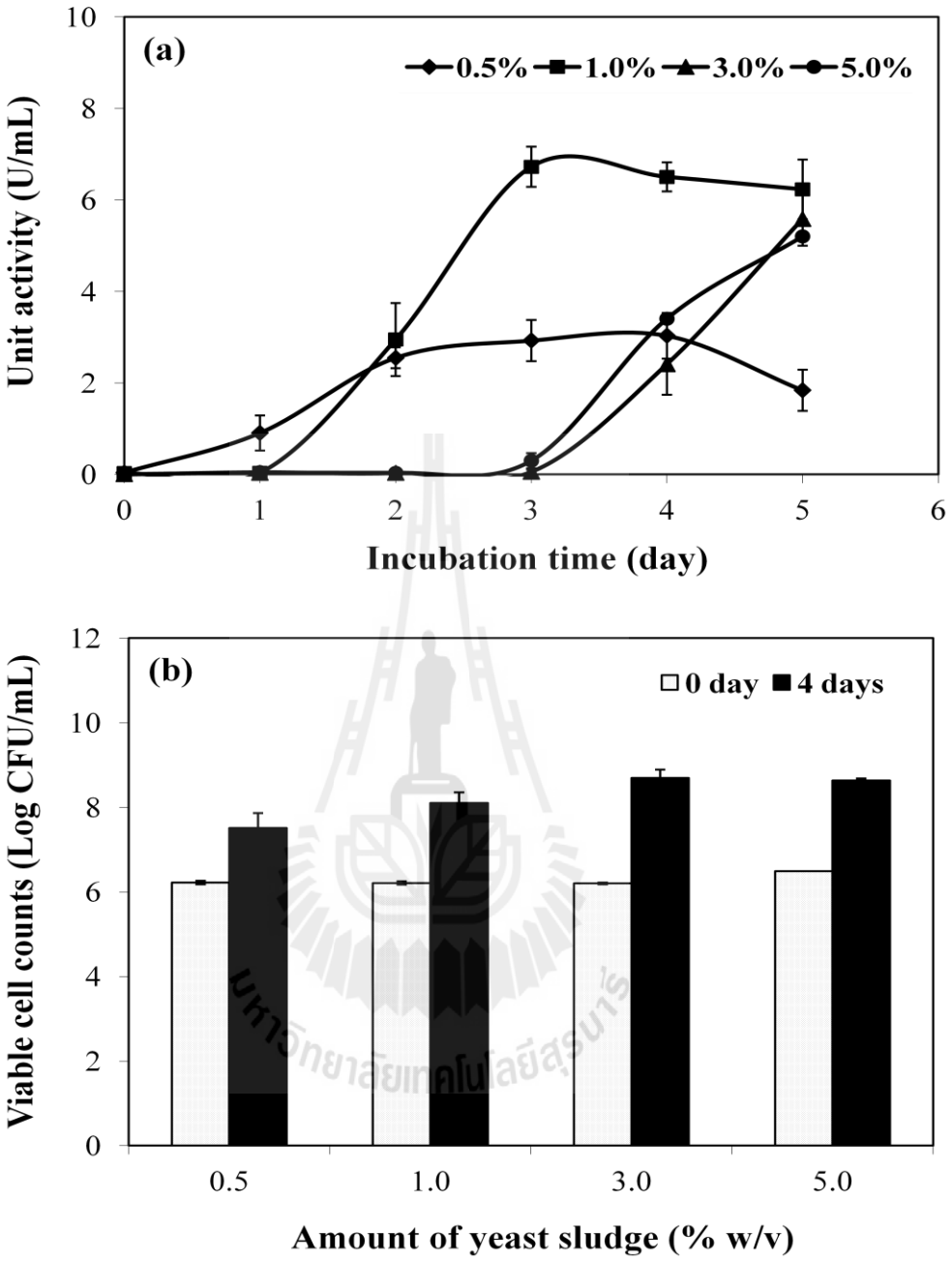

Figure 5.2 Effect of yeast sludge on proteinase production (a) and viable cell counts (b) of *Virgibacillus* sp. SK37. Media containing 5% NaCl, pH 7.0 and incubated at 35°C for 5 days.

($P < 0.05$) whereas cell counts in medium containing 1, 3, and 5% (w/v) Ys were similar at day 4 ($P > 0.05$) (Figure 5.2b). These results indicated that the content of the nitrogen source does not necessarily correspond with cell counts but it greatly affects proteinase production of *Virgibacillus* sp. SK37.

5.4.2.2 Temperature, pH, and NaCl concentration

The maximum proteinase activities of 6.80 ± 0.35 and 6.84 ± 0.34 U/mL were observed at 35 and 40°C, respectively at day 4 (Figure 5.3). The maximum cell count of 8.7 log CFU/mL was also observed at 40°C, indicating that 40°C was an optimum incubation temperature for both proteinase production and growth of *Virgibacillus* sp. SK37. These results are in agreement with Sinsuwan et al. (2008) who reported that 40°C was an optimum temperature for both growth and proteinase production of *Virgibacillus* sp. SK33 in the halobacterium broth. At a low temperature (30°C), proteinase production of *Virgibacillus* sp. SK37 was delayed, whereas early proteinase production was found at a high temperature of 45°C. However, growth of *Virgibacillus* sp. SK37 was not affected by temperatures ranging from 30 to 45°C.

pH strongly affects growth and proteinase production. Optimum pH for proteinase production and growth of *Virgibacillus* sp. SK37 was at pH 7.5 (Figure 5.4, $P < 0.05$). Proteinase production was not detected below pH 6.0 in spite of reasonable cell growth of 6.6 log CFU/mL. Optimum conditions of proteinase production from halophilic bacteria have been reported. The optimum temperature and pH for both growth and proteinase production of *Halobacterium* sp. PB407 and/or *H. halobium* ATCC 43214 were 37°C and pH 7.0, respectively (Kanlayakrit, Bovornreungroj, Oka, and Goto, 2004; Ryu and Dordick, 1994).

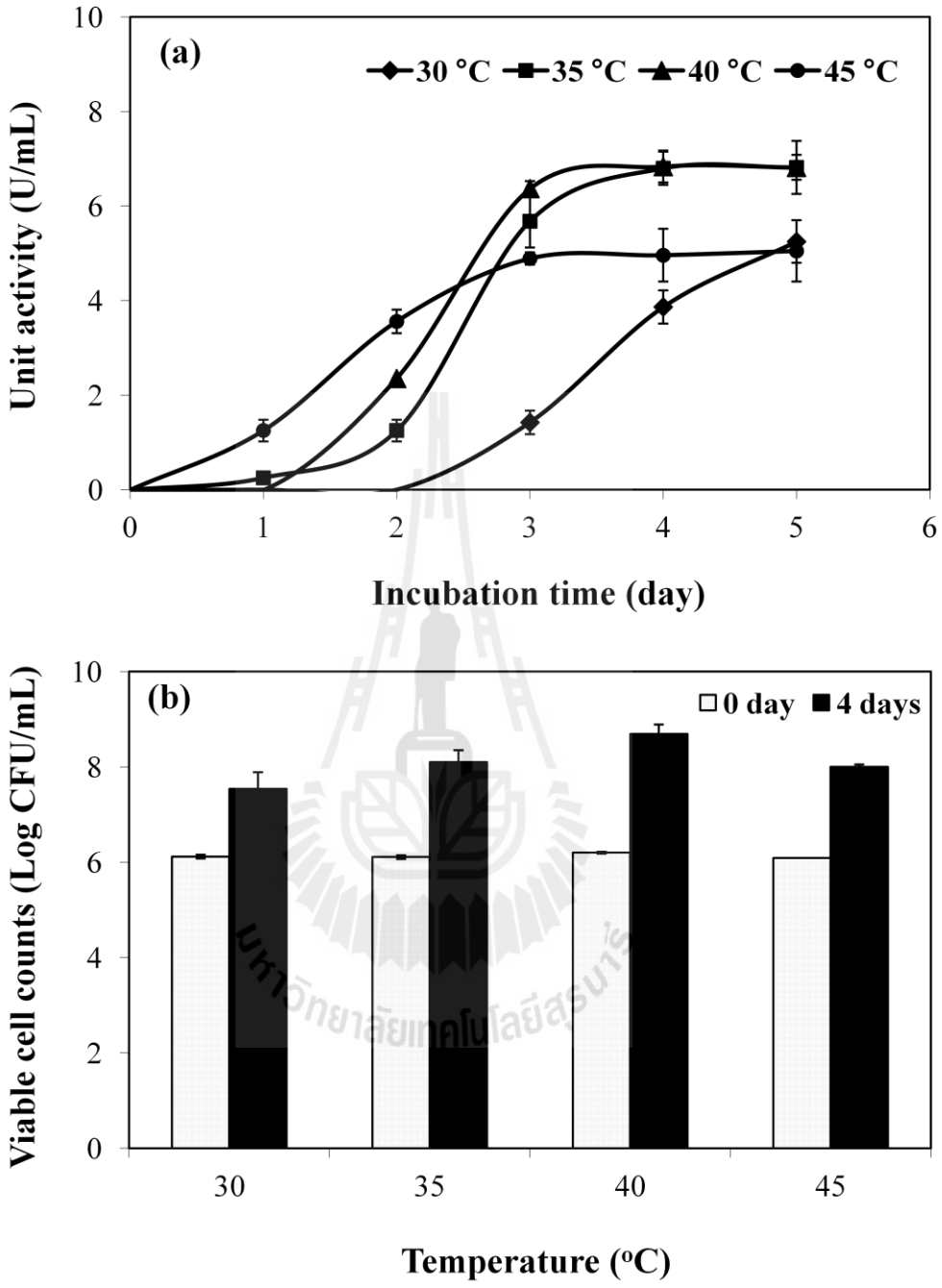

Figure 5.3 Effect of temperature on proteinase production (a) and viable cell counts (b) of *Virgibacillus* sp. SK37. Media containing 1% yeast sludge, 5% NaCl, pH 7.0 and incubated for 5 days.

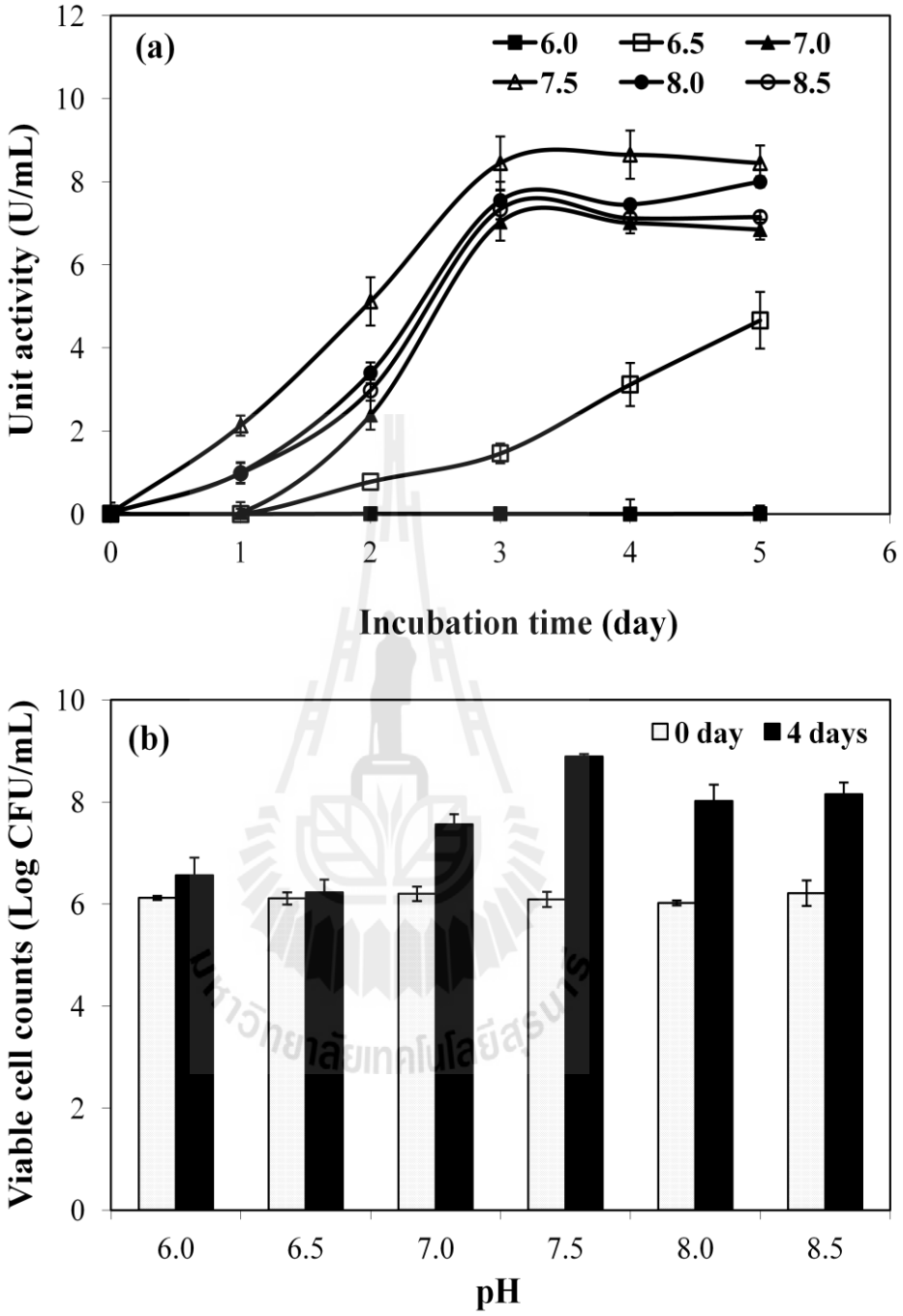

Figure 5.4 Effect of pH on proteinase production (a) and viable cell counts (b) of *Virgibacillus* sp. SK37. Media containing 1% yeast sludge, 5% NaCl and incubated at 40°C for 5 days.

Proteinase production greatly varied with salt content of the medium with the maximum at the addition of 2.5% NaCl (Figure 5.5a). However, growth of *Virgibacillus* sp. SK37 rapidly increased at day 1 in the medium containing 7.5% NaCl with the cell counts of 8.21 log CFU/mL, indicating moderately halophilic bacteria characteristic (Figure 5.5b). Whatmore, Chudek, and Reed (1990) reported that sodium chloride affect the accumulation of proline in *B. subtilis* cells. In addition, Ogura, Kawata-Mukai, Itaya, Takio, and Tanaka (1994) showed that requirement for proline biosynthesis is the presence of multiple copies of the *proB* gene, leading to enhanced *aprE* gene (encoding proteinase) expression. For this reason, proteinase production of *Virgibacillus* sp. SK37 appeared to decrease in the medium with no addition of NaCl, but increased in the presence of 2.5 and 5% NaCl. However, salt stress has been reported to decrease expression of the *aprE* gene which is controlled by the DegS-DegU two-component system (Kunst and Rapoport, 1995). In the case of *Virgibacillus* sp. SK37, salt stress has been effected in the medium containing >7.5% NaCl. Growth of *Virgibacillus* sp. SK37 at the optimum condition of 2.5% NaCl (in Ys medium) and 40°C gradually increased and reached maximum growth at day 4, corresponding to the early stationary phase (Figure 5.5b). At day 4, this medium provided activity about 1.7 times greater than that observed in the medium containing the commercial yeast extract (Figure 5.1a), implying the potential use of spent brewery yeast sludge as an inexpensive nitrogen source in the culture medium for proteinase production. Viable cell counts of *Virgibacillus* sp. SK37 decreased slowly after day 5, corresponding to the death phase. Our results demonstrated that proteinase productivity of *Virgibacillus* sp. SK37 was the highest at the end of log phase. When compared to other bacilli, *Virgibacillus* sp. SK37 exhibited lower optimum NaCl

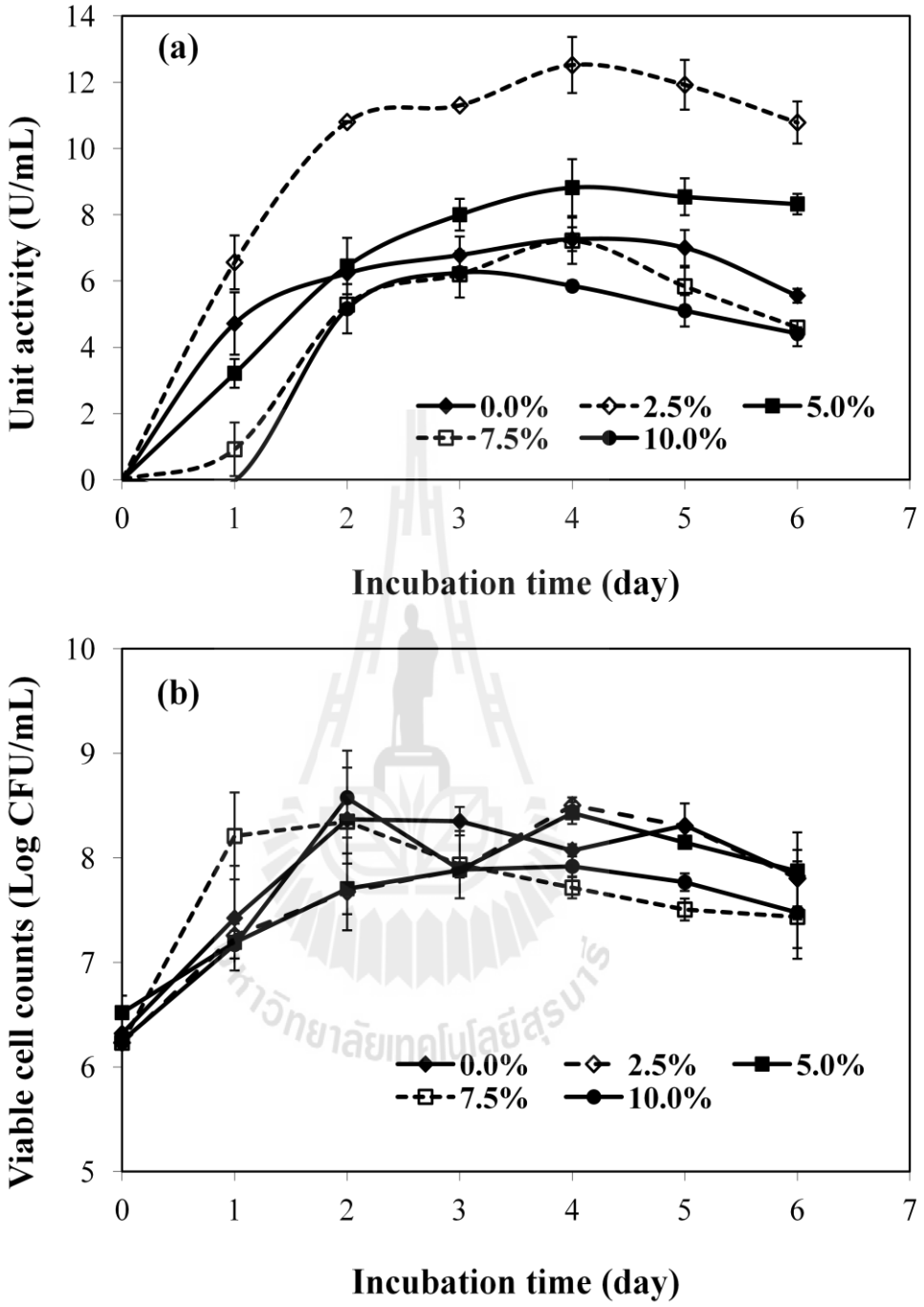

Figure 5.5 Effect of NaCl concentration on proteinase production (a) and viable cell counts (b) of *Virgibacillus* sp. SK37. Media containing 1% yeast sludge, pH 7.5 and incubated at 40°C for 6 days.

concentration for proteinase production. The optimum growth and proteinase production of *B. halophilus* and *B. salexigens* were observed at 15 and 10% NaCl, respectively (Garabito, Arahal, Mellado, Márquez, and Ventosa, 1997; Ventosa, García, Kamekura, Onishi, Ruiz-Berraquero, 1989).

5.4.3 Zymographic assay

Molecular weight (Mw) of crude proteinase from *Virgibacillus* sp. SK37 was estimated to be 19, 24, 28, 32, 42, 44, 60, 61, 63, and 64 kDa, based on activity staining of all substrates (fibrin, casein, and gelatin) (Figure 5.6).

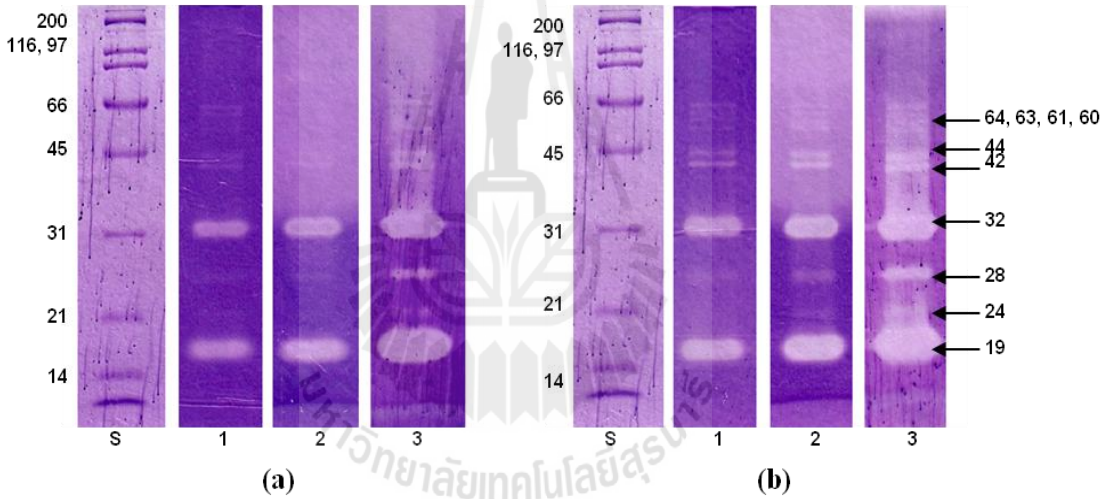

Figure 5.6 Activity staining (SDS-PAGE, 12% T) of *Virgibacillus* sp. SK37 proteinases using fibrin (1), casein (2), and gelatin (3) as a substrate by incubating at 37°C for 30 min (a) and at 60°C for 30 min (b). S, molecular weight standard.

Two major proteinases with Mw of 19 and 32 kDa showed fibrinolytic, caseinolytic, and gelatinolytic activity when incubated at both 37 and 60°C for 30

min, while minor proteinases can be activated at 60°C. *Bacillus* sp. from different traditional fermented foods have been found to produce fibrinolytic enzymes and identified as an effective source of thrombolytic agents. Therefore, *Virgibacillus* sp. SK37 could be employed to use as effective sources of thrombolytic agents.

5.4.4 Degradation of fibrinogen

The Aα-chain of fibrinogen was first cleaved within 10 min, followed by Bβ- and γ-chains within 50 min (Figure 5.7a). Similarly, when fibrinogen was incubated with plasmin, the Aα-chain of fibrinogen was hydrolyzed faster than Bβ- and γ-chains (Figure 5.7b). All chains were observed in the control throughout 60 min hydrolysis (Figure 5.7c). Fibrinolytic enzymes from *B. subtilis* TP-6 isolated from Indonesian fermented soybean showed similar pattern to *Virgibacillus* sp. SK37 proteinases (Kim et al., 2006). Fibrinolytic enzyme from *B. subtilis* DC33 and *Bacillus* sp. SK006 showed a different mode of hydrolysis of fibrinogen from *Virgibacillus* sp. SK37 proteinases or plasmin. For fibrinolytic pattern of *B. subtilis* DC33, Bβ-chains of fibrinogen were firstly cleaved, followed by Aα- and γ-chains while Bβ-chains of fibrinogen were firstly cleaved by *Bacillus* sp. SK006, followed by γ-chains. The Aα-chains were typically more resistant to enzyme digestion (Hua et al., 2008; Wang et al., 2006). Degradation products produced by *Virgibacillus* sp. SK37 proteinases consisted of the fragments with Mw of 39, 41, and 42 kDa, while peptides with mass of 41, 42, and 44 kDa were observed in plasmin-digested samples. In addition, a peptide with Mw of 32 kDa produced by *Virgibacillus* sp. SK37 proteinases was also observed within 40 min hydrolysis. These results implied that *Virgibacillus* sp. SK37 proteinases and plasmin showed different specificity toward fibrinogen.

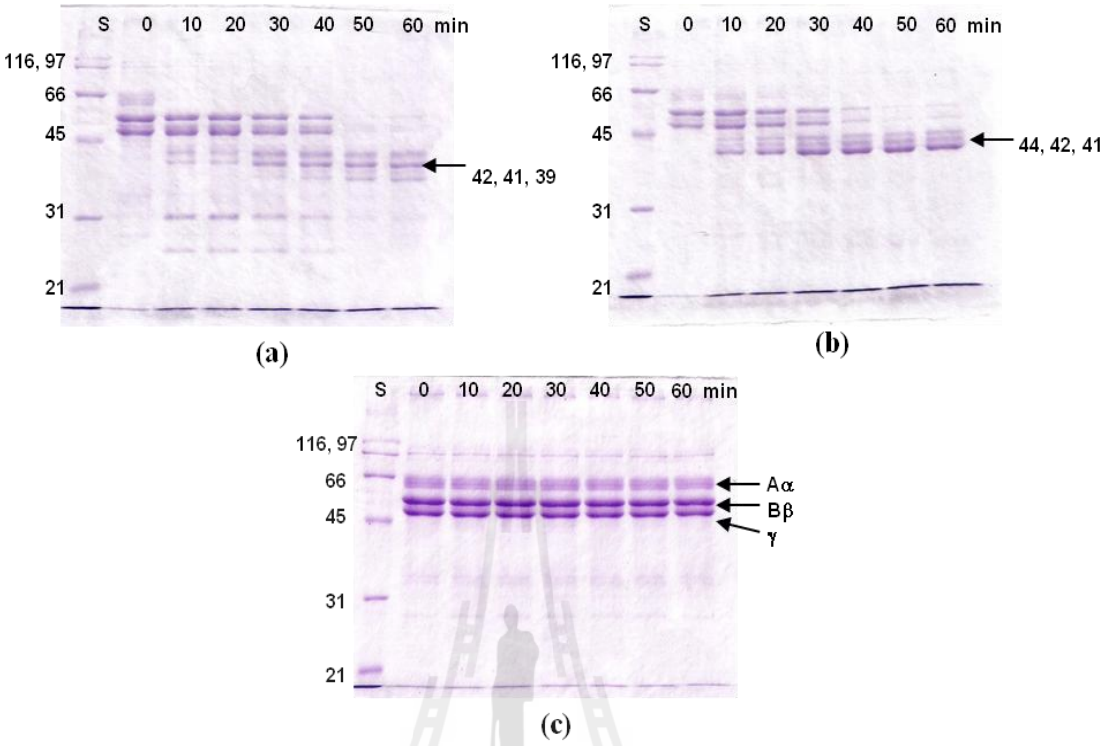

Figure 5.7 Degradation pattern of fibrinogen incubated with *Virgibacillus* sp. SK37 proteinases (88.2 U/mL) (a), plasmin (150 μg/mL) (b), and deionized water (control) (c) at 37°C. S, molecular weight standard.

5.5 Conclusions

Spent brewery yeast sludge is an inexpensive nitrogen source that can be used to replace the more expensive yeast extract to promote growth and induce proteinase production of *Virgibacillus* sp. SK37. Optimal conditions for the highest proteinase yield were 1% (w/v) yeast sludge medium containing 2.5% NaCl, pH 7.5, incubated at 40°C for 4 days. In addition, *Virgibacillus* sp. SK37 could be a promising source of fibrinolytic enzyme that can be further developed as a functional food.

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CHAPTER VI

PRODUCTION AND PURIFICATION OF ANTIOXIDANT PEPTIDES FROM MUNGBEAN MEAL HYDROLYSATE BY *VIRGIBACILLUS* SP. SK37 PROTEINASES

6.1 Abstract

Antioxidant peptides of mugbean meal hydrolyzed by *Virgibacillus* sp. SK37 proteinases (VH), Alcalase (AH), and Neutrase (NH) were investigated. The antioxidant activities based on 2,2′-azinobis (3-ethyl-benzothiazoline-6-sulphonate) (ABTS) radical scavenging, ferric reducing antioxidant power (FRAP), and metal chelation of VH were comparable to those of NH. VH was purified using ultrafiltration, ion exchange, and gel filtration chromatography. The purified peptide (F37) from VH showing the highest specific antioxidant activity consisted of four peptides containing Arg residue at their C-termini. In addition, ABTS radical scavenging activity of the purified peptides (F42) at 0.148 mg/mL was comparable to that of 1 mM of butylated hydroxytoluene (BHT). These two fractions were stable over a wide pH (4-10) and temperature (25-121°C) range. *Virgibacillus* sp. SK37 proteinases are potential possessing aid for production of a mungbean meal hydrolysate with antioxidative properties.

Keywords: Mungbean meal, Antioxidant peptide, *Virgibacillus* sp., hydrolysate,

proteinase

6.2 Introduction

Mungbean (*Vigna radiata* (L.) Wilczek) or green gram is commonly eaten as bean sprouts, and mungbean starch is a major raw material of glass noodle production. After starch extraction, the meal consisting of 72-80% protein is typically discarded and utilized as animal feed (Jantawat, Chinprahast, and Siripatrawan, 1998; Sonklin, Laohakunjit, and Kerdchoechuen, 2011). In Thailand, the quantity of mungbean supplied to glass noodle industry is estimated to be 200,000 tons/year and 27% of raw materials which is rich in protein are disposed as mungbean meal (Jantawat et al., 1998). The efficient utilization of this byproduct as food should be sought.

Major storage proteins of mungbean are 8S globulin or vicilin consisting of 89% of total protein, followed by 11S globulin or legumin (7.6%), and basic 7S (3.4%) (Mendoza, Adachi, Bernardo, and Utsumi, 2001). Mendoza et al. (2001) showed that 8S vicilin, 11S legumin, and basic 7S have high homology with soybean *β*-conglycinins, soybean glycinin, and soybean basic 7S, respectively. Chen, Muramoto, and Yamauchi (1995) isolated six antioxidant peptides from digested *β*-conglycinin using proteinase from *Bacillus* sp. These peptides composed of 5-16 amino acid residues, including Pro, His, or Tyr within the sequences and Val/Leu or hydrophobic amino acids at the N-termini. Some histidine-containing peptides can act as a metal-ion chelator, a singlet oxygen quencher, and a hydroxy radical scavenger (Chen, Muramoto, Yamauchi, Fujimoto, and Nokihara, 1998). Mungbean proteins could be a good source for antioxidant peptides. Several studies have demonstrated

the antioxidant activity of soy protein hydrolysates (Beermann, Euler, Herzberg, and Stahl, 2009; Chen et al., 1995; Moure, Domínguez, and Parajó, 2006; Peña-Ramos and Xiong, 2001; Zhang, Li, and Zhou, 2010). Very few research works have reported the bioactive potential of mungbean protein hydrolysate.

Peptides with different antioxidant activities are based on amino acid composition of their sequence, which varies upon proteinases used (Peña-Ramos and Xiong, 2001). Enzymatic hydrolysis of various food proteins have been reported to possess antioxidant potential. Different commercial proteinases have been used for production of bioactive hydrolysates, such as Alcalase, Neutrase, pepsin, trypsin, chymotrypsin, papain, bromelain, and subtilisin. Recently, *Virgibacillus* sp. SK37 (GenBank accession number DQ910840) isolated from 1-month-old Thai fish sauce mashes produced subtilisin-like proteinase with halotolerant characteristics (Phrommao, Rodtong, and Yongsawatdigul, 2010; Sinsuwan, Rodtong, and Yongsawatdigul, 2007). In addition, proteinases from different strains, *Virgibacillus* sp. SK33, hydrolysed threadfin bream surimi wastes, yielding high antioxidant activity towards HepG2 cell (Wiriyaphan, Chitsomboon, and Yongsawatdigul, 2012). Sinsuwan, Rodtong, and Yongsawatdigul (2010) demonstrated that these proteinases have broad specificity toward oxidized insulin B with the dominant cleavage sites at Tyr^{16}-Leu^{17} and Phe^{25}-Tyr^{26}. Efficacy of *Virgibacillus* sp. SK37 proteinase as a processing-aid for bioactive peptides with high antioxidant activity has never been reported.

The objectives of this study were to evaluate the antioxidant activities of mungbean meal hydrolysates produced by *Virgibacillus* sp. SK37 proteinase in comparison with commercial proteinases, namely Alcalase and Neutrase. In addition,

purification and characterization of active antioxidant mungbean peptides derived from *Virgibacillus* sp. SK37 proteinases were investigated.

6.3 Materials and Methods

6.3.1 Materials

Mungbean meal was obtained from a glass noodle plant (Thai Wah Food Products Public Co., Ltd., Nakhon Pathom, Thailand). Alcalase 2.4L and Neutrase 0.8L were gifted from Novozymes (Bagsvaerd, Denmark). 2,2′-Azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were purchased from Biochemika (Buchs, Switzerland). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) and trinitrobenzenesulfonic acid (TNBS) were obtained from Sigma Chemical Co. (St. Louis, MO). Suc-Ala-Ala-Pro-Phe-amino-7-methylcoumarin (AMC) was obtained from Bachem A.G. (Bubendorf, Switzerland). Other chemicals and reagents used were of analytical grade.

6.3.2 Proximate analyses

Proximate compositions (moisture, crude fat, crude protein, crude fiber, and ash) of mungbean meal were analyzed according to AOAC (2000). Carbohydrate composition was calculated from the differences using following the equation: carbohydrate = 100-(percentage of moisture, crude fat, crude protein, crude fiber, and ash).

6.3.3 *Virgibacillus* sp. SK37 proteinase production

The *Virgibacillus* inoculum was prepared by inoculating a loopfull of pure culture of *Virgibacillus* sp. SK37 into a 200 mL of modified halophilic medium (1%

yeast extract, 0.3% trisodium citrate, 0.2% KCl, 2.5% $MgSO_4.7H_2O$) containing 5% NaCl, pH 7 at 35°C for 1 day (Sinsuwan, Rodtong, and Yongsawatdigul, 2008). The inoculum was then subsequently transferred to the modified medium (1,800 mL) containing 1% brewer yeast sludge, 2.5% NaCl, 0.3% trisodium citrate, 0.2% KCl, 2.5% $MgSO_4.7H_2O$, pH 8 and incubated at 40°C for 4 days with a shaking speed of 100 rpm. This medium and condition were found to be optimal for proteinase production of the strain. Cells (approximately 10^8 CFU/mL) were harvested using tangential flow filtration equipped with a 0.45-μm membrane cassette installed in a LV centramate™ holder (Pall Co., East Hills, NY). The cell-free supernatant was further concentrated using ultrafiltration (UF) membrane cassette with molecular weight cut-off (MWCO) 30 kDa (Pall Co., East Hills, NY). The retentate was dialyzed against 50 mM Tris-HCl, pH 8.0 at 4°C. The dialysate was referred to as *Virgibacillus* sp. SK37 proteinase and stored at 4°C throughout the study. Proteinase activity was assayed according to Sinsuwan et al. (2007) using succinyl(Suc)-Ala-Ala-Pro-Phe-7-amino-4-methylcoumarin(AMC) as a substrate. One unit (U) was defined as the amount of enzyme that released AMC in nmol per min.

6.3.4 Production of protein hydrolysate

Ten grams of mungbean meal in 90 mL of buffer were hydrolysed using various enzymes under their respective optimal condition: Mcllvain buffer (0.2 M trisodium phosphate, 0.1 M trisodium citrate) pH 8.5, 65°C for *Virgibacillus* sp. SK37; Tris-HCl, pH 8, 65°C for Alcalase; Tris-HCl, pH 7, 50°C for Neutrase. The ratio of Alcalase and Neutrase to substrate was 0.25:100 and 0.5:100 (v/w), respectively, which was equivalent to about 50 mAU (AU=Anson Unit). *Virgibacillus* sp. SK37 proteinase was added to the mixture at the final unit of 440 U. Samples were taken at

various time intervals of 0, 1, 2, 4, 6, 8, 12, and 24 h and heated at 90ºC for 15 min to inactivate the enzyme. Subsequently, it was cooled and centrifuged at 10,000 x g for 20 min at 4ºC and used for further analyses.

6.3.5 Degree of hydrolysis (DH)

DH was performed according to Adler-Nissen (1979). The hydrolysate (50 μL) was mixed with 0.5 mL of 0.2125 M phosphate buffer, pH 8.2 and 0.5 mL of 0.05% TNBS reagent. The mixture was incubated at 50ºC for 1 h in a water bath. The reaction was stopped by adding 1 mL of 0.1 N HCl and left at room temperature for 30 min. Absorbance was monitored at 420 nm. L-Leucine was used as a standard. To determine total amino acid content, mungbean meal was hydrolyzed with 6 N HCl with sample to acid ratio of 1:100 at 120ºC for 24 h. The degree of hydrolysis was calculated following the equation (Benjakul and Morrissey, 1997):

$$\% \text{ DH} = [(A_t - A_0)/A_{max} - A_0)] \times 100$$

Where, A_t was the amount of α-amino group released at time t. A_0 was the amount of α-amino group in the supernatant at 0 h. A_{max} was the total amount of α-amino group obtained after acid hydrolysis.

6.3.6 Antioxidant activity of protein hydrolysate

6.3.6.1 ABTS radical scavenging activity assay

ABTS radical scavenging activity assay was performed according to Re et al. (1999) with some modifications. ABTS radical cation ($ABTS^{\bullet+}$) stock solution was produced by reacting 7 mM ABTS with 2.45 mM potassium persulfate (final concentration) in 10 mM phosphate buffer (pH 7.4) and allowing the mixture in dark at room temperature for 12-16 h. Fresh $ABTS^{\bullet+}$ working solution was prepared by diluting the radical $ABTS^{\bullet+}$ stock solution in 10 mM phosphate buffer (pH 7.4) to

attain absorbance of 0.70 (± 0.02) at 734 nm. Hydrolysates (20 µL) were mixed with 1980 µL of fresh $ABTS^{\bullet+}$ working solution. Then, the reaction mixture was kept in dark for 5 min and absorbance was monitored at 734 nm. ABTS radical scavenging activity of sample at each time interval was expressed as mg Trolox equivalents increment of hydrolysis at 0 h.

6.3.6.2 Ferric reducing antioxidant power assay (FRAP assay)

FRAP assay was performed according to Benzie and Strain (1996) with some modifications. FRAP reagent was prepared freshly and warmed at 37ºC for 10 min before used by mixing 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of 10 mM TPTZ solution in 40 mM HCl, and 2.5 mL of 20 mM $FeCl_3.6H_2O$ solution. Hydrolysates (40 µL) were mixed with 200 µL of distilled water and 1800 µL of FRAP reagent. The absorbance of the reaction mixture was monitored at 593 nm after incubating at 37ºC for 10 min. FRAP value of each time interval was expressed as mM $FeSO_4.7H_2O$ increment of hydrolysis at 0 h.

6.3.6.3 Metal chelating activity assay

Metal chelating activity assay was performed according to Decker and Welch (1990) with some modifications. Hydrolysates (100 µL) were mixed with 1,400 µL of distilled water and 100 µL of 2 mM $FeCl_2.4H_2O$. The reaction mixture was incubated at room temperature for 3 min. Then, the reaction mixture was added with 400 µL of 5 mM ferrozine and incubated at room temperature for 10 min. The absorbance was monitored at 562 nm. Metal chelating activity of sample at each time interval was expressed as mM EDTA increment of hydrolysis at 0 h.

6.3.7 Purification of antioxidant peptides

6.3.7.1 Peptide fractionation

The selected mungbean protein hydrolysate was sequentially fractionated using ultrafiltration membranes with MWCO 30 and 5 kDa installed in a LV centramate^{TM} holder. Three fractions were obtained, namely fraction I with molecular weight (Mw) > 30 kDa (retentate of a 30-kDa membrane), fraction II with Mw 5-30 kDa (retentate of a 5-kDa membrane), and fraction III with Mw < 5 kDa (permeate of a 5-kDa membrane). Each fraction was collected for determination of ABTS radical scavenging activity. Peptide concentration was determined according to Lowry method (Lowry, Rosebrough, Farr, and Randall, 1951) and expressed as mg bovine serum albumin (BSA) equivalents.

6.3.7.2 Ion exchange chromatography

The fraction III showing the highest antioxidative specific activity (mg Trolox/mg) was selected for further purification using an AKTA fast protein liquid chromatography (FPLC) system controlled by the UNICORN^{TM} software version 3.2 (GE Healthcare, Uppsala, Sweden). The sample was loaded onto a DEAE-Sephacel anion exchange column equilibrated with buffer A (50 mM Tris-HCl buffer, pH 8). The unbound peptides were washed with two column volumes of buffer A. Subsequently, the bound peptides were eluted with linear gradient of buffer B (1 M NaCl in 50 mM Tris-HCl buffer, pH 8) from 0-100% with three column volumes at a flow rate of 1 mL/min. Fractions of 5 mL were collected for determination of peptide content at 220 nm and specific antioxidant activity. Fractions having the highest specific antioxidant activity was lyophilized and used for further purification.

6.3.7.3 Gel filtration chromatography

The lyophilized fraction was loaded onto Superdex Peptide 10/300 GL gel filtration column (GE Healthcare, Uppsala, Sweden). The column was equilibrated and eluted with 150 mM NaCl in 50 mM Tris-HCl buffer pH 8 in an isocratic mode at a flow rate of 0.4 mL/min. Fractions of 500 µL were collected and specific antioxidant activity was determined. Antioxidant activity of 1 mM butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and ascorbic acid was also determined and compared with purified peptides of fraction 37 and 42 (F37 and F42).

6.3.8 Identification of peptide sequences

Two fractions of purified peptides (F37 and F42) were separated on a nanocolumn (Acclaim PepMap 100 C18, 3 µm, 100A, 75 µm i.d. x 150 mm) connected to Ultimate 3000 LC System (Dionex, Bannockburn, IL) coupled with ESI-Ion Trap MS (HCT Ultra PTM Discovery System, Bruker, Germany) at a flow rate of 300 nl/min. A solvent gradient (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in 80% acetronitrile) was started from 10-70% solvent B at 0-13 min, 90% solvent B at 13-15 min, and 10% B at 15-20 min. Acquired LC-MS raw data were analyzed with PepNovo software for De novo peptide sequencing (http://proteomics.ucsd.edu/LiveSearch/). Mw and theoretical isoelectric point (pI) of each peptide and of each fraction were estimated using the Compute pI/MW tool (http://web.expasy.org/compute_pi/).

6.3.9 Stability of purified peptides

6.3.9.1 Thermal stability

Thermal stability of the purified peptides was determined by incubating at 25, 37, 50, 60, 70, 80, 90, and 100ºC in a water bath and at 121ºC using an

autoclave for 30 min. Then, the heated samples were immediately cooled in iced water. The remaining specific antioxidant activity was determined.

6.3.9.2 pH stability

The effect of pH on the stability of the purified peptides was investigated at pH 4 (0.1 M sodium acetate), pH 7 (0.1 M phosphate buffer), and pH 10 (0.1 M sodium tetraborate). The samples were mixed with the buffer at a ratio of 1:1. The mixtures were incubated at 37ºC for 30 min. When incubation was reached, the remaining specific antioxidant activity was determined. The effect of each buffer on individual antioxidant assays was also carried out using distilled water.

6.3.10 Statistical analyses

Protein hydrolysis experiments and all chemical analyses were carried in duplicate and mean values were presented. Analysis of variance (ANOVA) was carried out using SPSS program (SPSS version 14, Windows version). Differences among mean values were established using Duncan Multiple Range Test (DMRT) at $P < 0.05$.

6.4 Results and discussion

6.4.1 Degree of hydrolysis (DH)

Protein is a major component of mungbean meal (Table 6.1). Mendoza et al. (2001) reported that the major seed proteins of mungbean are storing globulins of the basic 7S globulins (~3.4%), 8S vicili (~89%) and 11S legumin (~7.6%). The major 8S globulins consist of three homologous isoforms, namely 8Sα′, 8Sα, and 8Sβ. Bernardo et al. (2004) found that both α and β subunits of soybean β-conglycinin (7S globulin) showed the highest homology of 58-61% with 8Sα′, 59-61% with 8Sα, and 66-68%

with 8Sβ.

Table 6.1 Proximate composition of mungbean meal powder (% wet basis).

Composition	Content (%)
Moisture	6.73 ± 0.02
Crude protein	76.79 ± 0.59
Crude fat	1.32 ± 0.17
Crude fiber	0.21 ± 0.01
Ash	2.11 ± 0.32
Carbohydrate (by difference)	12.85 ± 0.72

In order to compare efficacy of *Virgibacillus* sp. SK37 proteinase with commercial proteinases, the amount of *Virgibacillus* sp. SK37 proteinase was added to attain comparable DH to the commercial enzymes. DH of mungbean hydrolysates increased with hydrolysis time in all proteinases ($P < 0.05$, Figure 6.1). DH of Alcalase-hydrolyzed mungbean meal (AH) rapidly increased within the first hour and slowly increased thereafter. At any time of hydrolysis, AH showed the highest DH ($P < 0.05$), whereas *Virgibacillus* sp. SK37 proteinase-hydrolyzed mungbean meal (VH) and Neutrase-hydrolyzed sample (NH) showed comparable DH ($P > 0.05$). As expected, DH of AH showed higher than NH. Alcalase appeared to hydrolyze mungbean proteins to a greater extent than Neutrase. Peña-Ramos and Xiong (2002) also showed that Alcalase hydrolyzed soy protein isolate to a greater extent than did Flavourzyme and Protamex. Alcalase is a subtilisin-like endoproteinase having broad specificity toward aromatic (Phe, Trp, and Tyr), acidic (Glu), sulfur-containing (Met), aliphatic (Leu and Ala), hydroxyl (Ser), and basic (Lys) residues (Doucet, Otter,

Gauthier, and Foegeding, 2003). In contrast, Neutrase is a metallo-proteinase, which preferably cleaves peptide bonds with hydrophobic amino acids (Rao, Tanksale, Ghatge, and Deshpande, 1998).

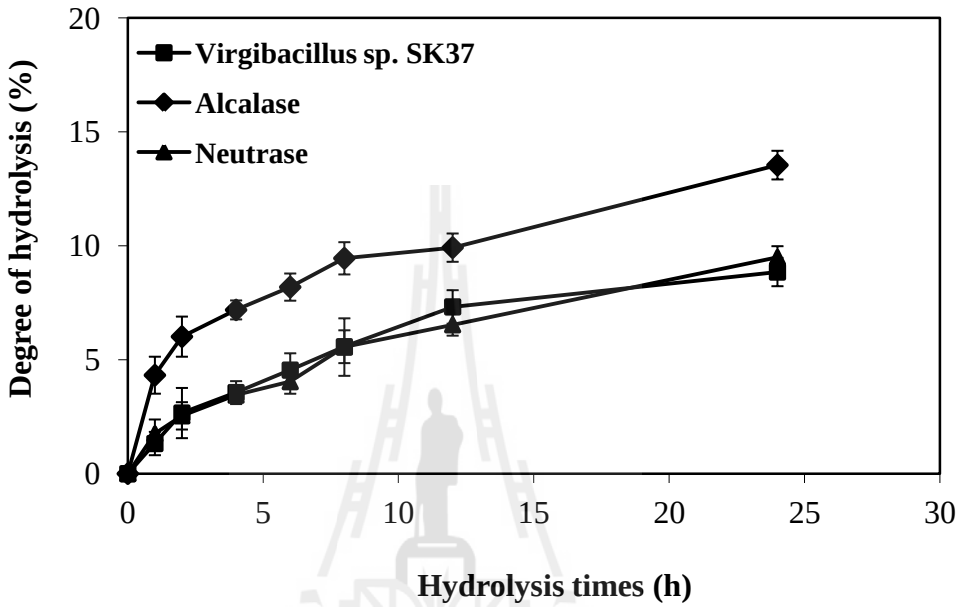

Figure 6.1 Degree of hydrolysis of mungbean meal hydrolysate treated with *Virgibacillus* sp. SK37 proteinase, Alcalase, and Neutrase.

6.4.2 Antioxidant activities of mungbean protein hydrolysates

ABTS radical scavenging activity of VH, AH, and NH increased with DH ($P < 0.05$, Figure 6.2a). ABTS radical scavenging activity of VH, AH, and NH were comparable ($P > 0.05$) within 12-h hydrolysis. At 24-h hydrolysis, VH and NH showed higher ABTS radical scavenging activity than AH despite that DH of the former two (8.9, 9.5%) was lower than the latter (13.5%) ($P < 0.05$). *Virgibacillus* sp. SK37 proteinase could be used as a processing-aid to produce peptides with high radical scavenging activity as compared to Neutrase. VH and NH might contain

specific amino acids in peptide sequences which are more important factor for antioxidant activity than the length of peptides governed by DH. Several amino acids, such as Trp, Tyr, His, Met, Leu, and Cys have been reported to possess radical scavenging activity (Chen, Muramoto, Yamauchi, and Nokihara, 1996; Park, Jung, Nam, Shahidi, and Kim, 2001). Trp, Tyr, and His residue contained the indolic, phenolic, and imidazole group, respectively, which serve as hydrogen donors. Aromatic amino acid (Tyr and Phe) have ability to donate protons easily to electron deficient radicals, while maintain their stability via resonance structures. In addition, Met and Cys have ability to donate their sulfur hydrogen (Ajibola, Fashakin, Fagbemi, and Aluko, 2011; Hernández-Ledesma, Dávalos, Bartolomé, and Amigo, 2005; Rajapakse, Mendis, Byun, and Kim, 2005). Thus, these amino acids are considered as potent radical scavengers.

FRAP of VH, AH, and NH rapidly increased during the first hour of hydrolysis (Figure 6.2b). As DH increased, the reducing power of VH did not change ($P > 0.05$), while reducing power of NH and AH increased after 12- and 24-h hydrolysis, respectively. However, the reducing power of all samples at 24-h hydrolysis was comparable ($P > 0.05$). This method is based on the ability of a compound to donate one electron to Fe^{3+} to reduce it to Fe^{2+}, whereas ABTS radical scavenging assay is involved both electron and hydrogen atom transfer. At 24-h hydrolysis, VH seemed to have higher hydrogen atom transfer ability than AH and showed comparable electron and hydrogen atom transfer ability to NH.

An increase in reducing power as DH increased has also been reported in Alcalase-hydrolysed porcine plasma protein (Liu, Kong, Xiong, and Xia, 2010) and canola protein hydrolysate (Cumby, Zhong, Naczk, and Shahidi, 2008). Raghavan,

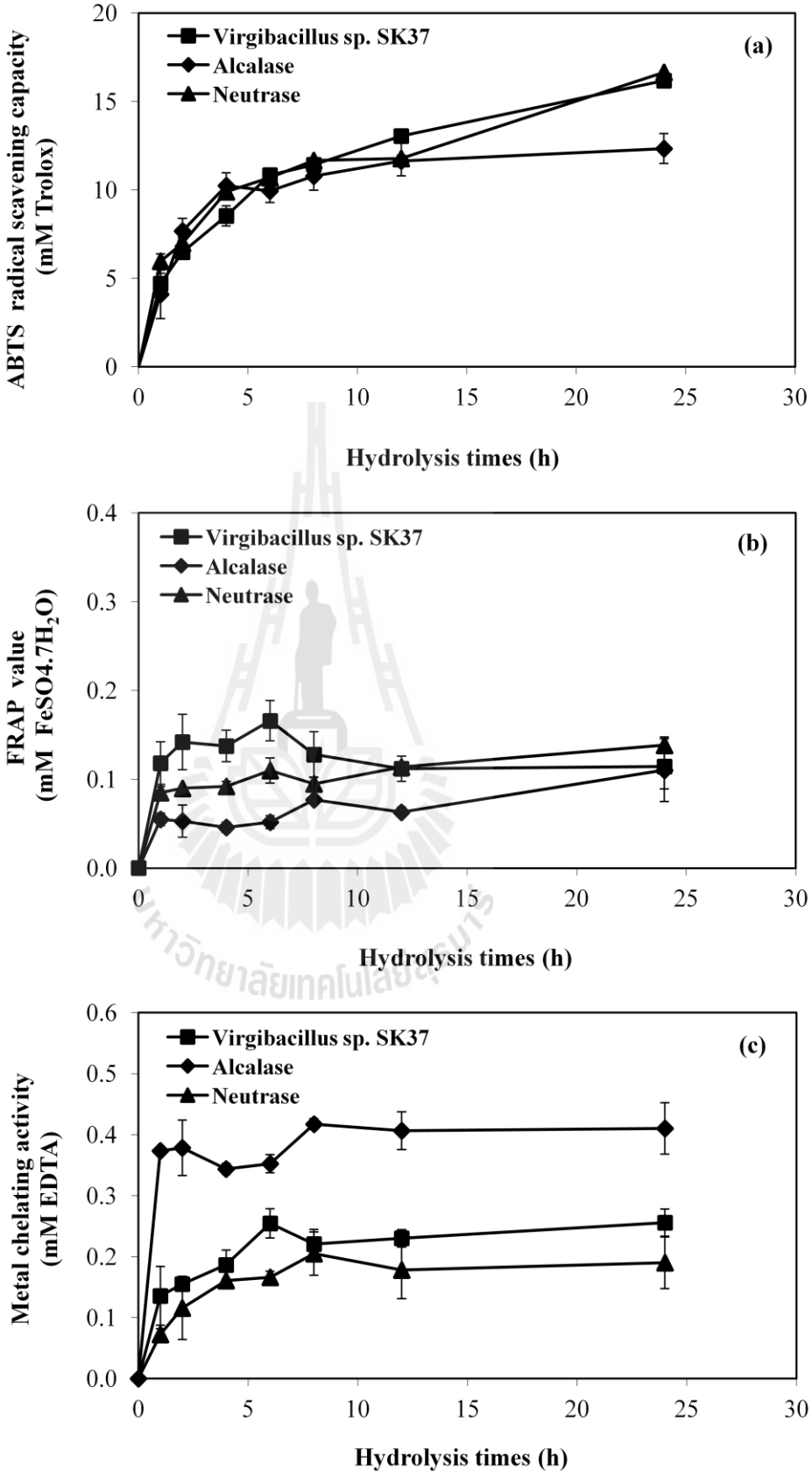

Figure 6.2

(see next page)

Figure 6.2 ABTS radical scavenging activity (a), ferric reducing antioxidant power (b), and metal chelating activity (c) of mungbean meal hydrolysates prepared by *Virgibacillus* sp. SK37 proteinase, Alcalase, and Neutrase.

Kristinsson, and Leeuwenburgh (2008) also reported that the reducing power of tilapia hydrolysates prepared from Flavourzyme increased with an increase in %DH, whereas reducing power of those prepared from *Bacillus subtilis* proteinase did not vary with the extent of DH. These results demonstrated that reducing power ability of protein hydrolysate vastly depends on the type of proteinase.

The highest chelating activity was found in AH ($P < 0.05$), whereas chelating activity of VH and NH were comparable ($P > 0.05$) at any time of hydrolysis (Figure 6.2c). Chelating activity of all hydrolysates well correlated with DH ($P < 0.05$). Higher ferrous chelating activity was also reported when DH of silver carp hydrolysate increased (Dong et al., 2008). Transition metals may act as catalysts that promote the generation of the first few radicals, which initiate the oxidative chain reaction (Liu et al., 2010). The carboxylic and amino groups in branches of acidic (Asp and Glu) and basic (Arg, Lys, and His) amino acids have been reported to enhance metal chelation through their charged residues properties (Liu et al., 2010; Rajapakse et al., 2005; Saiga, Tanabe, and Nishimura, 2003). At high pH, carboxyl residues of acidic amino acids are charged to form anions. These residues should be involved in the formation of complexes with metal ions.

For basic amino acids, amino nitrogen at side chain loses its proton nitrogen, resulting in nitrogen with unshared pairs of electrons which can also bind to the metal atoms (Zaida, Farooqui, and Janrao, 2012). In addition, the position of these amino

acids was also reported to be important for chelating activity. Chen et al. (1998) demonstrated that peptides containing His residues at the N-terminus showed higher affinity than those with His residues at the C-terminus. The chelating of transition metal ions by these amino acids would reduce available transition metals, rendering the inhibition of the radical-mediated oxidative chain reactions.

6.4.3 Purification of antioxidant peptide

Since VH showed the highest ABTS radical scavenging activity at 24-h hydrolysis, it was selected for peptide purification. Ultrafiltration yielded a fraction with Mw < 5 kDa (fraction III) which showed the highest specific antioxidant activity (Table 6.2).

Table 6.2 Purification of antioxidative peptides from *Virgibacillus* sp. SK37-hydrolyzed mungbean meal.

Steps	Fraction	Peptide content (mg/mL)	Antioxidant activity (mg Trolox/mL)	Specific activity (mg Trolox/mg)	Fold
Ultrafiltration	Crude	3223.93	363.29	0.11	1.00
	> 30 kDa	289.91	41.42	0.14	1.27
	5-30 kDa	111.64	4.26	0.04	0.36
	< 5 kDa	1726.66	270.84	0.16	1.45
Ion exchange chromatography	F4-F7	1.13	0.28	0.25	2.27
	F18-F21	0.43	0.08	0.18	1.64
Gel filtration chromatography	F37	0.03	0.07	2.47	22.45
	F42	0.15	0.28	1.87	17.00

Lower-Mw peptides have been reported to exhibit better radical scavenging activities than the higher-Mw counterparts (Ajibola et al., 2011; Aluko and Monu, 2003; Girgih, Udenigwe, and Aluko, 2011). Therefore, this fraction was selected for further purification and characterization. Ion exchange chromatography on DEAE-Sephacel column of fraction III resulted in unbound and bound fraction (Figure 6.3). Specific antioxidant activity of unbound fractions (F4-F7) was higher than that of bound counterparts (F18-F21).

Since ion exchange chromatography separates samples based on charge characteristics, peptides in unbound fractions possessed a net positive charge at pH 8 and were speculated to have isoelectric point (pI) higher than 8. These peptides were likely to contain various residues of Arg or Lys based on pI value of each amino acid. There are two amino acids containing pI above 8, such as Arg (pI=10.8) and Lys (pI=9.7). As described above, pIs of unbound peptides should be higher than 8. Thus, these peptides likely contained many Arg or Lys residues. The potent unbound fraction pool was further subjected to gel filtration chromatography. After gel filtration chromatography (Figure 6.4), specific antioxidant activity of fraction 37 (F37) was higher than that observed in fraction 42 (F42) (Table 6.2). This method based on different peptide size during flow through a gel filtration medium. The bigger proteins cannot penetrate inside the matrix and are dispersed through the mobile phase. The smaller peptides can penetrate inside the matrix and stay longer in the column. This result confirmed that peptide size is not the only factor governing antioxidative capacity as size of peptides of F37 was higher than that of F42. Amino acid composition of peptides played a more important role for antioxidant activity than its mass. As purification fold of F37 and F42 increased about 20 times, they were

selected for peptide sequence identification. In F37, it is observed that all peptides contained Arg residue at the C-termini. High specific antioxidant activity for this fraction might be associated with the presence of C-terminal Arg.

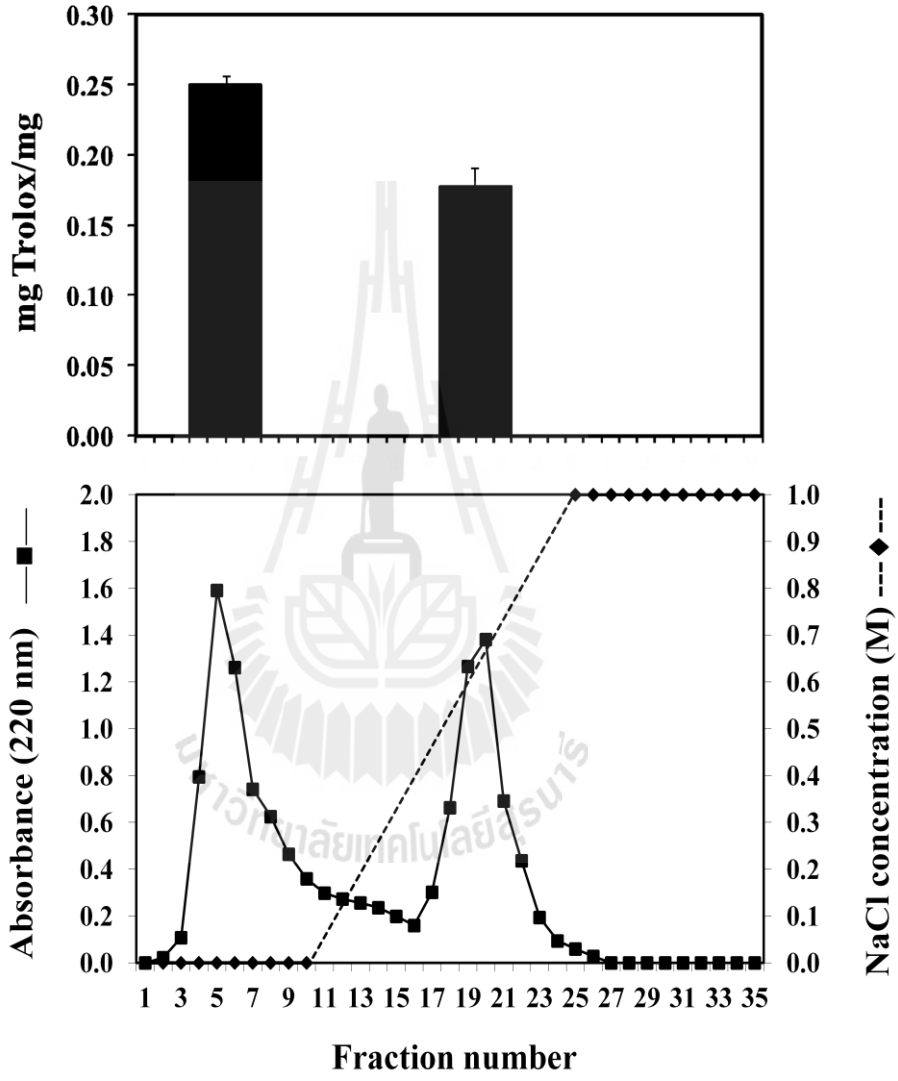

Figure 6.3 Ion exchange chromatography on DEAE-Sephacel column (25x50 mm) of mungbean meal hydrolysate. Specific antioxidant activity (mg Trolox/mg) of unbound fraction pool (F4-F7) and bound fraction pool (F18-F21) are indicated as solid bars in upper panel.

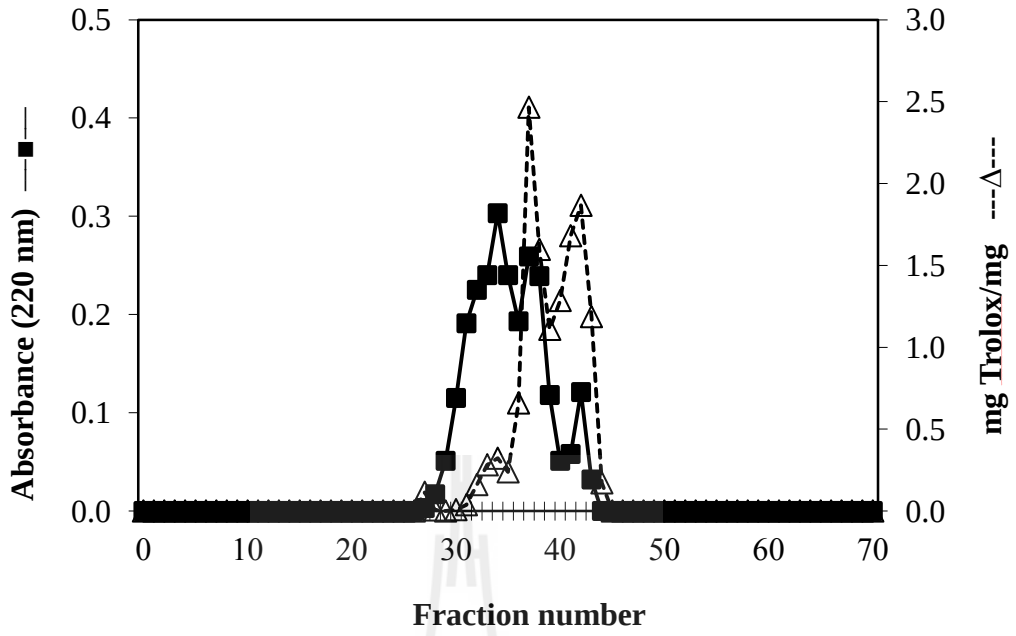

Figure 6.4 Gel filtration chromatography on Superdex Peptide 10/300 GL column (10x300 mm) and specific antioxidant activity (mg Trolox/mg) of unbound fraction pool (F4-F7) obtained from DEAE-Sephacel column.

6.4.4 Identification of peptide sequences

Four predominant peptides composing of 7-11 amino acid residues were found in F37 and F42 (Table 6.3). Lys and Arg were found to distribute in all peptides, contributing to basic pI. The theoretical pI values of F37 and F42 were estimated to be 9.69 and 9.99, respectively. This was in agreement with a positive charge characteristic which did not bind to DEAE-Sephacel column at pH 8. Arg^{22} of oxidized insulin B is one of preferred cleavage sites of *Virgibacillus* sp. SK33 proteinase (Sinsuwan et al., 2010). The purified peptide, Gln-Gly-Ala-Arg, from porcine skin collagen hydrolysates with C-terminal Arg was reported to have the highest radical scavenging activity (Li, Chen, Wang, Ji, and Wu, 2007).

Table 6.3 Amino acid sequence and theoretical Mw/pI of of antioxidative peptides from *Virgibacillus* sp. SK37-hydrolyzed mungbean meal.

Fraction	Sequence	Mass (Da)	pI	
37	Phe-Leu-Gly-Ser-Phe-Leu-Tyr-Glu-Tyr-Ser-Arg	1380.67	6.00	9.69
	Ala-Val-Lys-Pro-Glu-Pro-Ala-Arg	866.50	8.79	
	Gly-Val-Gly-Leu-Phe-Val-Arg	746.44	9.75	
	His-Asn-Val-Ala-Met-Glu-Arg	855.40	6.75	
42	Leu-Gly-Ser-Phe-Leu-Tyr-Glu-Tyr-Ser-Arg	1233.60	6.00	9.99
	Leu-Leu-Pro-His-Leu-Arg-Arg	903.58	12.00	
	Phe-Asn-Val-Pro-Ala-Thr-Lys	775.42	8.75	
	Ser-Gly-Val-Val-Pro-Gly-Tyr	677.34	5.24	

Arg, a diamine monocarboxylic amino acid with a guanidinium group, has been confirmed to have the antioxidant effect (Lass, Suessenbacher, Wölkart, Mayer, and Brunner, 2002; Lin, Yang, Chen, Huang, and Lee, 2005).

Antioxidant peptides, namely Phe-Pro-Leu-Glu-Met-Met-Pro-Phe and His-Asn-Gly-Asn, were obtained from extruded corn gluten and porcine plasma protein hydrolysates prepared by Alcalase, respectively (Liu et al., 2010; Zheng et al., 2006). The presence of Phe and His at N-terminal might contribute to high radical scavenging radical scavenging activity of peptide via its ability to donate proton of benzene ring and imidazole group, respectively. Therefore, Phe-Leu-Gly-Ser-Phe-Leu-Tyr-Glu-Tyr-Ser-Arg, His-Asn-Val-Ala-Met-Glu-Arg, and Phe-Asn-Val-Pro-Ala-Thr-Lys can be expected to be important antioxidative peptide in F37 and F42. In addition, Tyr and Met which are considered as hydrogen donors were also found in these mungbean peptides.

Two out of the four peptides in F42 were observed to have Leu residue at N-termini. The presence of N-terminal Leu was shown to be associated with antioxidant activity of soybean peptide prepared by proteinase S from *Bacillus* sp. (Chen et al., 1995). In addition, hydrolysate from another sources of peptides containing Leu residue at their N-terminal position have been reported to play an important role in antioxidant activity, including lecithin-free egg yolk hydrolysate (Park et al., 2001), bullfrog skin (Qian, Jung,, and Kim, 2008), and bigeye tuna (*Thunnus obesus*) (Je, Qian, Lee, Byun, and Kim, 2008). However, Leu residue at C-terminal has also been reported to be associated with radical scavenging activity of buckwheat peptide (Ma, Xiong, Zhai, Zhu, and Dziubla, 2010). High antioxidative potency of Leu is due to long aliphatic side chain, which can increase interaction between peptides and fatty acids (Mendis, Rajapakse, and Kim, 2005; Tang et al., 2010). In addition, Beermann et al. (2009) reported that three out of the nine peptides of soybean protein hydrolysate prepared by trypsin, including Thr-Thr-Tyr-Tyr, Leu-Tyr, and Ile-Tyr, revealed radical scavenging properties. Peptides with Tyr at C-terminal also possessed huge radical scavenger. Therefore, the mungbean peptide, Ser-Gly-Val-Val-Pro-Gly-Tyr, is also speculated to contribute to radical scavenging activity of F42.

It should be noted that F42 at the concentration of 0.148 mg/mL showed comparable ABTS radical scavenging activity to 1 mM BHT ($P > 0.05$, Figure 6.5) and higher than that of 1 mM ascorbic acid ($P < 0.05$). However, F42 showed lower ABTS radical scavenging activity than 1 mM of BHA ($P < 0.05$). As compared to F37, higher radical scavenging activity of F42 is likely due to its high peptide content. It should be noted that specific antioxidant activity of F37 was estimated to be higher

than that of F42 (Table 6.2). Both fractions (F37 and F42) showed potential as natural antioxidant peptides with high radical scavenging activity.

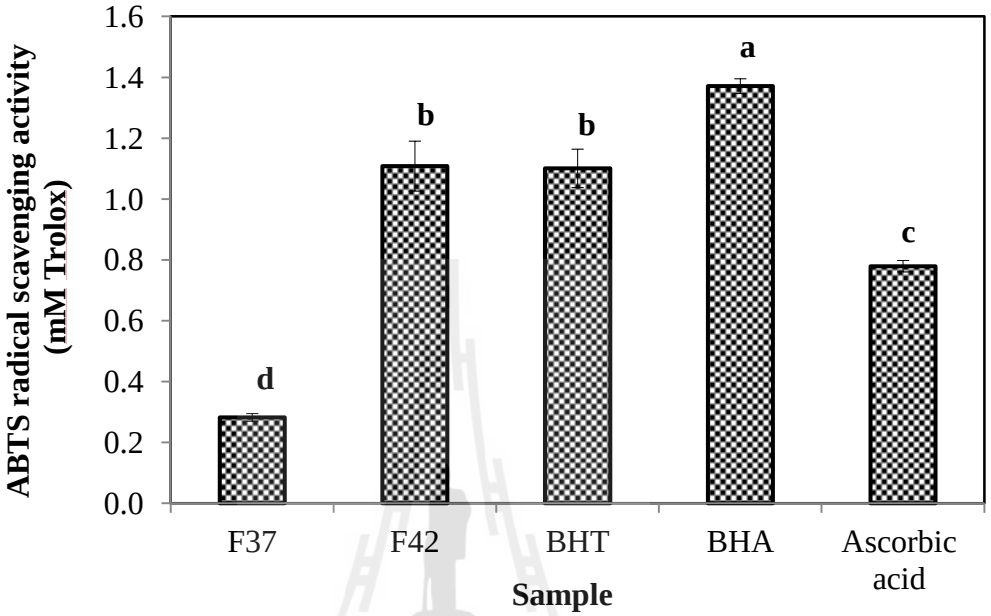

Figure 6.5 ABTS radical scavenging activity of purified peptides in comparison with 1 mM of BHT, BHA, and ascorbic acid. The concentration of sample F37 and F42 were 0.027 and 0.148 mg/mL, respectively. Letters indicate significant differences at $P < 0.05$.

6.4.5 Thermal and pH stability

Peptides in F37 and F42 showed high thermal stability at studied temperatures, particularly under sterilization (Figure 6.6a). This result suggested that these peptides could be used in thermally-processed food without a reduced antioxidant activity. Tong, Sasaki, McClements, and Decker (2000) demonstrated that high Mw whey proteins aggregated and lost almost 90% of free radical scavenging activity after heating at 70°C for 15 min. Antioxidant protein from curry leaves showed a reduced

activity after being subjected to 65 and 95°C for 20 min (Ningappa and Srinivas, 2008). Based on this study, mungbean peptides with relatively small masses (677-1,380 Da) did not undergo aggregation under thermal treatment and their antioxidant activity remained unchanged.

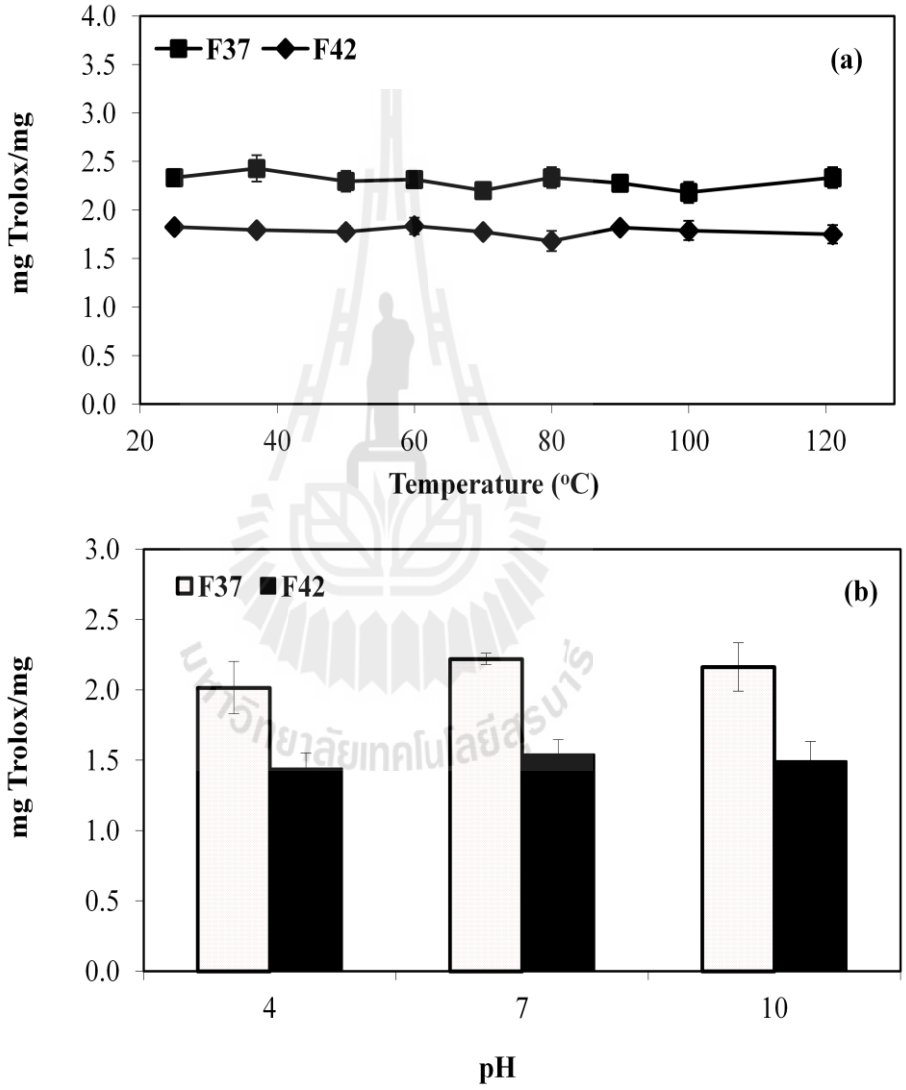

Figure 6.6 ABTS radical scavenging activities of purified peptides (F37 and F42) from mungbean meal hydrolysate after incubation at different temperatures (a) and pHs (b) for 30 min.

The influences of pH on the ABTS radical scavenging activity of purified peptides were shown in Figure 6.6b. Activity of F37 and F42 showed comparable at all pHs studied ($P > 0.05$). This result suggested that these 2 fractions could be applied in a wide pH range without affecting the antioxidant activity. Liu et al. (2010) demonstrated that the Alcalase-hydrolyzed plasma protein were soluble over a wide range pH 3.0-8.0 and exhibited significantly higher solubility and radical scavenging ability than that of non-hydrolyzed plasma protein. This might be because the cleavage of peptide bonds enhances levels of free amino and carboxyl groups resulting in enhanced solubility which positively affect the antioxidant activity. As compared to native protein, peptides with the short chains of amino acids are not much affected by charge modification governed by pH changes and might also can further its function. Radical scavenging activity of protein hydrolysates from yellow stripe trevally (*Selaroides leptolepis*) was reported to be stable over a wide pH range (2-12) (Klompong, Benjakul, Kantachote, Hayes, and Shahidi, 2008). The higher solubility of small peptide over a wide range of pH might positively affect the antioxidant activity.

6.5 Conclusions

Virgibacillus sp. SK37 proteinases have a potential to produce peptides with ABTS radical scavenging activity, reducing power, and metal chelating activity as compared to Neutrase. Peptides from mungbean hydrolysate prepared from *Virgibacillus* sp. SK37 proteinase possess high antioxidant potency which might be associated with the presence of C-terminal Arg and consisted of amino acids which serves as hydrogen donors, including Tyr, Phe, Met, and His. In addition, the purified

peptides showed high ABTS radical scavenging as compared to synthetic antioxidant and were stable over a wide pH and temperature range. Mungbean meal hydrolysate could be a promising source of bioactive peptides.

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CHAPTER VII

SUMMARY

Odor-active compounds in two commercial Thai fish sauce were performed by aroma extract dilution analysis (AEDA) and static headspace dilution analysis (SHDA) using gas chromatography-olfactometry (GC-O) and GC-mass spectrometry (GC-MS). Quantitative data using stable isotope dilution assays (SIDA) and their odor activity values (OAVs) revealed that 6 compounds, including methanethiol, 2-methylpropanal, 3-methylbutanal, dimethyl trisulfide, 3-(methylthio)propanal, and butanoic acid showed the highest OAVs (>500). Results from the ranking test revealed that sulfur-containing compounds had the highest influence on the overall aroma of fish sauce, followed by acids and aldehydes.

Addition of *Virgibacillus* sp. SK37 at 15% salt content increased protein hydrolysis only at the early stage of fermentation, whereas it did not accelerate protein hydrolysis at 20% salt content at any time during fermentation. *Virgibacillus* sp. SK37 under reduced salt addition of 15-20% appeared to increase the content of 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, acetic acid, and 2-methylpropanoic acid. However, only aldehyde compounds were found to have an effect on the overall aroma of fish sauce based on high OAV values, suggesting that samples inoculated with *Virgibacillus* sp. SK37 under reduced salt content of 15-20**%** contributed to stronger malty and/or dark chocolate note.

Optimal conditions for the highest proteinase yield were 1%(w/v) spent

brewery yeast sludge medium containing 2.5% NaCl, pH 7.5, incubated at 40^{o}C for 4 days. In addition, *Virgibacillus* sp. SK37 could be a promising source of enzyme that has the potential to produce peptides with 2,2′-azinobis (3-ethyl-benzothiazoline-6-sulfonate) (ABTS) radical-scavenging activity, reducing power, and metal-chelating activity similarly to Neutrase. Peptides from mungbean hydrolysates prepared from *Virgibacillus* sp. SK37 proteinase possess a high antioxidant potency which might be associated with the presence of Arg at C-termini. In addition, the purified peptides showed high ABTS radical-scavenging activity compared to synthetic antioxidants and are stable over a wide pH (4-10) and temperature (25-121°C) range.

Virgibacillus sp. SK37 could be a potential strain to be used as a starter culture in fish sauce fermentation at 15-20% salt content and promising source of proteinase for the production of mungbean meal hydrolysate with antioxidant properties.

APPENDIX

Supporting materials for Chapter III and IV

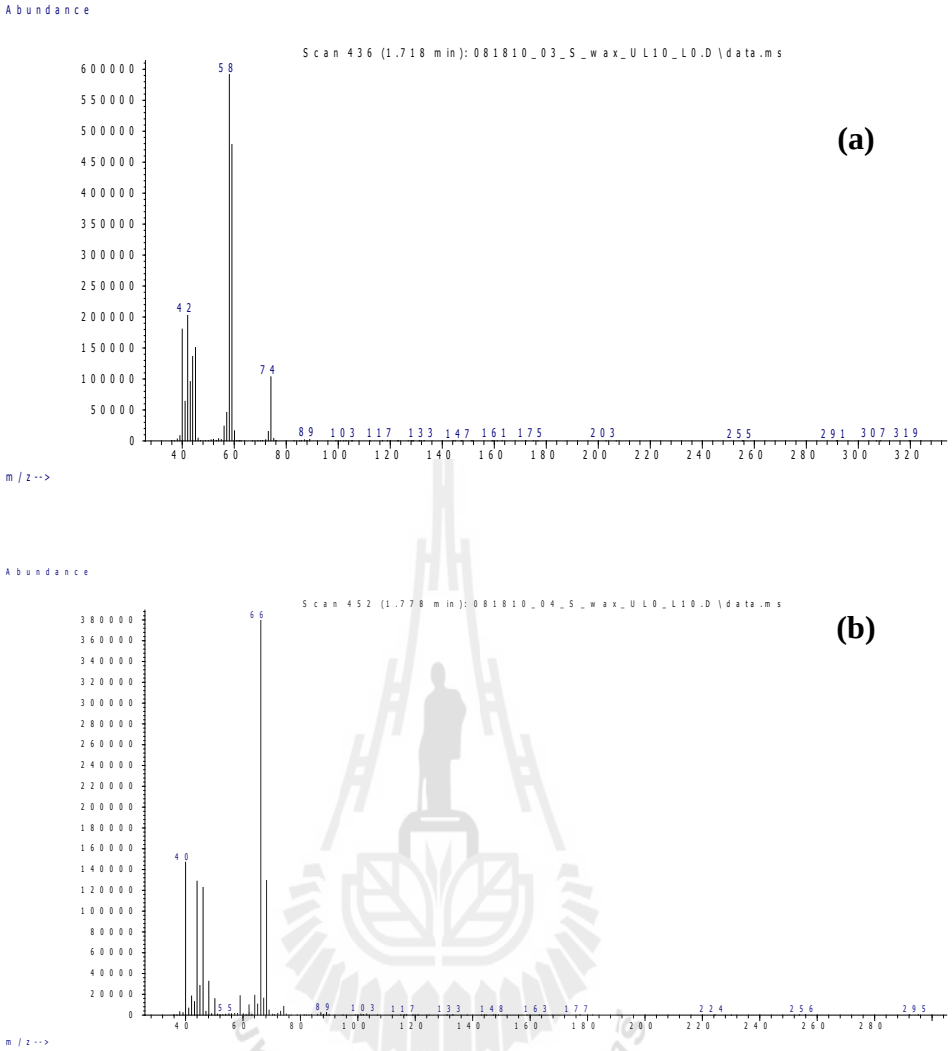
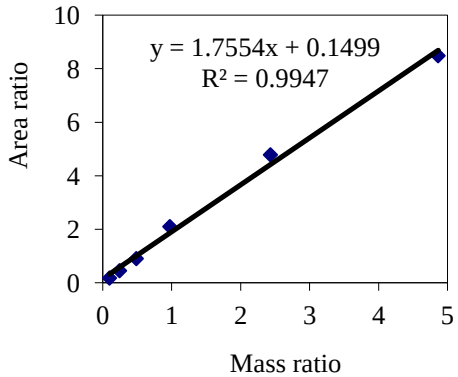


Figure 1A Mass spectra of trimethylamine (a) and [$^2H_9$]-trimethylamine (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	4.862	8.479	
2	2.431	4.776	
3	0.972	2.093	1/1.7554
4	0.486	0.898	**= 0.57**
5	0.243	0.445	
6	0.097	0.170	

Figure 1B Standard curve for determining response factor of trimethylamine.

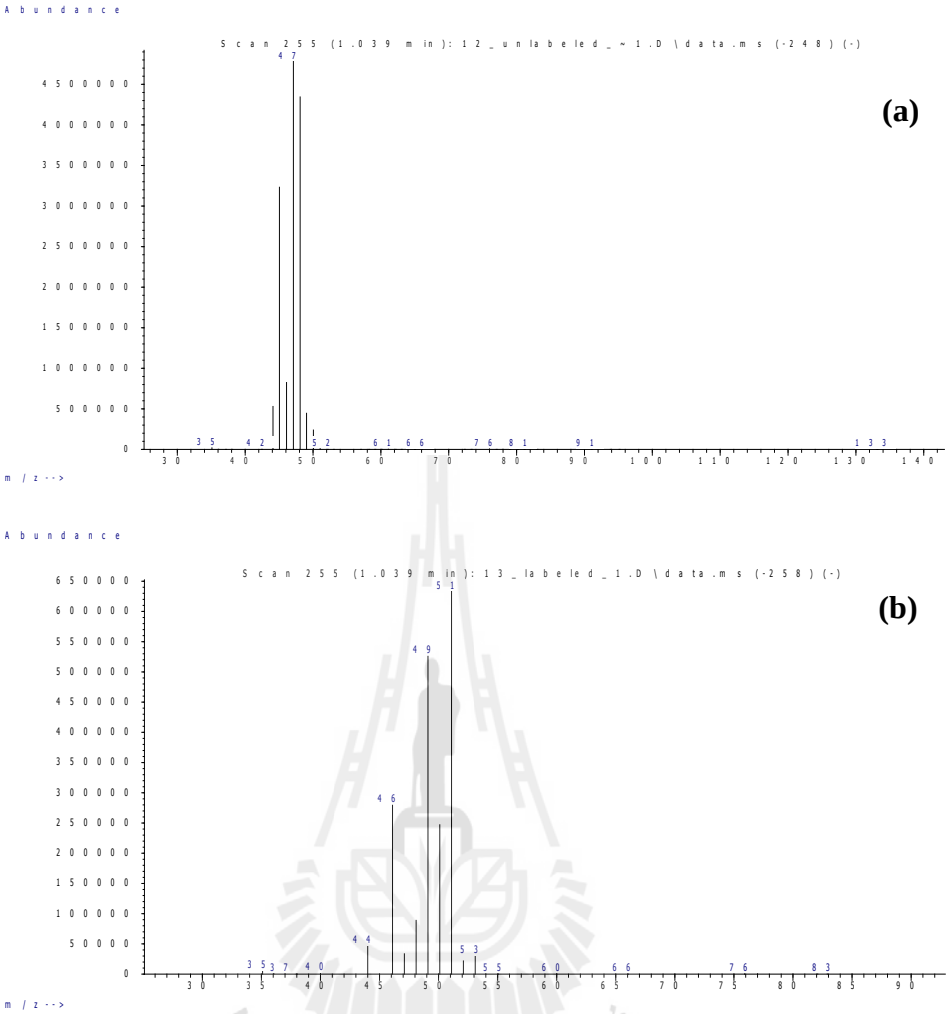
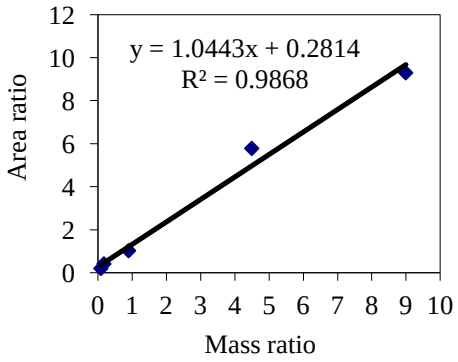


Figure 2A Mass spectra of methanethiol (a) and [$^{2}H_3$]-methanethiol (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	8.994	9.295	1/1.0443 = **0.96**
2	4.497	5.780	
3	0.899	1.027	
4	0.180	0.413	
5	0.090	0.202	

Figure 2B Standard curve for determining response factor of methanethiol.

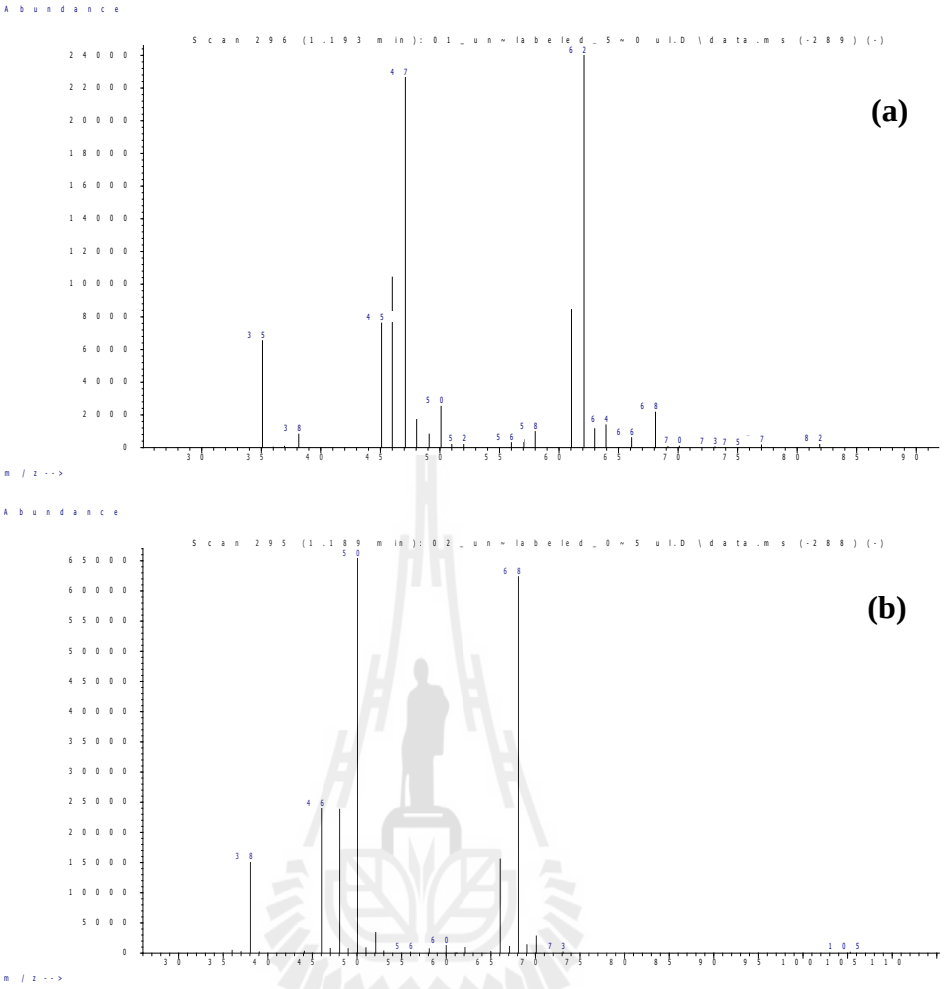
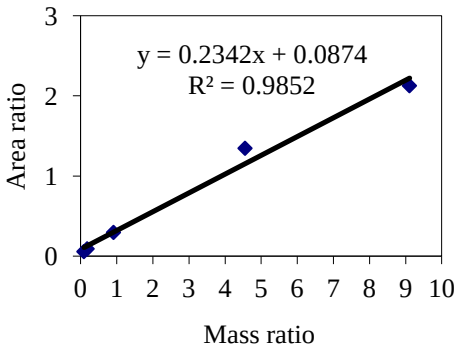


Figure 3A Mass spectra of dimethyl sulfide (a) and [$^2H_6$]-dimethyl sulfide (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	9.108	2.126	1/0.2342 = **4.27**
2	4.554	1.345	
3	0.911	0.295	
4	0.182	0.091	
5	0.091	0.056	

Figure 3B Standard curve for determining response factor of dimethyl sulfide.

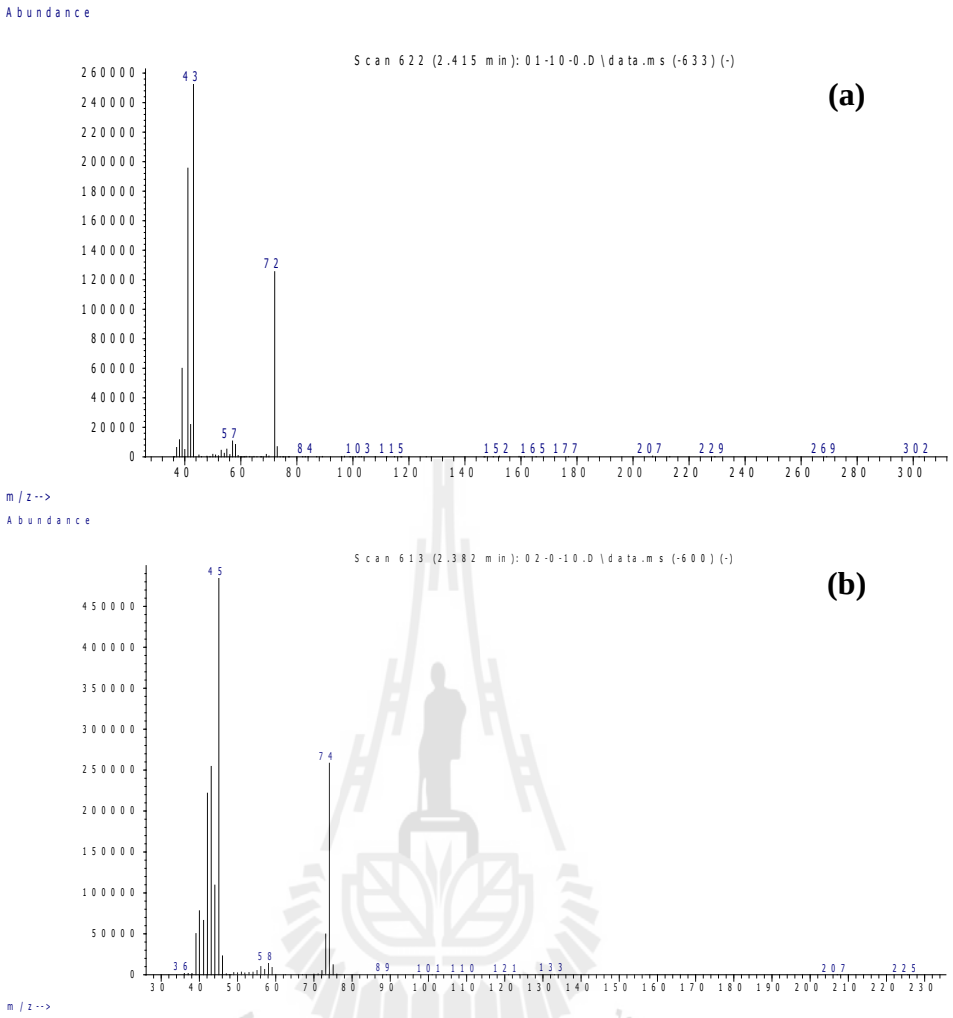
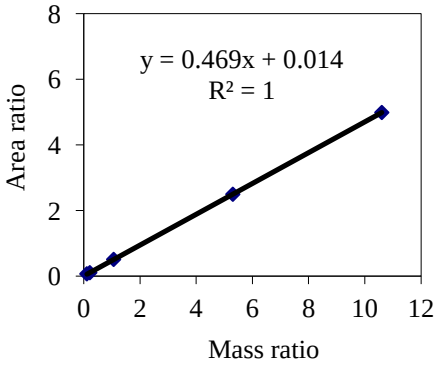


Figure 4A Mass spectra of 2-methylpropanal (a) and [$^{2}H_{2}$]-2-methylpropanal (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.106	0.072	1/0.469 = **2.13**
2	0.212	0.107	
3	1.060	0.511	
4	5.302	2.497	
5	10.604	4.988	

Figure 4B Standard curve for determining response factor of 2-methylpropanal.

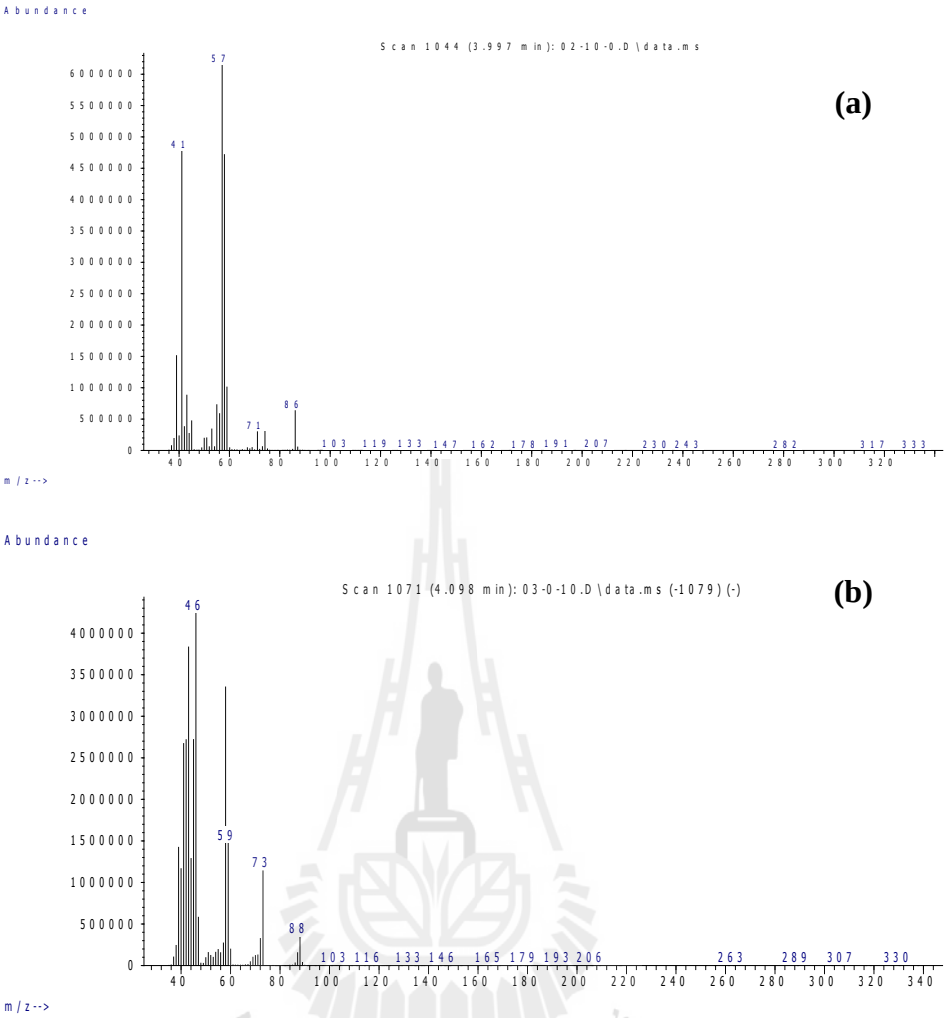
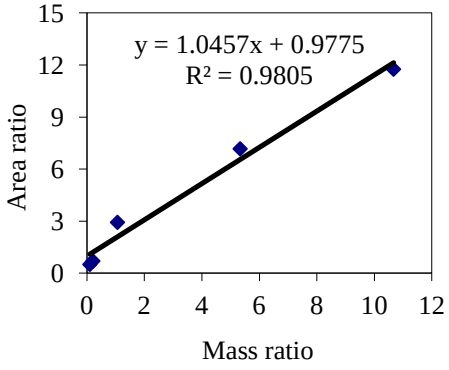


Figure 5A Mass spectra of 2-methylbutanal (a) and [$^{2}H_{2}$]-3-methylbutanal (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.107	0.507	1/1.0457 = **0.96**
2	0.213	0.705	
3	1.067	2.932	
4	5.335	7.171	
5	10.671	11.760	

Figure 5B Standard curve for determining response factor of 2-methylbutanal.

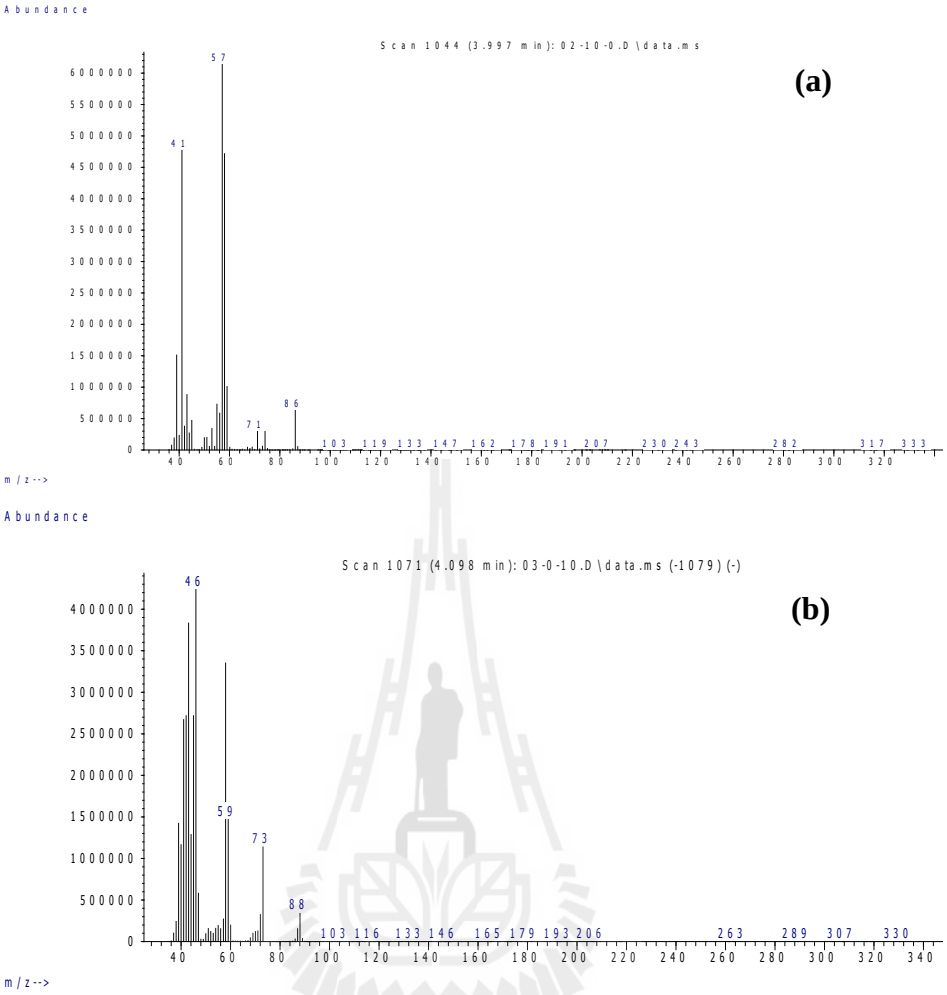
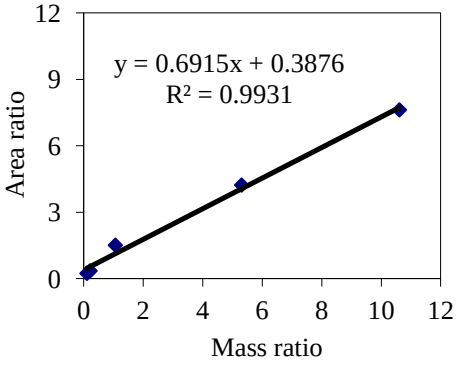


Figure 6A Mass spectra of 3-methylbutanal (a) and [$^2H_2$]-3-methylbutanal (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.106	0.228	1/0.6915 = **1.45**
2	0.212	0.341	
3	1.061	1.500	
4	5.306	4.216	
5	10.611	7.614	

Figure 6B Standard curve for determining response factor of 3-methylbutanal.

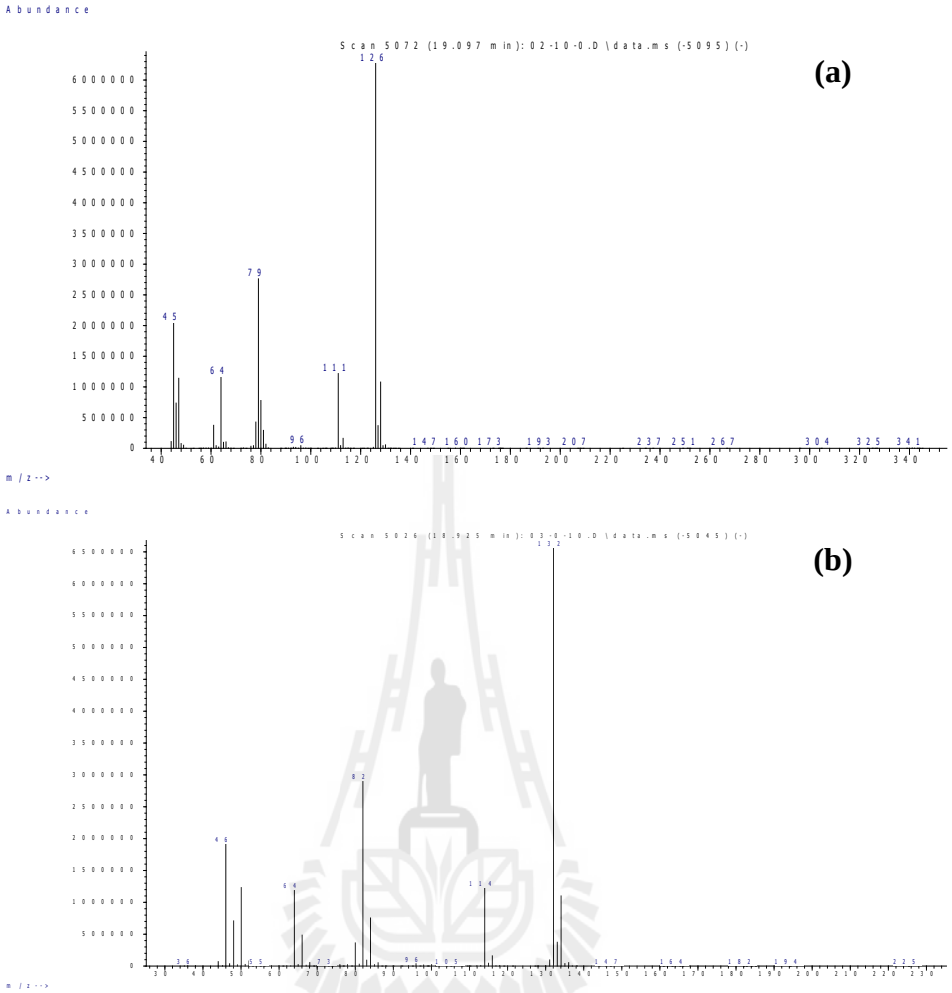
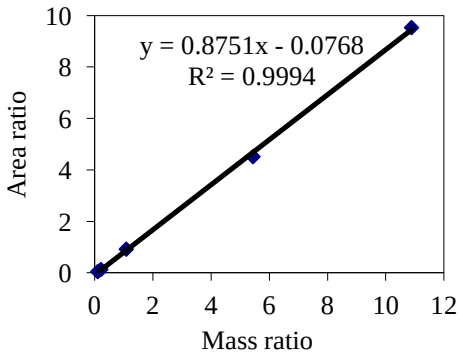


Figure 7A Mass spectra of dimethyl trisulfide (a) and [$^2H_6$]-dimethyl trisulfide (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.109	0.049	1/0.8751 = **1.14**
2	0.218	0.130	
3	1.089	0.920	
4	5.444	4.516	
5	10.888	9.532	

Figure 7B Standard curve for determining response factor of dimethyl trisulfide.

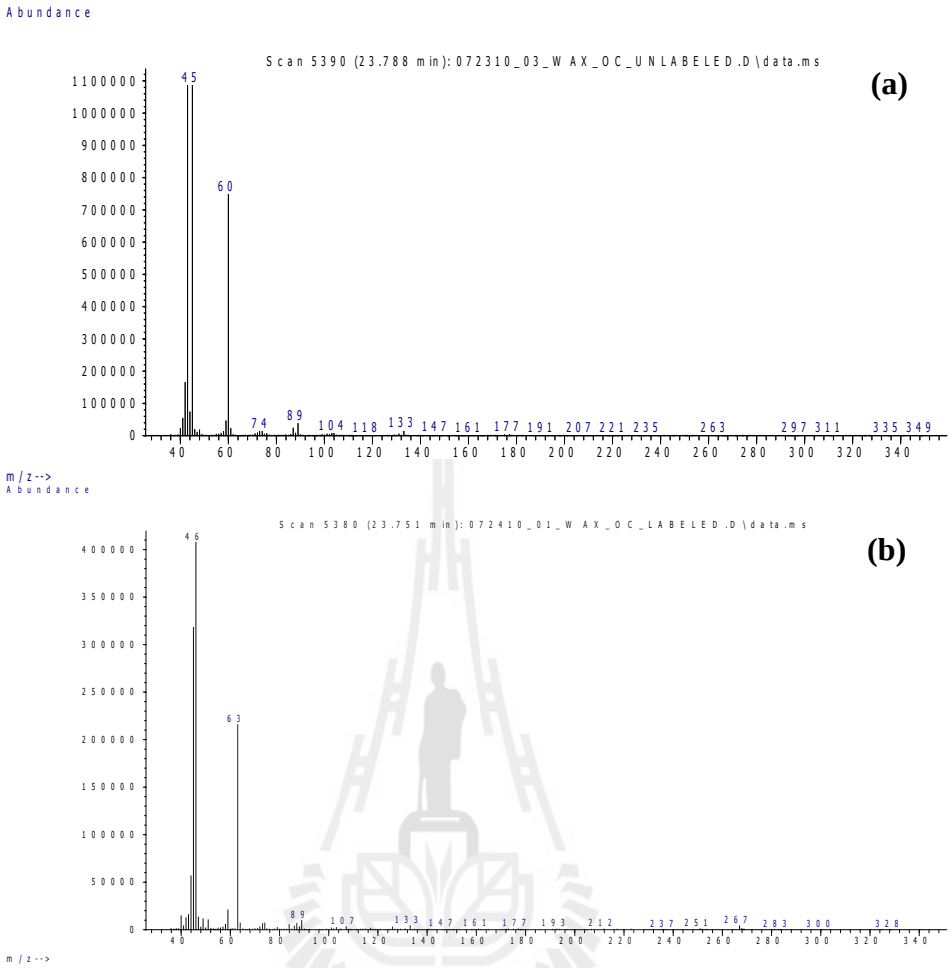
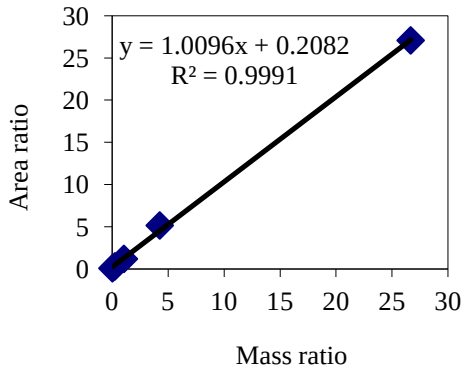


Figure 8A Mass spectra of acetic acid (a) and [$^{2}H_{3}$]-acetic acid (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.043	0.052	1/1.0096 = **0.99**
2	0.267	0.279	
3	1.067	1.164	
4	4.268	5.126	
5	26.676	27.049	

Figure 8B Standard curve for determining response factor of acetic acid.

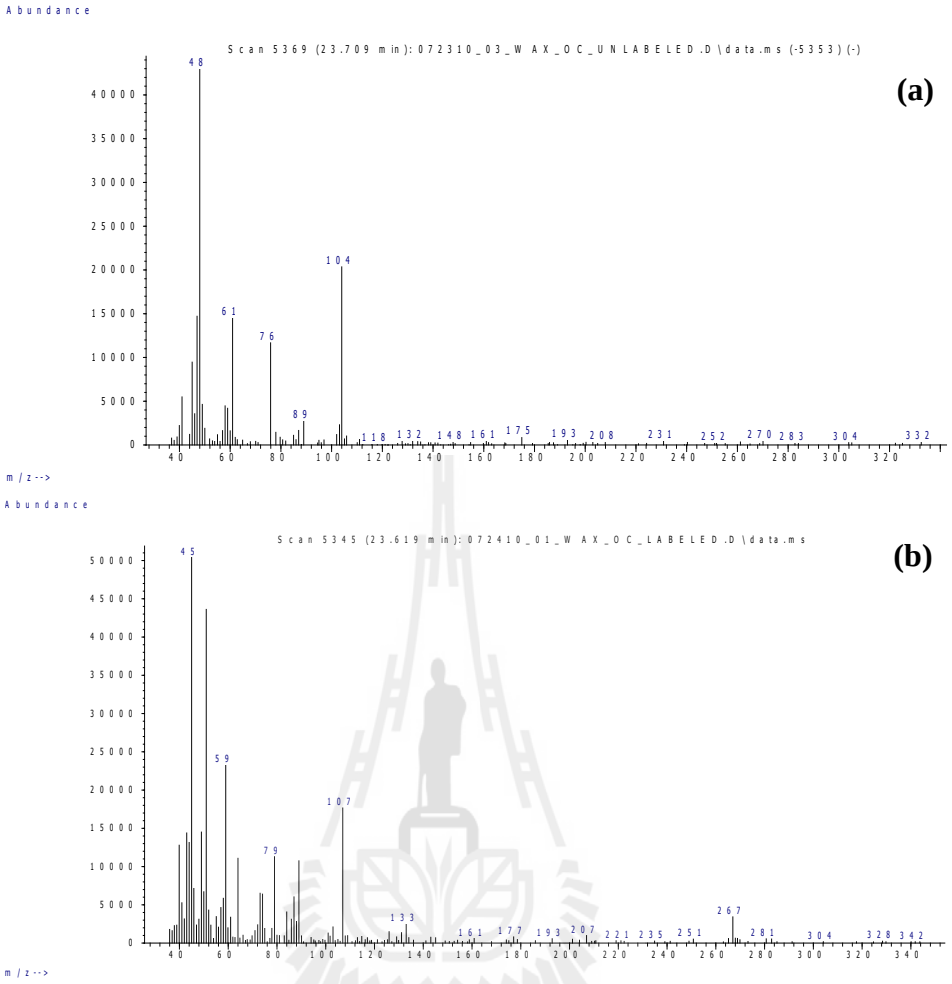
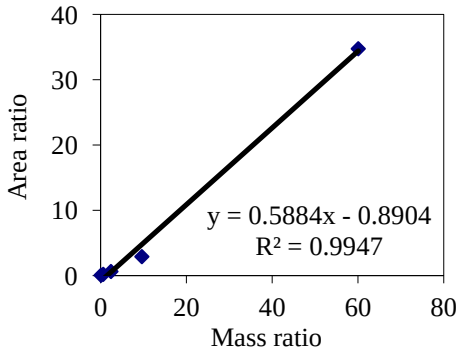


Figure 9A Mass spectra of 3-(methylthio)propanal (a) and [$^2H_3$]-3-(methylthio) propanal (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.096	0.011	1/0.5884 = **1.70**
2	0.601	0.155	
3	2.402	0.590	
4	9.609	2.866	
5	60.053	34.737	

Figure 9B Standard curve for determining response factor of 3-(methylthio)propanal.

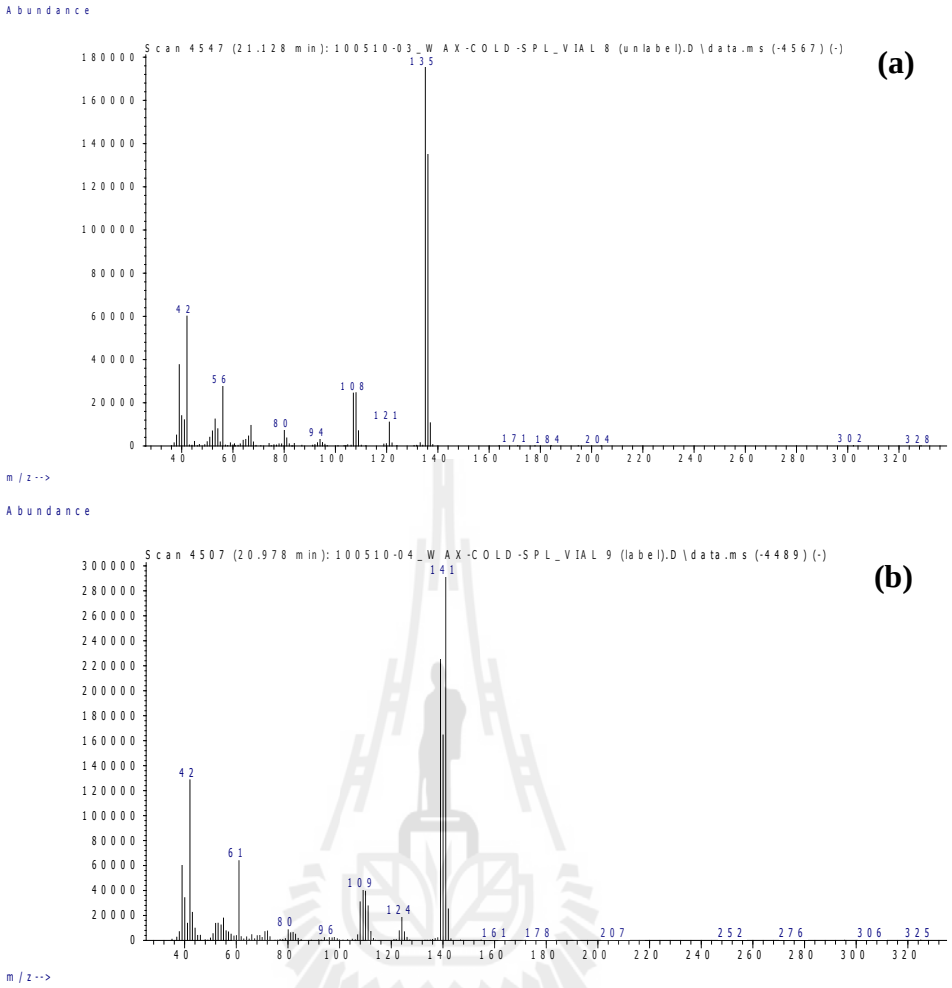
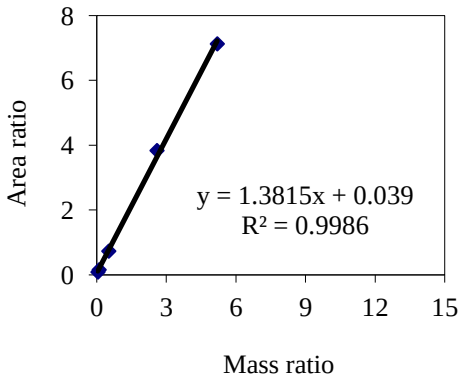


Figure 10A Mass spectra of 3,6-dimethyl-2-ethylpyrazine (a) and [$^2H_5$]-3,6-dimethyl -2-ethylpyrazine (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.052	0.077	1/1.3815 = **0.72**
2	0.104	0.146	
3	0.519	0.722	
4	2.597	3.827	
5	5.195	7.120	

Figure 10B Standard curve for determining response factor of 3,6-dimethyl-2-ethylpyrazine.

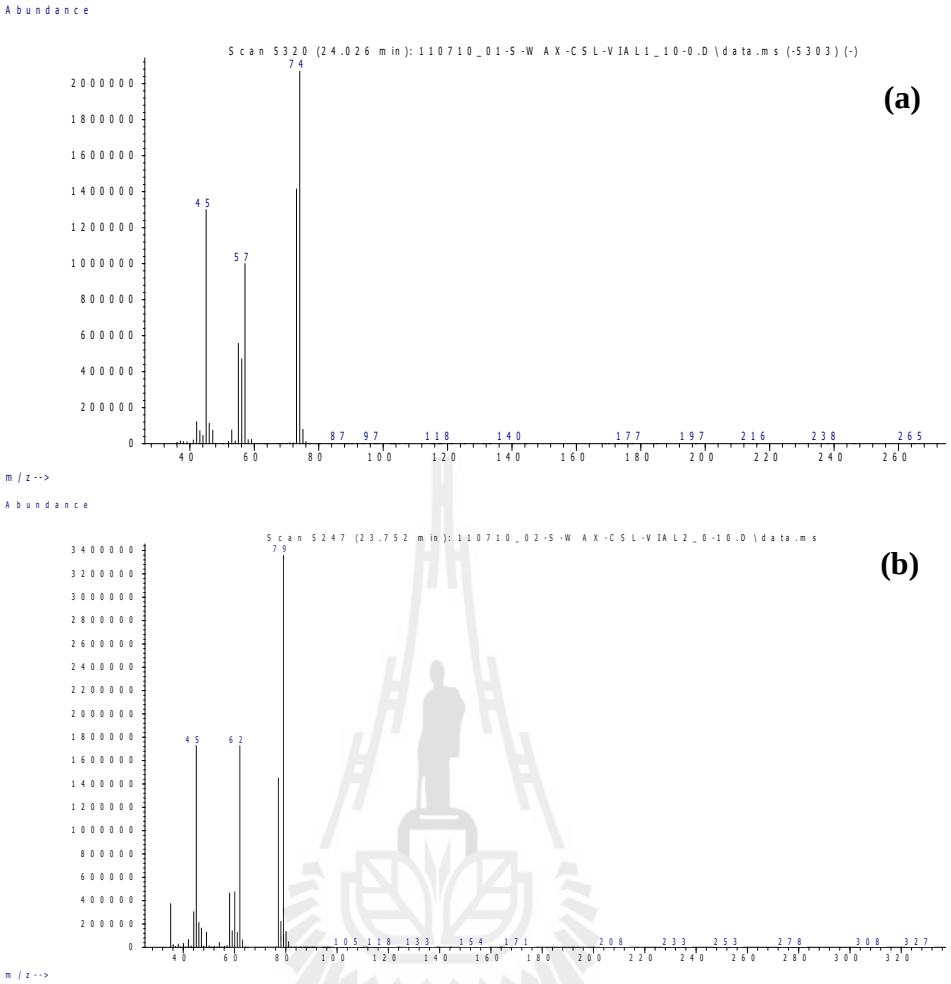
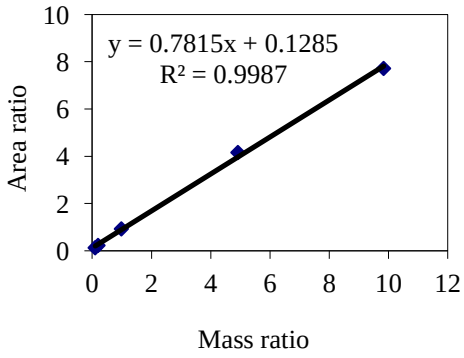


Figure 11A Mass spectra of propanoic acid (a) and [$^{2}H_5$]-propanoic acid (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.098	0.130	1/0.7815 = **1.28**
2	0.197	0.230	
3	0.983	0.931	
4	4.916	4.160	
5	9.831	7.715	

Figure 11B Standard curve for determining response factor of propanoic acid.

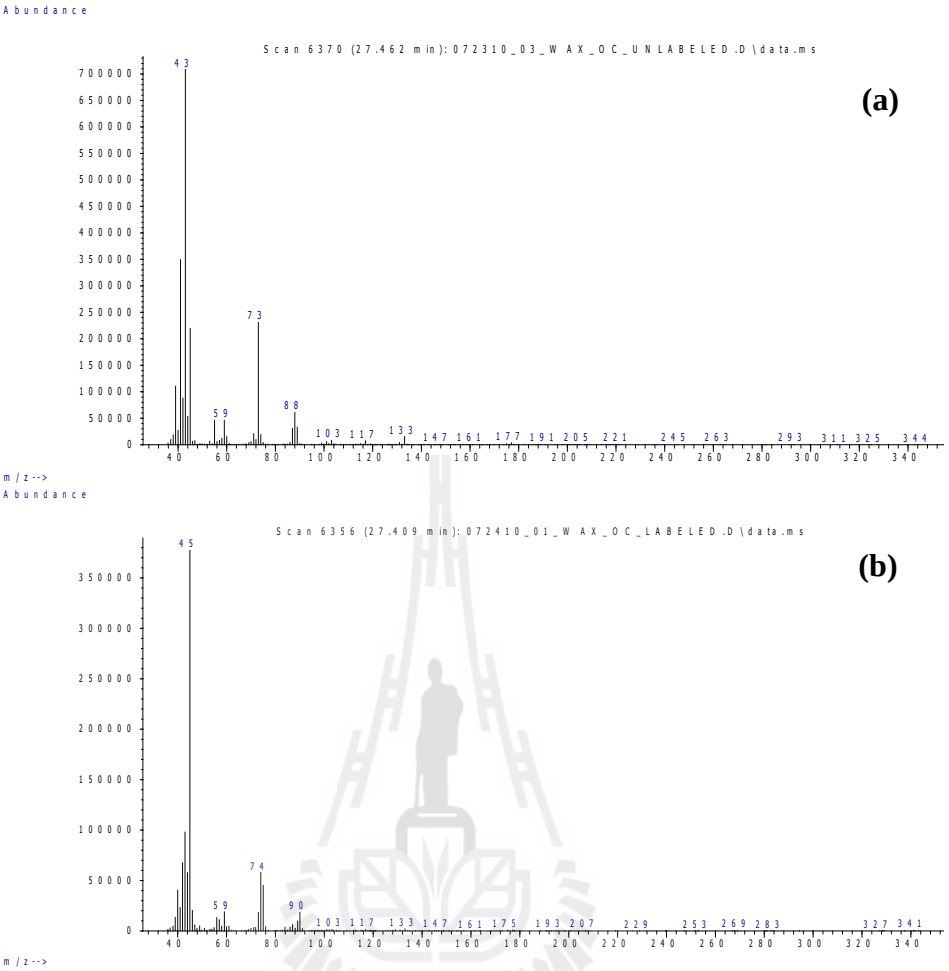
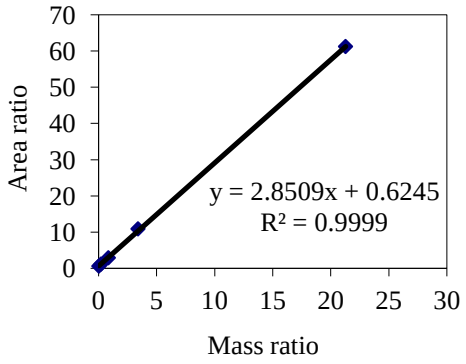


Figure 12A Mass spectra of 2-methylpropanoic acid (a) and [$^2H_2$]-2-methylpropanoic acid (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.034	0.576	1/2.8509 = **0.35**
2	0.213	1.113	
3	0.851	2.866	
4	3.403	10.850	
5	21.271	61.192	

Figure 12B Standard curve for determining response factor of 2-methylpropanoic acid.

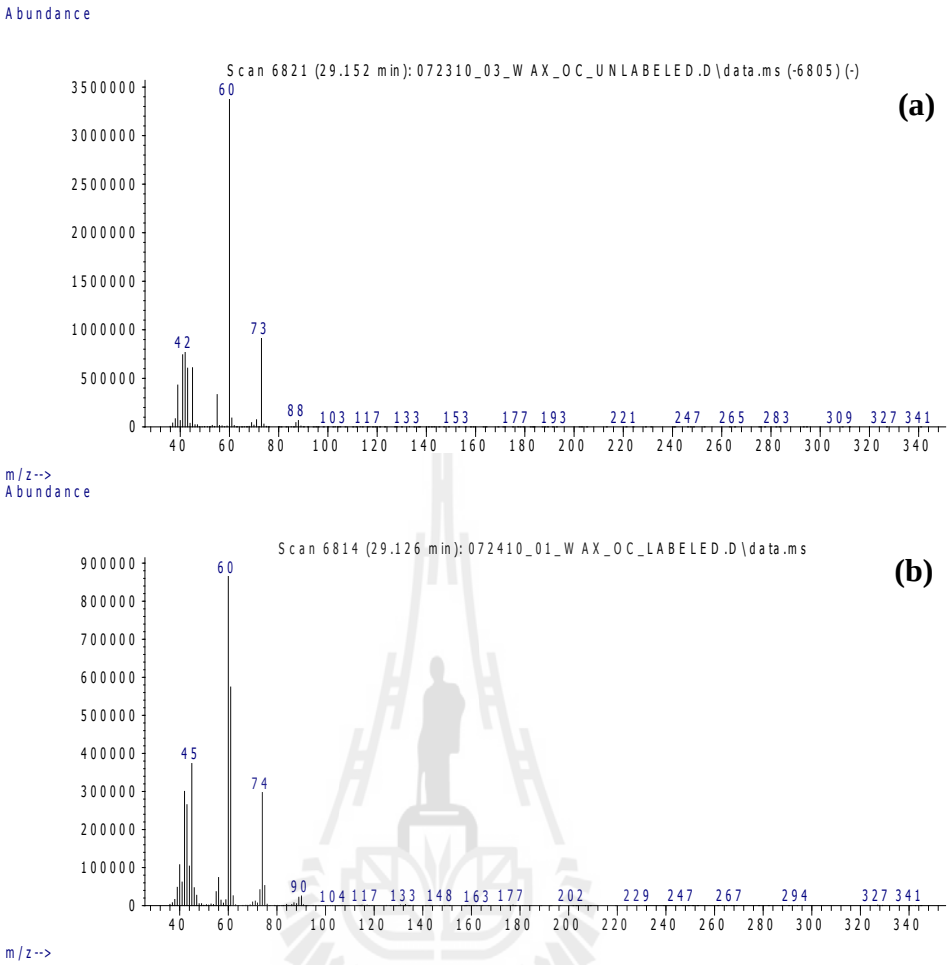
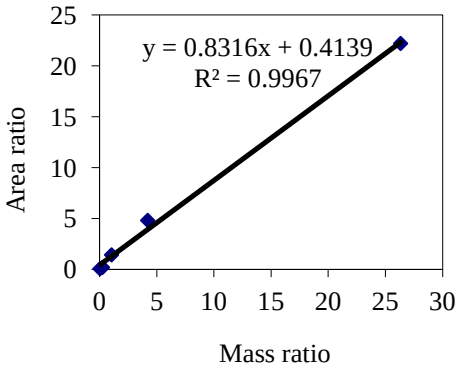


Figure 13A Mass spectra of butanoic acid (a) and [$^{2}H_{2}$]-butanoic acid (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.042	0.047	1/0.8316 = **1.20**
2	0.263	0.179	
3	1.054	1.407	
4	4.215	4.798	
5	26.342	22.179	

Figure 13B Standard curve for determining response factor of butanoic acid.

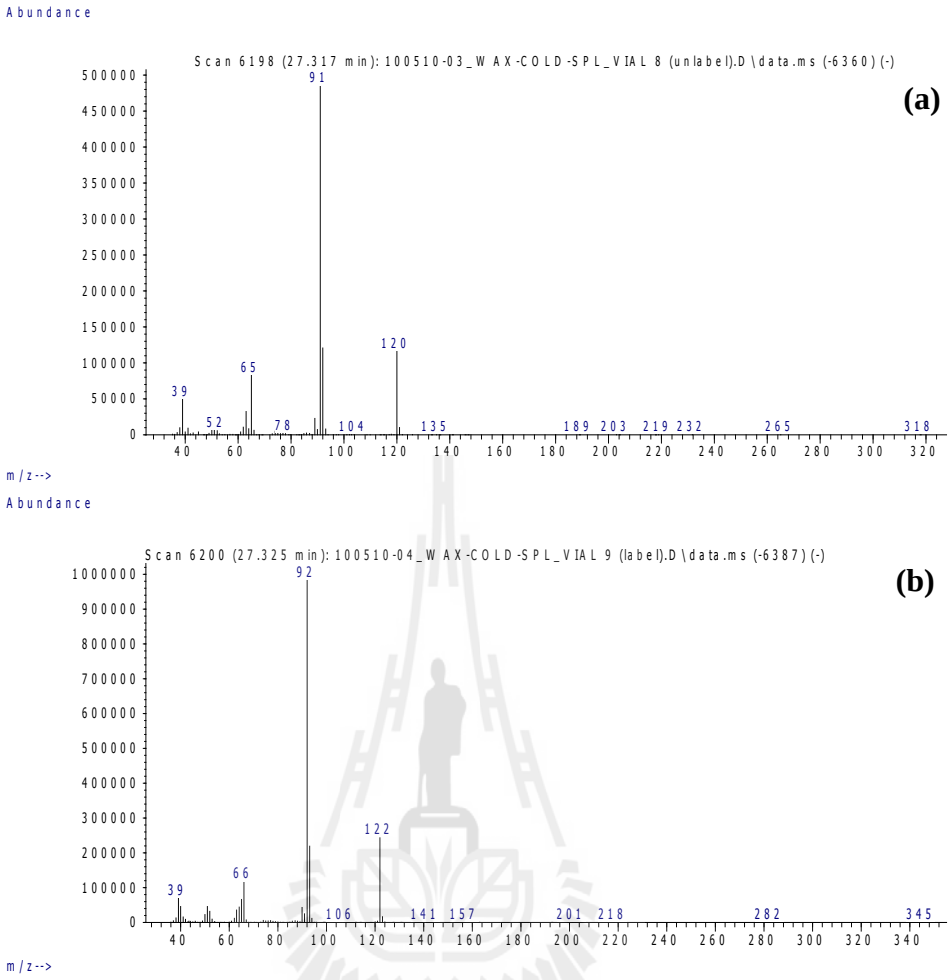
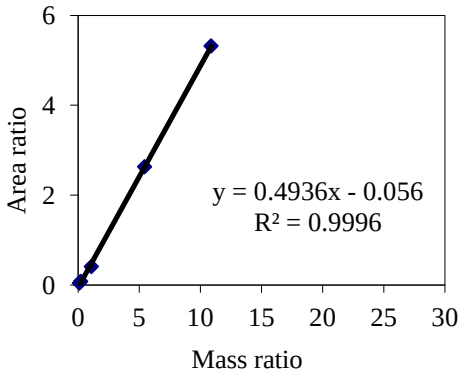


Figure 14A Mass spectra of phenylacetaldehyde (a) and [$^{13}C_2$]-phenylacetaldehyde (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.109	0.040	1/0.4936 **=2.03**
2	0.218	0.076	
3	1.088	0.407	
4	5.439	2.629	
5	10.877	5.319	

Figure 14B Standard curve for determining response factor of phenylacetaldehyde.

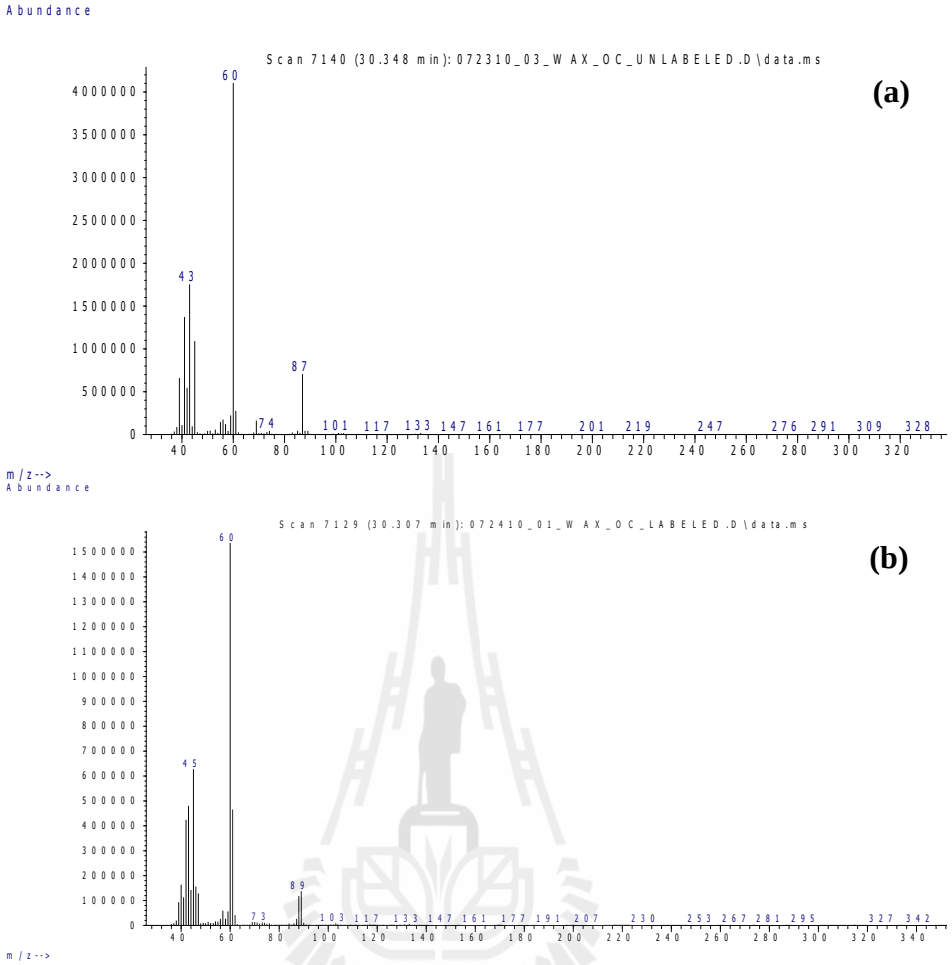
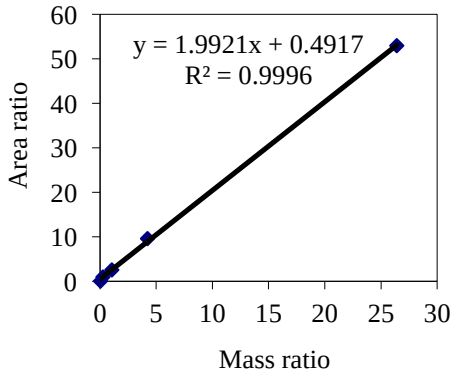


Figure 15A Mass spectra of 3-methylbutanoic acid (a) and [$^2H_2$]-3-methylbutanoic (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.042	0.054	1/1.9921 = **0.50**
2	0.264	0.979	
3	1.056	2.567	
4	4.225	9.606	
5	26.405	52.983	

Figure 15B Standard curve for determining response factor of 3-methylbutanoic acid.

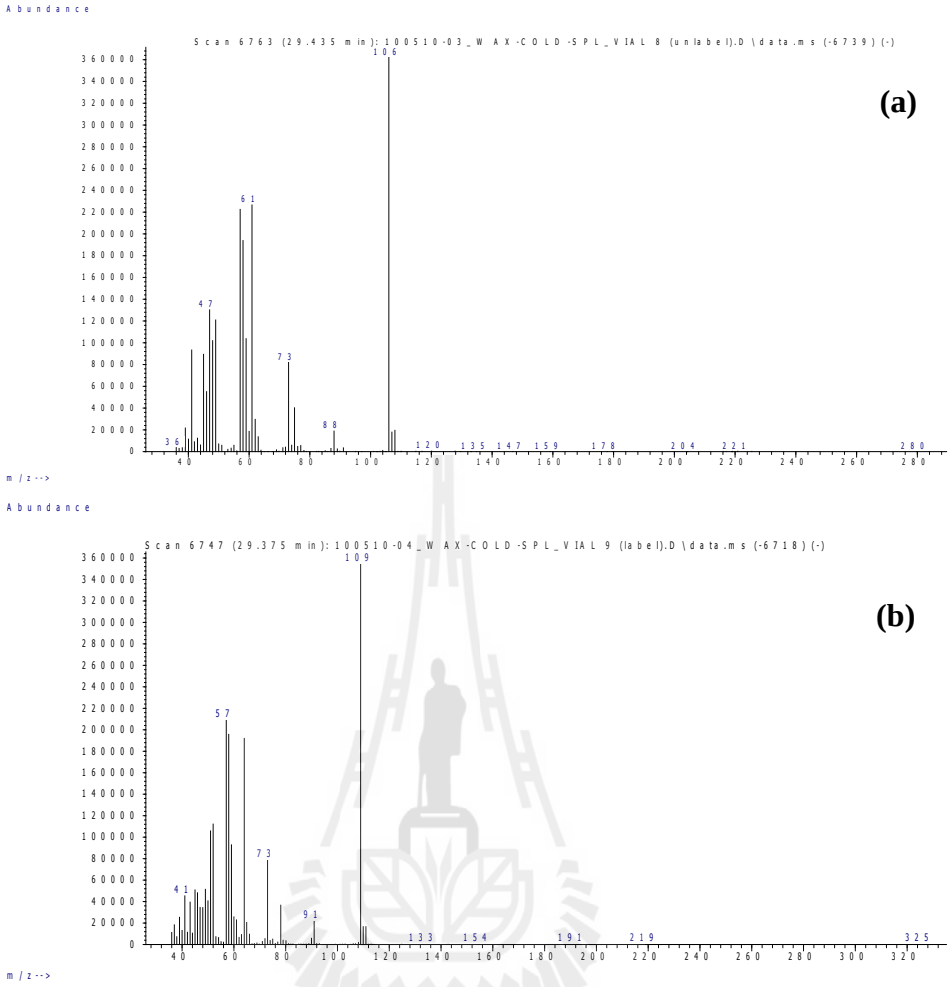
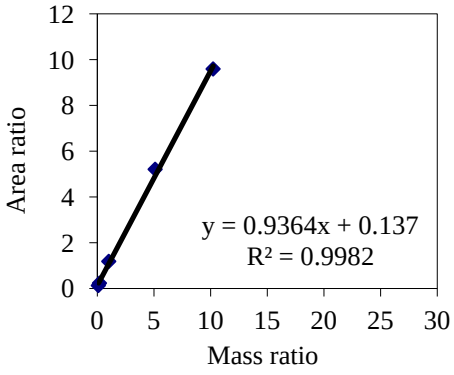


Figure 16A Mass spectra of 3-(methylthio) propanol (a) and [$^{2}H_{3}$]-3-(methylthio) propanol (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.102	0.123	1/0.9364 = **1.07**
2	0.205	0.227	
3	1.024	1.178	
4	5.118	5.199	
5	10.235	9.581	

Figure 16B Standard curve for determining response factor of 3-(methylthio) propanol.

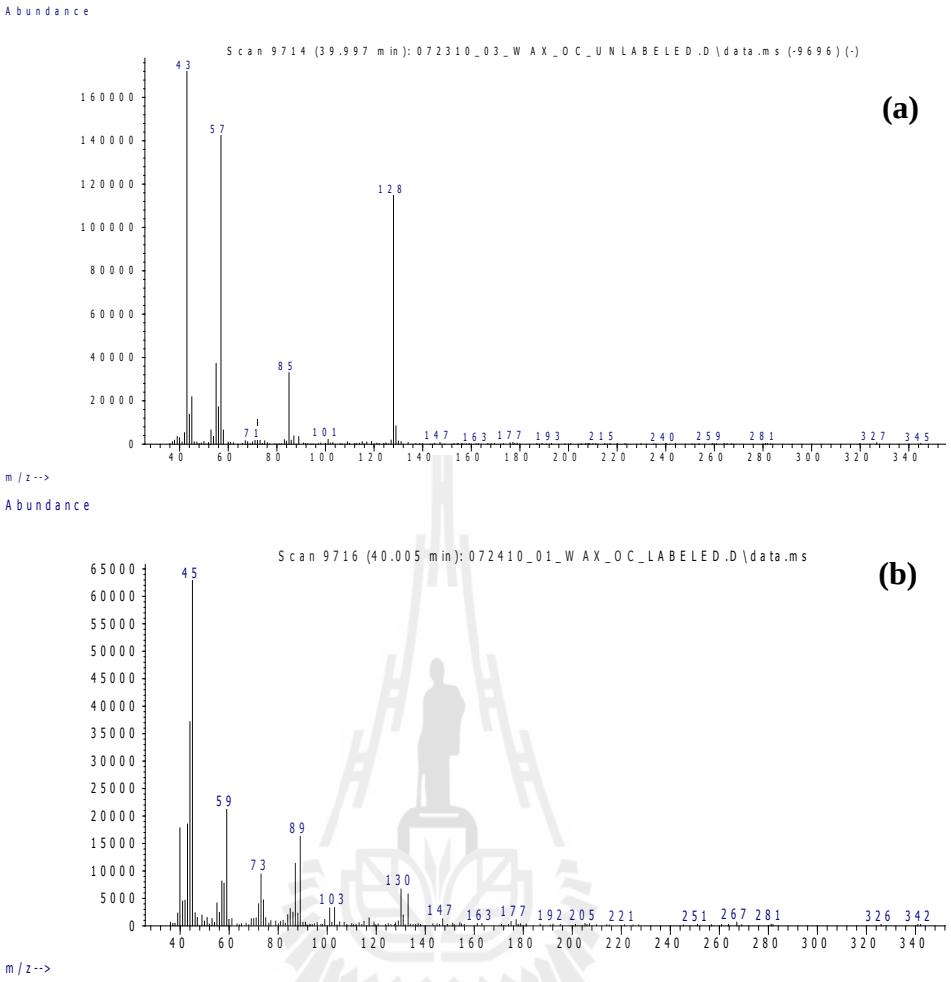
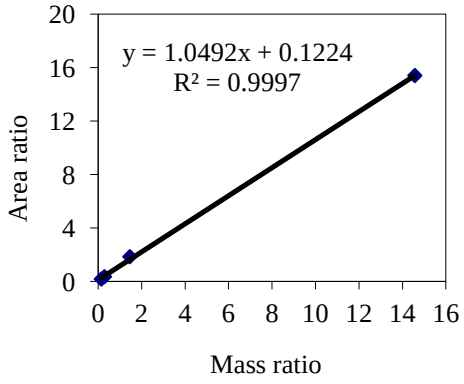


Figure 17A Mass spectra of 4-hydroxy-2,5-dimethyl-3(2H)-furanone (a) and [$^{13}C_2$]-4-hydroxy-2,5-dimethyl-3(2H)-furanone (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.146	0.175	1/1.0492 = **0.95**
2	0.291	0.353	
3	1.457	1.846	
4	7.286	10.112	
5	14.571	15.394	

Figure 17B Standard curve for determining response factor of 4-hydroxy-2,5-dimethyl-3(2H)-furanone.

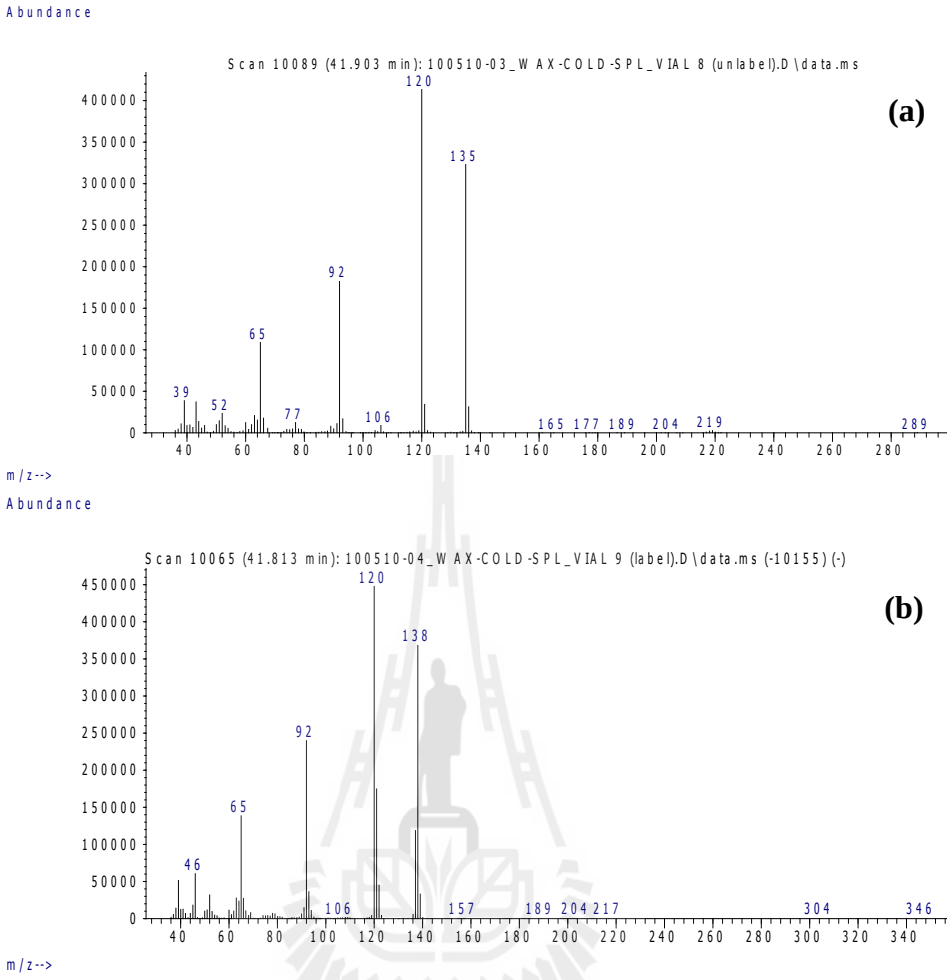
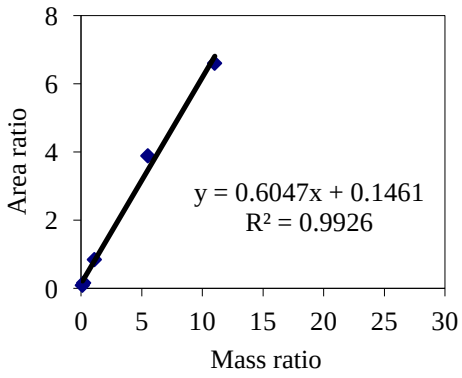


Figure 18A Mass spectra of o-aminoacetophenone (a) and [$^2H_3$]-o-aminoacetophenone (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.110	0.092	1/0.6047 = **1.65**
2	0.220	0.161	
3	1.102	0.845	
4	5.508	3.888	
5	11.015	6.602	

Figure 18B Standard curve for determining response factor of o aminoacetophenone.

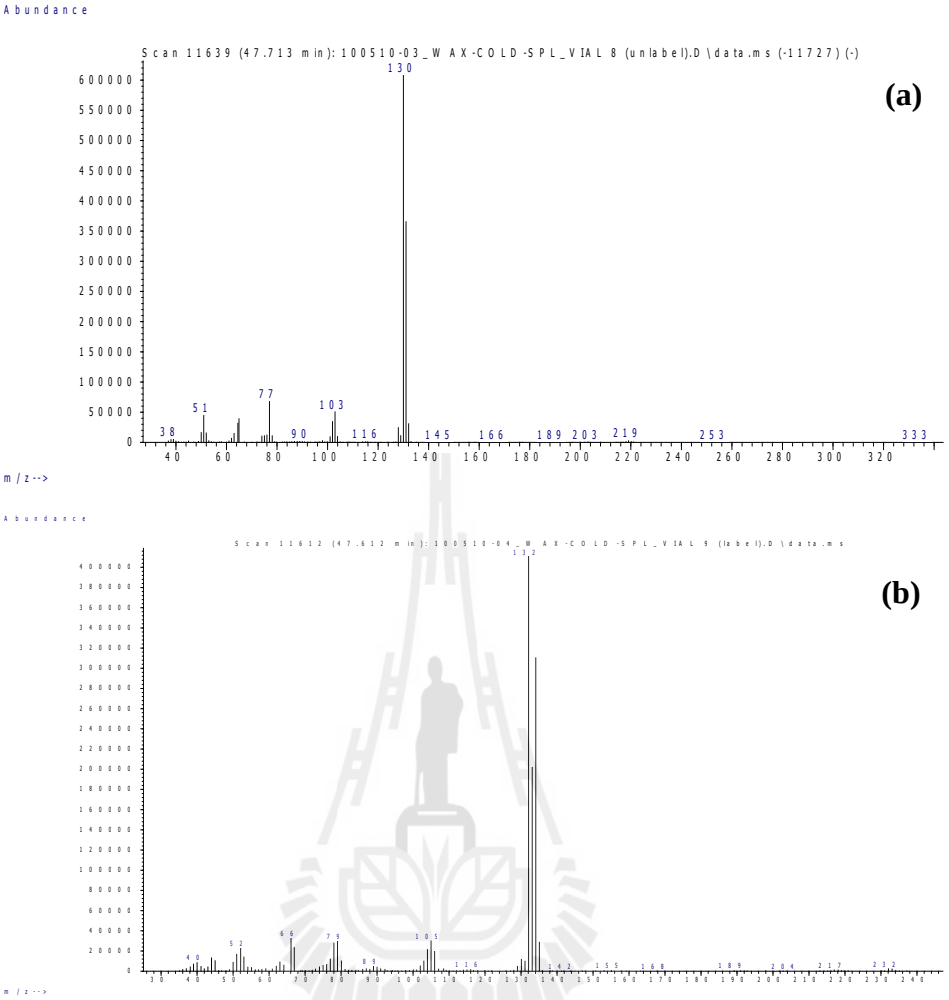
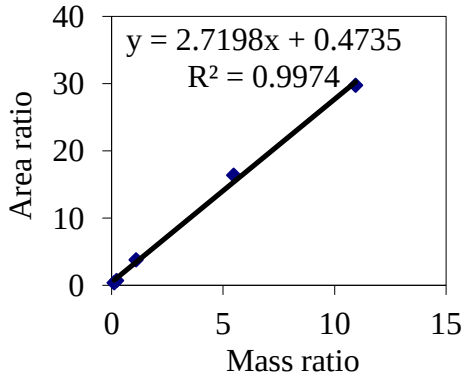


Figure 19A Mass spectra of skatole (a) and [$^2H_3$]-skatole (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.109	0.353	1/2.7198 **=0.37**
2	0.219	0.687	
3	1.094	3.771	
4	5.472	16.356	
5	10.945	29.721	

Figure 19B Standard curve for determining response factor of skatole.

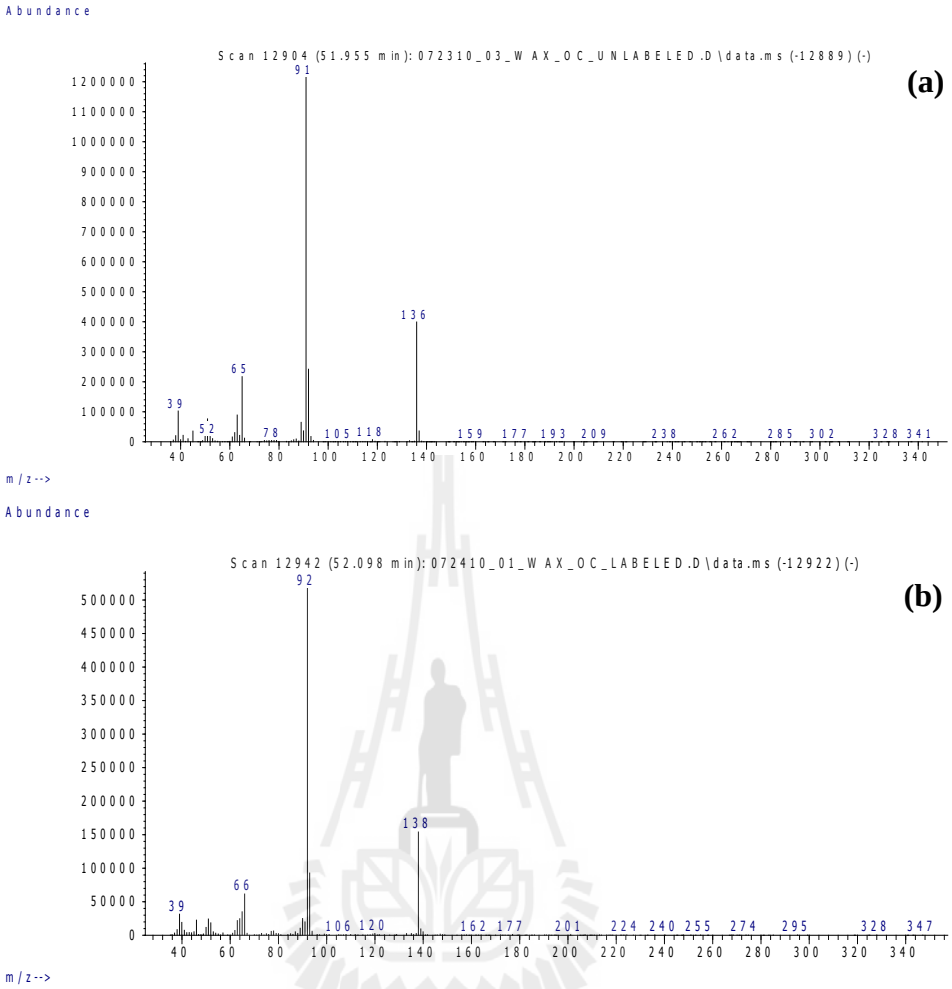
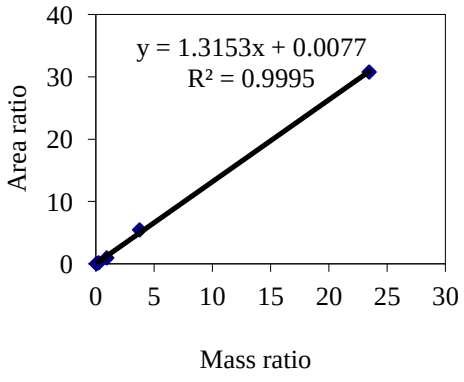


Figure 20A Mass spectra of 2-phenylacetic acid (a) and [$^{13}C_2$]-2-phenylacetic aci (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.038	0.024	1/1.3153 **=0.76**
2	0.234	0.178	
3	0.938	0.967	
4	3.751	5.457	
5	23.445	30.774	

Figure 20B Standard curve for determining response factor of 2-phenylacetic acid.

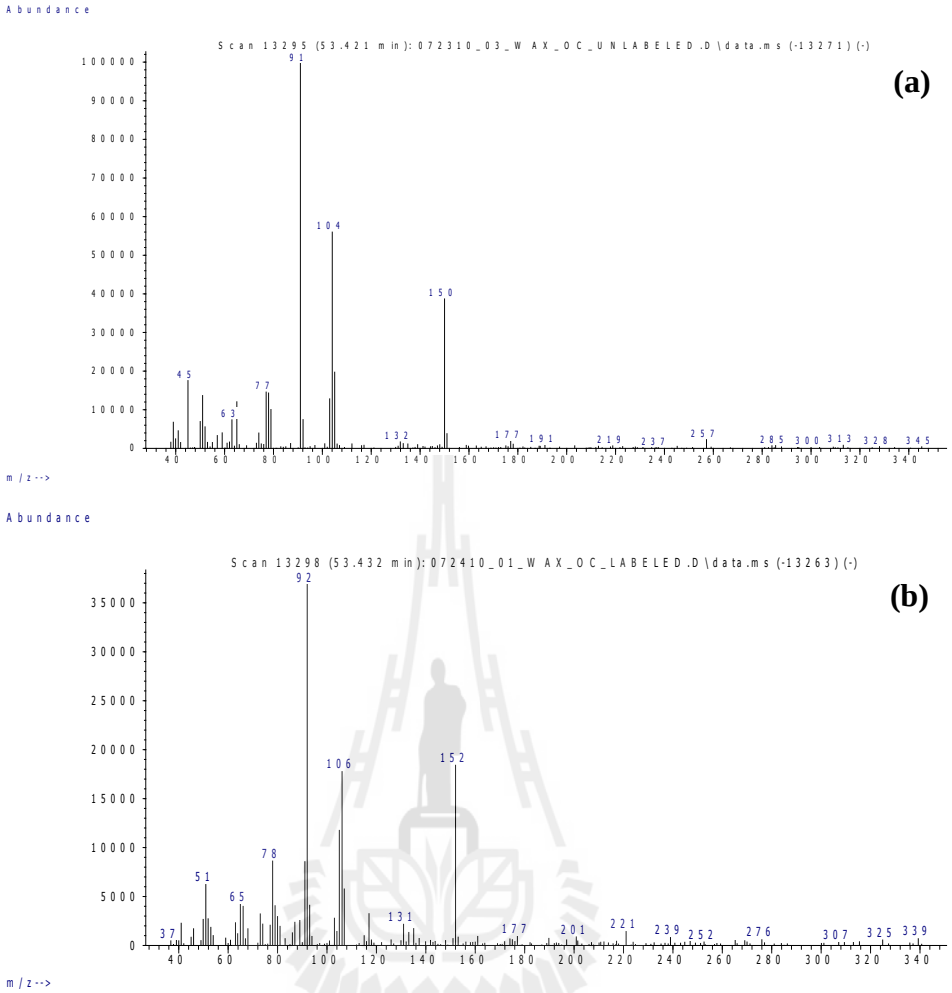
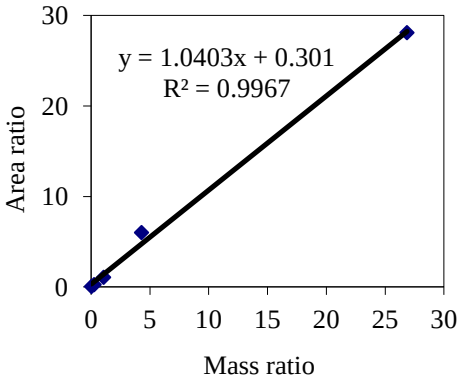


Figure 21A Mass spectra of 3-phenylpropanoic acid (a) and [$^2H_2$]-3-phenylpropanoic acid (b).



No.	Unlabeled/ isotope		Response factor
	Mass ratio	Area ratio	
1	0.043	0.031	1/1.0403 = **0.96**
2	0.269	0.232	
3	1.074	1.043	
4	4.298	5.986	
5	26.860	28.067	

Figure 21B Standard curve for determining response factor of 3-phenylpropanoicacid.

CURRICULUM VITAE

Nawaporn Lapsongphon was born in January 2nd, 1984 in Prachuap Khiri Khan, Thailand. She studied for her high school diploma at Samroiyodwittayakom School (1999-2001). In 2005, she received the degree of Bachelor of Science (Food Technology) with first class honor from Suranaree University of Technology, Nakhon Ratchasima, Thailand. In 2006-2012, she received the Royal Golden Jubilee Scholarship from Thailand Research Fund to study for the degree of Doctor of Philosophy (Food Technology) at Suranaree University of Technology. During her graduate study, she obtained opportunities to present her research works including IFT annual meeting and Food expo (New Orleans, LA, June 11-14th 2011) under the title of "Aroma characterization of premium Thai fish sauce" and EFFoST Annual Meeting 2012 (Montpellier, France, November, 21-23, 2012) under the title of "Purification and characterization of antioxidant mungbean peptides derived by *Virgibacillus* sp. SK37 proteinase". She also published her research work under the title of "Spent brewery yeast sludge as a single nitrogen source for fibrinolytic enzyme production of *Virgibacillus* sp. SK37" in Food Science and Biotechnology (Vol. 22, No.1, page 71-78) in 2013.