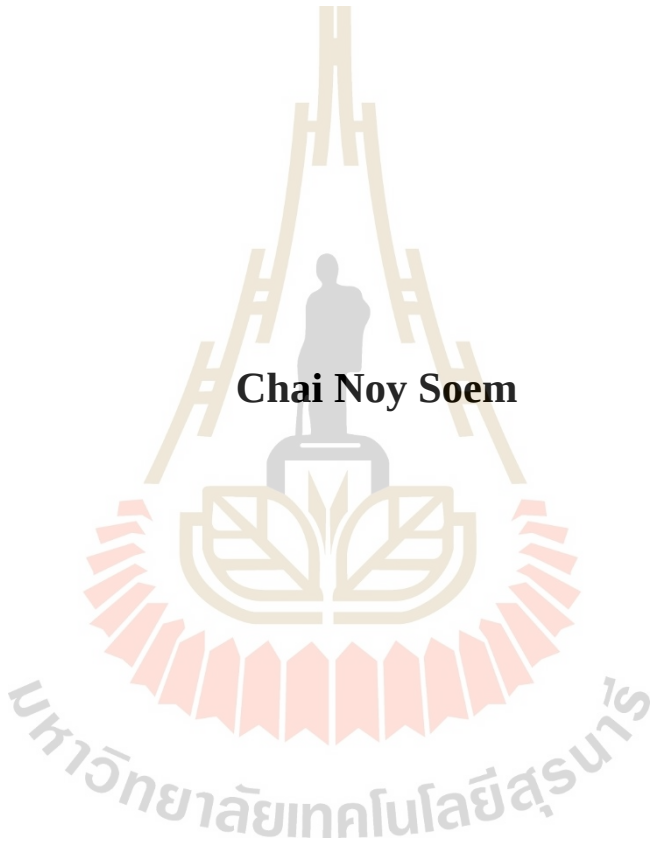
BIOLOGICAL ACTIVITIES OF CHITOSAN OLIGOSACCHARIDES (CHOS) TOWARD COSMECEUTICAL INNOVATIONS

Chai Noy Soem



A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2019

การศึกษาฤทธิ์ของไคโตซานโอลิโกแซคคาไรด์ (คอซ) เพื่อพัฒนานวตกรรมเวชสำอาง

นายชายน้อย เซิ่ม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2562

BIOLOGICAL ACTIVITIES OF CHITOSAN OLIGOSACCHARIDES (CHOS) TOWARD COSMECEUTICAL INNOVATIONS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

Thesis Examining Committee

(Assoc. Prof. Dr. Apichat Boontawan)
Chairperson

(Prof. Dr. Montarop Yamabhai)
Member (Thesis Advisor)

(Prof. Dr. Dietmar Haltrich)
Member

ramona suharoschi
(Prof. Dr. Ramona Suharoschi)
Member

(Asst. Prof. Dr. Paiboon Jitprasertwong)
Member

(Assoc. Prof. Flt. Lt. Dr. Kontorn Chamniprasart)
Vice Rector for Academic Affairs and Internationalization

(Prof. Dr. Neung Teaumroong)
Dean of Institute of Agricultural Technology

นายชายน้อย เชิ่ม : การศึกษาฤทธิ์ของไคโตซานโอลิโกแซคคาไรด์ (คอซ) เพื่อพัฒนานวตกรรมเวชสำอาง (BIOLOGICAL ACTIVITIES OF CHITOSAN OLIGOSACCHARIDES (CHOS) TOWARD COSMECEUTICAL INNOVATIONS) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร.ภญ.มณฑารพ ยมาภัย, 103 หน้า.

ไคโตซานโอลิโกแซคคาไรด์หรือคอซ เกิดจากกระบวนการเร่งปฏิกิริยาย่อยสลายในน้ำ (hydrolysis) ของไคโตซาน ให้ได้ได้เป็น ไคโตโอลิโกแซคคาไรด์ หรือ คอซ ซึ่งมีคุณสมบัติหลากหลายทั้งในทางชีวภาพและเคมี (physicochemical properties) อันเป็นประโยชน์ต่อทั้งสุขภาพและความงามของมนุษย์ ในงานวิทยานิพนธ์นี้ได้นำเทคโนโลยีเอนไซม์ได้พัฒนามาแล้วในห้องปฏิบัติการมาใช้ ได้แก่การปรับแต่งพันธุกรรมเอนไซม์ไคโตซาเนส จากแบคทีเรียแลคโตบาซิลลัส แพลนทารัม (*Lactobacillus plantarum*) TGL02 ซึ่งเป็นสายพันธุ์สำหรับใช้ในอาหารและจากระบบการแสดงออกของเอนไซม์ที่มีประสิทธิภาพในแบคทีเรียเอสเชอริเชีย โคไล (*Escherichia coli* : *E. coli*) TOP10 เพื่อผลิตให้ได้คอซที่มีความสามารถในการละลายน้ำได้ดี จากการย่อยไคโตซานที่เตรียมจากกุ้งและเปลือกกุ้งในตัวทำละลายกรดแลคติก โดยได้ทำการทดสอบสภาวะที่เหมาะสมต่างๆ ต่อกระบวนการผลิต ดังนี้ ความเข้มข้นของไคโตซาน ร้อยละ 1 - 2 มวลต่อปริมาตร (1-2 mg/ml) ระดับปริมาตร (100 และ 250 มิลลิลิตร) ความเข้นข้นของเอนไซม์ไคโตซาเนส (0.1, 0.5, 1 และ 3 ยูนิตต่อมิลลิตร) ระดับอุณหภูมิ (37 และ 50 องศาเซลเซียล) ระยะเวลา (6, 12, 24 และ 48 ชั่วโมง) และตัวทำละลาย (1% กรดไฮโดรคลอริกและ 2% กรดแลคติก) รวมไปถึงการประเมินผลวิธีการการจัดเก็บผลิตภัณฑ์คอซที่ได้ ผลการศึกษาพบว่าสภาวะที่เหมาะสมในการผลิตคอซด้วยกระบวนการแปรรูปทางชีวภาพ (bioconversion) ได้แก่ ไคโตซานมีความเข้มข้นเป็น ร้อยละ 1 มวลต่อปริมาตร ในตัวทำละลาย กรดแลคติกความเข้มข้น ร้อยละ 2 โดยใช้เวลาย่อยสลาย 48 ชั่วโมง ในปริมาตร 100 - 250 มิลลิลตร ในสภาวะเช่นนี้ให้ผลผลิตทางชีวภาพเป็น ร้อยละ 100 จากนั้นทำการวิเคราะห์โครงสร้างสารผลิตภัณฑ์ด้วยวิธีการต่างๆ ได้แก่ การวัด ^{1}H NMR การวิเคราะห์ด้วยแมสสเปกโตรเมทรี (MS) การวิเคราะห์น้ำหนักโมเลกุลของพอลิเมอร์ (SEC) และวิธีโครมาโตกราฟฟีแบบแผ่นบาง (TLC) ผลการวิเคราะห์ผลิตภัณฑ์พบว่าส่วนใหญ่ผลิตภัณฑ์สุดท้ายหลังจากกระบวนการแปรรูปทางชีวภาพ ประกอบด้วยหน่วยย่อย 2 (dimer) และ 3 หน่วย (trimer) เป็นส่วนใหญ่ และมีโอลิโกแซคคาไรด์คาไรด์สายยาว ขนาด 4 และ 5 หน่วยย่อยด้วย จากผลการเปรียบเทียบทางชีวภาพของผลิตภัณฑ์ในรูปแบบของเหลวหรือรูปแบบผงแห้งจากวิธีการเยือกแข็ง (lyophilized form) ที่ความเข้มข้นต่างๆ ได้แก่ 25, 50 และ 100 ไมโครกรัมต่อมิลลิลิตร พบว่าไม่มีความเป็นพิษต่อเซลล์แมคโครฟาจของมนุษย์ (differentiated human macrophages) โดยผลการ

ทดลองที่สำคัญจากการศึกษาในครั้งนี้คือพบว่าผลิตภัณฑ์ที่ผลิตได้จากกระบวนการย่อยนี้สามารถยับยั้งกระบวนการอักเสบที่เกิดจากการชักนำด้วยสาร LPS ต่อเซลล์โมโนไซต์ที่โตเต็มวัยของมนุษย์ (human mature monocytes; THP-1) อย่างไรก็ตามพบว่าฤทธิ์การยับยั้งการอักเสบของคอซจะลดลงหลังจากกระบวนการทำให้แห้งแบบเยือกแข็ง เนื่องจากโอลิโกแซคคาไรด์ คอซ ที่ผลิตได้นั้นสามารถละลายน้ำได้ดีและมีฤทธิ์ทางชีวภาพ ดังนั้นงานวิจัยชิ้นนี้จึงมีความน่าสนใจ เพราะอาจสามารถประยุกต์ใช้ในงานด้านเภสัชผลิตภัณฑ์และเวชสำอางอย่างหลากหลาย

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2562 ลายมือชื่ออาจารย์ที่ปรึกษา _____

CHAINOY SOEM : BIOLOGICAL ACTIVITIES OF CHITOSAN OLIGOSACCHARIDES (CHOS) TOWARD COSMECEUTICAL INNOVATIONS. THESIS ADVISOR : PROF. MONTAROP YAMABHAI, Ph.D., 103 PP.

CHITOSAN OLIGOSACCHARIDES/CHOS/COSMECEUTICAL/ANTI-INFLAMMATION/CHITOSANASE/ENZYME TECHNOLOGY/ BIOCONVERSION/WASTES/CHITIN/PRAWN/SHRIMP

Chitosan oligosaccharides (CHOS), hydrolyzed oligomers of chitosan, have been shown to have several biological and physicochemical properties which are beneficial to human health and beauty. In this study, we used an established enzyme technology, recombinant chitosanase produced from food grade expression system of *Lactobacillus plantarum* TGL02 and efficient *Escherichia coli* : *E.coli* TOP10 expression system, for the production of soluble CHOS from chitosan prepared from shrimp and prawn shells, dissolved in lactic acid solution. To determine the optimal bioconversion reaction, different conditions, namely chitosan concentrations (1 and 2%), reaction volumes (100 mL and 250 mL), enzyme concentrations (0.1, 0.5, 1 and 3 U/mL), temperatures (37 and 50 ^{o}C), reaction times (6, 12, 24 and 48 hours), solvents (1% HCL and 2% LAC), as well as the condition for the storage of the final products were evaluated. The results indicated that the optimal condition for the bioconversion is 1% chitosan in 2% LAC, hydrolyzed for 48 hours at 100-250 mL scale . At these conditions, the bioconversion yields were 100%. ^{1}H NMR, Mass spectrometry (MS), Size exclusion chromatography (SEC) and Thin Layer Chromatography (TLC) were employed to analyze the final

hydrolysis product. Product analysis revealed that the majority of the final product after bioconversion are dimers and trimers with combination of small numbers of longer saccharides such as tetramers and pentamers. Comparison of bioactivity of the products kept in liquid or lyophilized form were investigated, and it was shown that this product was not toxic to differentiated human macrophages at various concentrations, i.e., 25, 50, 100 μg/mL. Most importantly, the hydrolytic products also showed anti-inflammation activity against LPS-induced inflammation of human mature monocytes (THP-1). However, there were some loss of anti-inflammatory activity of certain CHOS samples after lyophilization. Since the oligosaccharides generated in this thesis are water soluble and bioactive, the expansion of its application to a wide a variety of pharmaceutical and cosmeceutical products is attractive.

School of Biotechnology

Academic Year 2019

Student's Signature _____

Advisor's Signature _____

ACKNOWLEDGEMENTS

The completion of this thesis was made possible through the support and inspiration of several individuals all of whom have my gratitude.

Firstly, I would like to express my gratitude to thesis advisor Prof. Dr. Montarop Yamabhai, for the support of my study and related research. I appreciate her contributions of time, ideas, and funding.

I express my very profound gratitude to my parents, my sisters, and my girlfriend for providing me with unfailing support, emotionally, financially and continuous encouragement as well as extra push throughout my years of study. This accomplishment would not have been possible without them.

I would like to express my gratitude to Prof. Vincent Eijsink who provided me an opportunity to work at NMBU for two months. He guided, funded and shared his expertise in the field.

I would like to thank all committees for their valuable time, attention, comments and really thoughtful correction to my thesis.

I would like to thank MY lab members; P' Daw, P' Otto, P' Tae Tae, P' Kun, P' Bee, P'Pang, P' Kik, N'Nan and especially P Mun for her help in translating my Thai abstract. I am grateful for their good help, ideas, comments, suggestion and friendship as well as all the fun memories.

I would like to thank the member from protein engineering and proteomics group (PEP) at NMBU that have contributed immensely to my personal and professional time

at NMBU, Norway. The group has been a source of friendships as well as good advice and collaboration, especially all the work they have done for me even when I left.

I would like to thank Prof. Ramona and the member from Life science group at USAMV, Cluj-Napoca that have contributed immensely to my personal and professional time at USAMV, Romania. The group has been a source of friendships as well as good advice and collaboration

I would like to thank Marine Bioresource co, ltd for their support in material (chitosan and chito-oligosaccharides) for the whole study.

Finally, I would like to say thank to all friends I made during my study in SUT, especially those Cambodian companions who have been and are still really real good friends, seniors and juniors. They have been there during the fun time, the hard time and every time. I am very grateful for them.

Chai Noy Soem

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF TABLES

Table **Page**

LIST OF ABBREVIATIONS

CHOS	=	chitosan oligosaccharides
^{o}C	=	degree Celsius
%	=	percent
DNA	=	deoxyribonucleic acid
DD	=	degree of Deacetylation
DA	=	degree of Acetylation
F_A	=	fraction of acetylation
DP	=	degree of Polymerization
bp	=	base pair
LAC	=	Lactic acid
MAPK	=	Mitogen-activated protein kinase
min	=	minute
mins	=	minutes
mm	=	millimeter
NF-κB	=	Nucleus factors kappa-light-chain-enhancer of activated B cells

LIST OF ABBREVIATIONS (Continued)

µg	=	microgram
µl	=	microliter
g	=	gram
et al	=	Et alia (And other)
UV	=	ultraviolet
IL-1β	=	Interleukin 1 Beta
IL-6	=	Interleukin 6
IκB	=	Inhibitory kappa B
TNF-Alpha	=	Tumor Necrosis Factor Alpha
NMR	=	Nuclear magnetic resonance
SEC	=	Size exclusive chromatography
Maldi TOF/MS	=	Matrix-Assisted Laser Desorption Ionization mas Spectrometry
DPPH	=	1,1-diphenyl-2-picrylhydrazyl

CHAPTER I

INTRODUCTION

1.1 Background and significant of this study

Chitin, which is considered to be the second most abundant natural carbohydrate on earth after cellulose, is a special polysaccharide found in fungi, mushrooms, yeast cell walls, and shells of crustaceans and insects (Aam et al., 2010; Khoushab et al., 2010). The structure of this insoluble fiber is analogous to indigestible cellulose in plants. Chitin is a long chain polymer of N-acetylglucosamine which is shown to feeds vital populations in the human gut microorganisms selectively which are responsible for maintaining proper functioning of the gut, intestinal mucosa integrity and barrier control, immunoprotection and energy efficient metabolism. With its insoluble form, chitin exists in two crystalline polymorphic forms, alpha chitin, which is parallel or antiparallel chains as tightly packed sheets, and beta chitin, which is parallel chains. In nature, alpha form of chitin is more commonly found than the beta one. Chitin is applied in different fields such as food technology, material science, microbiology, agriculture, wastewater treatment, drug delivery systems, tissue engineering, bionanotechnology (Khoushab et al., 2010).

Chitosan, which is a deacetylated form of chitosan, is heteropolymer of glucosamine and D-glucosamine residues. Despite chitin insolubility, chitosan is soluble in acid solution. According to *N*-acetylation pattern (P_A), *N*-acetylation fraction

(F_A), degree of *N*-acetylation (DA), degree of polymerization (DP), molecular weight (M_W) and molecular weight distribution or Polydispersity (PD), chitosan is classified different from chitin. Chitosan is also described as continuum of soluble polymeric chitin derivatives. With its physicochemical properties, biological properties, non-toxicity, biocompatibility and biodegradability, chitosan is suitable for a wide variety of applications including agriculture, water treatment plant, cosmetic as well as pharmaceuticals (Aam et al., 2010; Muanprasat et al., 2017) (V.D., 1998).

Chitosan oligosaccharides (CHOS), which are expected to be utilized as functional foods, pharmaceuticals ingredient and bioactive compound, are hydrolyzed oligomers of chitosan. Chitosan oligosaccharides can be produced partially through enzymatic reaction or chemically hydrolyzed. At high temperature with strong acidic condition, chitosan is hydrolyzed, result in large amount of glucosamine (chitosan monomer) produced (Muanprasat et al., 2017). Down point of chemical reaction method is needs of highly convenient system to control the progress of the reaction. Even large amount of glucosamine produced, however, pentamers and hexamers yield is low. Chitosan oligosaccharides production using enzymatic hydrolysis has few advantaged over high performance chemical reaction such as reaction condition and low monosaccharide final product (Aam et al., 2010). The hydrolysis of chitosan using enzymatic methods is better than chemical reagent-catalyzed methods and high energy impact as it offers advantages on predictability and controllability (Lodhi et al., 2014). The enzymatic methods are highly specific and beneficial with minimal chemical modification of the products. Furthermore, the enzyme-catalyzed reactions are controllable, which leads to production of CHOS with specific chemical characteristics by modification of the production procedures. Enzyme concentrations, pH,

temperature, and reaction duration are critical factors in controlling the CHOS production using enzyme-catalyzed reaction.

Chitosan oligosaccharides are being proved to be future industrial applicable polysaccharides as currently, it is found to hold plenty of potential biological properties and physicochemical properties which are beneficial to human health and beauty. It is also reported to have anticancer activity, anti-inflammatory, anti-oxidation and especially, as well as a stand-out prebiotic for gut microbiota which are potential microorganism supporting human body (Muanprasat et al., 2017). Of particular importance, CHOS and its derivatives have been demonstrated to possess several biological activities including anti-inflammation, immunostimulation, anti-tumor, anti-obesity, antihypertension, anti-Alzheimer's disease, tissue regeneration promotion, drug and DNA delivery enhancement, anti-microbial, anti-oxidation and calcium-absorption enhancement (Dai et al., 2017; Jia et al., 2017; Muanprasat et al., 2017; XuWangQu et al., 2017; XuWangYang et al., 2017; Zou et al., 2017).

Cosmetics are "any substance or mixture intended to be placed in contact with the external parts of the human body or with the teeth and the mucous membranes of the oral cavity to clean them, change their appearance, protect them, keep them in good condition or correcting body odors. There are wide variety products which are included in the cosmetic category: make-up powders, toilet soaps, perfumes, shower preparations, depilatories, deodorants and antiperspirants, products for external intimate hygiene, skin, hair, oral and nail care products. Cosmetics are believed to have been used since the Egyptian times. The color from minerals and plants was applied as eye make-up while some of the substances were toxic, even though they were used to

treat eye and skin illnesses. Gelen, a Greek physician, invented the first cold cream (an emulsion of beeswax, vegetable oil and water) was invented in the 2nd century (Costa et al., 2017; Dubuisson et al., 2018; Lin, 2010; Nohynek et al., 2010).

Skin care products processes billions of euros in the cosmetic market. Consumer demand and requirements are increasing incredibly. For example, a facial cream, a product with hydrating function, should now has cleansing, smoothing, restoring and reinforcing as well as protecting properties in addition to its original invented purposes (Costa et al., 2017). According to Costa and Santos (2017), consumers demands are not universal and static, for instance, old people would normally want preventive and therapeutic effects instead of appearance changing effects which are requirements of younger consumers. Therefore, to develop a cosmetic product, approaching consumers perceptions and requirements are such as critical focusing points as formulation process and marketing strategies. Active ingredients are considered a potential factor that differentiate thousands type of products. There are variable types of active ingredients, instantly, biological active ingredients with natural benefits to consumers are more valuable than those synthesized components which actually has advantages in term of productivity, source and stability. By the way, as stated in cosmetic safety regulations of European union, America, Japan and East Asia, safety concerns of products as well as its active ingredients are manufacturer's responsibility (Nohynek et al., 2010).

Using natural ingredients in cosmetic formulations is an attracting interest among scientific community, cosmetic industry, and consumers in all around the globe. Natural emollients, UV-filters, antioxidants, or any other natural substances which has beneficial biological activities that could also add value to the product. Products process

anti-ageing properties with antioxidants in their formulation are increasingly taking over the cosmetic market (Costa et al., 2017). Huge skin care products producer like Vaseline, Nivea, Loreal and Secret Key are innovating their skin products with inspiration of nature. Some popular natural ingredient seen in many soap brands, body scrubs and creams products are curcumin, coconut oil, olive oil as well as some others such as honey, avocado, aloe vera, tea tree oil, sea salt, shea butter, lemon essential oil. While worldwide consumption of cosmetic products growth is unstoppable and expanding undeniable and keeping up with market demands, neither by new creation nor innovation, is crucial, and since Chitosan oligosaccharides have been proved to have many biological activities including anti-inflammation, immunostimulation, tissues regeneration promotion, anti-microbial, anti-oxidation and more, creating new skin care product with this value-added oligosaccharide is brightly an advantage for humanity.

1.2 Research Objective

The main objective of this thesis was to produce Chitosan oligosaccharides (CHOS) using enzyme technology as material toward novel cosmeceutical product(s). This study is also aimed to focus on investigation of chitosan oligosaccharide novel biological activities related to skin health, specifically, anti-inflammation, using human cell lines as model of study.

1.3 Scope and limitation

In this research chitosan oligosaccharides (CHOS) was produced using enzyme technology already established in the laboratory. Only *in vitro* experiments were conducted. Investigations of CHOS biological activities were performed on macrophage cell lines (THP-1).

CHAPTER II

LITERATURE REVIEWS

2.1 Chitin and Chitosan

Chitin, which is considered to be the second most abundant natural carbohydrate on earth after cellulose, is a special polysaccharide found in fungi, mushrooms, yeast cell walls, and shells of crustaceans and insects (Aam et al., 2010). The structure of this insoluble fiber is analogous to indigestible cellulose in plants. Chitin is a long chain polymer of N-acetylglucosamine which is shown to feeds vital populations in the human gut microorganisms selectively which are responsible for maintaining proper functioning of the gut, intestinal mucosa integrity and barrier control, immunoprotection and energy efficient metabolism. With its insoluble form, chitin exists in two crystalline polymorphic forms, alpha chitin, which is parallel or antiparallel chains as tightly packed sheets, and beta chitin, which is parallel chains. In nature, alpha form of chitin is more commonly found than the beta one (Berezina, 2016).

Chitosan, which is a deacetylated form of chitin, is heteropolymer of glucosamine and D-glucosamine residues. Despite chitin insolubility, chitosan is soluble in acid solution. According to *N*-acetylation pattern (P_A), *N*-acetylation fraction (F_A), degree of *N*-acetylation (DA), degree of polymerization (DP), molecular weight (M_W) and molecular weight distribution or Polydispersity (PD), chitosan is classified different

from chitin. Chitosan is also described as continuum of soluble polymeric chitin derivatives. With its physicochemical properties, biological properties, non-toxicity, biocompatibility and biodegradability, chitosan is suitable for a wide variety of applications including agriculture, water treatment plant, cosmetic as well as pharmaceuticals (Muxika et al., 2017).

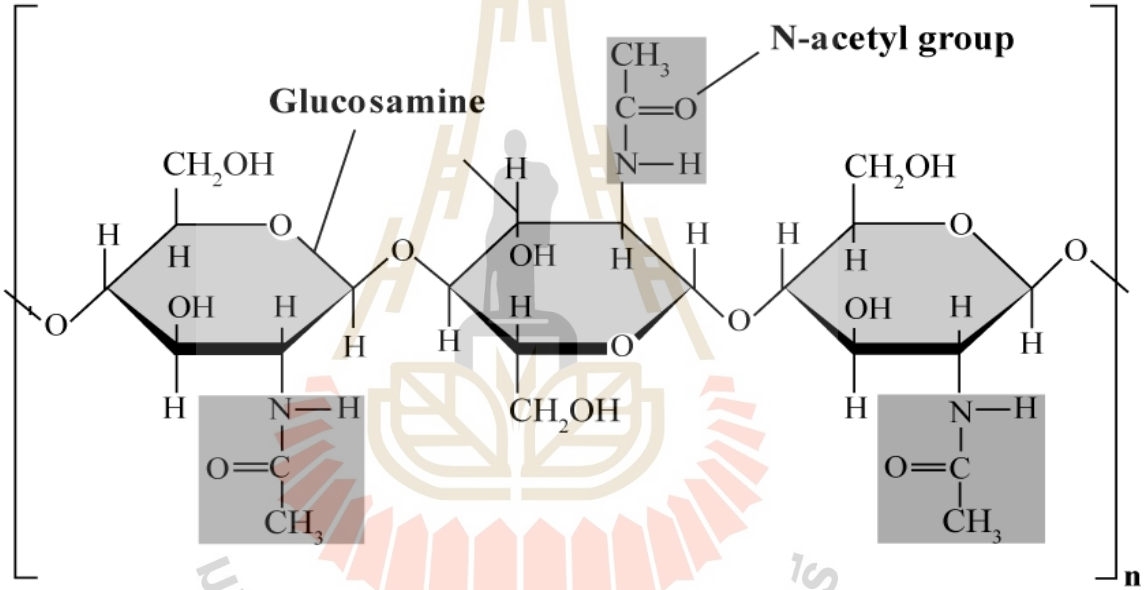


Figure 1. Chitin structure, source: (Berezina, 2016).

2.1.1 Discovery

Chitin was first discovered by a French scientist, professor Henri Braconnot, when studying mushroom in 1811 and named this product as chitin fungine, while Chitosan was discovered in 1859 by prof C. Rouget. Chitin and chitosan is used in several countries worldwide in variety of applications, and today there are more than 2000 applications of chitin and its derivatives (Primex).

2.1.2 Properties and Application

Chitin is a long chain polymer of N-acetylglucosamine that selectively feeds vital populations in the human gut microbiota which are responsible for maintaining proper functioning of the gut, intestinal mucosa integrity and barrier control, immunoprotection and energy efficient metabolism (Synowiecki et al., 2003). Chitin may play a role in preventing or reversing modern disorders such as diabetes and obesity, however, regarding its insolubility in water, chitin has a limit number of its bio application (Berezina, 2016; Elieh-Ali-Komi et al., 2016; G. Liu).

Chitosan, a natural derivative of chitin, is a polysaccharide that has been proven to possess a broad spectrum of antimicrobial activity that encompasses action against fungi, yeast and bacteria. Chitosan is found to process biological ability in decreasing obesity even though its water insolubility limits its absorption through intestine (Barroso Aranda et al., 2002; Cnubben et al., 2016; Egan et al., 2016; Egan et al., 2015; Pokhis et al., 2015; Trivedi et al., 2016; Walsh et al., 2013).

Anthocyanins as food additives have attracted increasing interest from the food industries, due to its potential health-related functionalities. Chitosan was studied in a trial application of formation and stability of anthocyanins-loaded nanocomplexes prepared with chitosan hydrochloride and carboxymethyl chitosan in the coming report of (Ge et al., 2018)

2.2 Chitosan Oligosaccharides (CHOS) and Production of CHOS

Chitosan oligosaccharides (CHOS) or Chito-oligosaccharides (CHOS) are homo-oligomers or heterooligomers of N-acetylglucosamine and D-glucosamine. While the

polysaccharides like chitin and chitosan are not water-soluble and therefore have only limited use as additives in food products, chitosan oligosaccharide is water-soluble, making it suitable for use as an ingredient in food products (Aam et al., 2010). Chitosan oligosaccharide (CHOS) is an oligomer of chitosan, which usually has a degree of polymerization (DP) b 50–55 and an average molecular weight (MW) b10,000 Da (Fig. 1). The chemical characteristics of CHOS can also be described by the degree of deacetylation (DD), which corresponds to the molar fraction of GlcN in the CHOS molecule, molecular weight distribution (polydispersity or PD) and the sequence or pattern of N-acetylation (PA). These chemical characteristics have great impacts on the physicochemical and biological properties of CHOS (Muanprasat et al., 2017).

CHOS is produced using chitosan which is a deacetylated form of chitin as a starting material, by enzymatic hydrolysis and chemical reaction methods or combination of both procedures. The chitosan hydrolysis reactions can be catalyzed by chemical reagents, enzymes and high-energy impact. Chemical reaction method can be done using chemical reagents that can induce chitosan hydrolysis including acids (e.g., hydrochloric acid, nitrous acid and phosphoric acid) and oxidative reductive agents (e.g., hydrogen peroxide/H2O2, ozone and persulfate). Hydrochloric Acid is the most common chemical used in production of chitosan oligosaccharides using chemical reaction. The commonly used conditions for the HCl-catalyzed CHOS production are 35% HCl at 80 °C for 1–2h. By the way, the acid concentration, temperature and treatment duration greatly impact the MW and DD of CHOS products (Lodhi et al., 2014; Muanprasat et al., 2017).

High-energy impact represents an alternative method for chitosan depolymerization through its effect to break O-glycosidic bonds within the polymers. The energy sources for this application are ultrasonication, gamma rays and microwave (Rong Huei Chen, 1997). Even the method is simple and environmentally friendly, still there are induction of side reactions and post-irradiation effects of the high energy impact, which further decrease the MW of CHOS in the case of irradiation in the solid state (Wenwei et al., 1993).

The hydrolysis of chitosan using enzymatic methods is better than chemical reagent-catalyzed methods and high energy impact as it offers advantages on predictability and controllability (Lodhi et al., 2014). The enzymatic methods are highly specific and beneficial with minimal chemical modification of the products. Furthermore, the enzyme-catalyzed reactions are controllable, which leads to production of CHOS with specific chemical characteristics by modification of the production procedures. Enzyme concentrations, pH, temperature, and reaction duration are critical factors in controlling the CHOS production using enzyme-catalyzed reaction (Petsrichuang et al., 2018). Chitosan can be specifically hydrolyzed by chitosanases and chitinases. Chitosanases are enzymes that catalyze the hydrolysis of the b-1,4 glycosidic bond of chitosan. Chitosanases and chitinases are very effective in CHOS production, however, their high cost limits their applications (Muanprasat et al., 2017). However, productive and cost effective recombinant chitosanase was developed and suitable for the production of CHOS (Pechsrichuang et al., 2016; Pechsrichuang et al., 2013; Petsrichuang et al., 2018; Sak-Ubol et al., 2016)

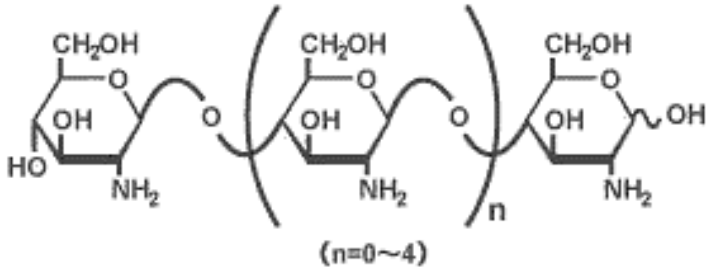


Figure 2. Chitosan oligosaccharides structure.

2.2.1 Chitosanase and Chitosanase production

Chitosanases are enzymes that catalyze the hydrolysis of the b-1,4 glycosidic bond of chitosan. Enzymes with chitosanase activity have been found in GH families 5, 7, 8, 46, 75 and 80 but only those in GH8 are found to have true chitosanase activity (Aam et al., 2010). According to extensive mutagenesis and sequence analysis, a group of researchers has established that Glu25 and Asp43 in chitosanase OU01 are essential for catalysis in the inverting hydrolysis reaction (Lyu et al., 2015). Glu25 helps position the substrate for catalysis by making a hydrogen-bond from its main-chain carbonyl group to the C-2amino group of the sugar in the+1 subsite and act as the general acid in the inverting hydrolysis reaction (Lyu et al., 2015). (Lyu et al., 2015) demonstrated that recognition and subsequent orientation of oligomeric/polymeric substrates for catalysis occurs predominantly through interactions formed with sugars in the −2 and −1 subsite. Report on efficiency conversion of chitosan with different degrees of acetylation into mixtures of CHOS with different properties such as chain lengths and compositions by chitosanase, specifically, *Bacillus subtilis* Chitosanase

46A. Researchers also reported that variation of conversion depends on the substrate and the reaction conditions (Petsrichuang et al., 2018).

(Pechsrichuang et al., 2013) reported suitable recombinant chitosanase enzyme from *Bacillus subtilis* for the biosynthesis of chitosan-oligosaccharides. Later on, the same group of researchers has successfully developed heterogenous secretion of *B. subtilis* chitosanase enzyme from E. coli expression system using OmpA signal peptides (Pechsrichuang et al., 2016). In-dept characterization of the BsCsn46A chitosanase characteristics was also carried out (Petsrichuang et al., 2018). These studies above leaded to better production of Chitosanase enzyme production as well as clearer understanding of the enzyme nature. A food-grade expression system for expression and secretion of Chitosanase enzyme using *Lactobacillus plantarum* TLG02 as expressing host was successfully developed by (Sak-Ubol et al., 2016). Chitosanase enzyme was secreted into fermentation broth and can be effectively used for the production of Chitosan oligosaccharides directly after purification and concentration step.

2.3 Chitosan Oligosaccharides biological properties

Chitosan oligosaccharides are found to hold plenty of potential biological properties and physicochemical properties which are beneficial to human health and beauty. It is also reported to have anticancer activity, anti-inflammatory, anti-oxidation and especially, it is shown to be a stand-out prebiotic for gut microbiota which are potential microorganism supporting human body (Muanprasat et al., 2017). Of particular importance, CHOS and its derivatives have been demonstrated to possess

several biological activities including anti-inflammation, immunostimulation, anti-tumor, anti-obesity, antihypertension, anti-Alzheimer's disease, tissue regeneration promotion, drug and DNA delivery enhancement, anti-microbial, anti-oxidation and calcium-absorption enhancement (Dai et al., 2017; Jia et al., 2017; Muanprasat et al., 2017; XuWangQu et al., 2017; XuWangYang et al., 2017; Zou et al., 2017).

2.3.1 Anti-inflammation activity

Inflammation is a biological reaction that protects our body from harmful stimuli. In general, inflammatory responses are triggered by foreign substances and products of tissue damage which lead to production of proinflammatory cytokines, immune cell recruitment and activation, and free radical production (Karin et al., 2016; Takeuchi et al., 2010). Normally, these inflammatory processes causes eradication of foreign bodies and pathogens and tissue regeneration (Karin et al., 2016). In some disorders, the inflammation becomes excessive, causing acute or chronic inflammatory reactions as well as tissue/organ damage. Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacteria that is involved in the pathogenesis of several inflammation-associated disease conditions including septic shock, vascular inflammation/atherosclerosis, neurodegenerative disorders, metabolic syndrome and inflammatory bowel disease (Molteni et al., 2016; Paul-Clark et al., 2012). This component binds to toll-like receptor 4 and subsequently induces inflammatory responses via either inhibitory kappa B (IκB) degradation or mitogen-activated protein kinase (MAPK)-dependent pathways. The IκB degradation triggers inflammatory responses by enabling the translocation of nuclear factor-kappa B (NFκB) into the nucleus, which induces the transcription of proinflammatory genes (Molteni et al., 2016). MAPK is composed of three downstream mediators including p38 MAPK,

extracellular signal-regulated kinase (ERK1/2) and C-Jun N-terminal kinase (JNK1/2). These three mediators promote the nuclear translocation of activator protein 1 (AP-1), which induces the transcription of proinflammatory genes.

CHOS has extensively been demonstrated to inhibit the inflammatory responses induced by LPS or other stimuli in various types of cells and animal models (Fernandes et al., 2010; Yoon et al., 2007; Yousef et al., 2012). The mechanisms of actions of CHOS have been found to involve the modulation of several important pathways including the suppression of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPK) and the activation of AMP-activated protein kinase (AMPK) (Lee et al., 2009; Muanprasat et al., 2017).

2.3.2 Anti-oxidation activity

Nine kinds of hetero-chitosan oligosaccharides (hetero-CHOS) with relatively higher molecular weights, medium molecular weights, and lower molecular weights were prepared from partially deacetylated hetero-chitosan (90, 75 and 50% deacetylated chitosan) were found to have scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, superoxide and carbon-centered radicals using electron spin resonance (ESR) spin-trapping technique (Je et al., 2004). The same group of researchers stated that the radical scavenging activity of hetero-CHOS increased with a dose-dependent manner, and it was dependent on their degree of deacetylation values and molecular weights. (Chang et al., 2017) demonstrated that chitosan molecular weight effects on its antioxidant and antimutagenic properties.

Skin photoaging, which causes skin coarse wrinkling, dryness, irregular pigmentation and laxity, is a skin-aging damage when skin is exposed to Ultra Violet

(UV) radiation repeatedly, mainly UVA and UVB. Skin photoaging is well characterized by oxidative stress and inflammatory disequilibrium. In the current research, potential preventive effect of CHOS against UV-caused damage in hairless mouse dorsal skin were investigated (Kong et al., 2017). Topical application after each UV-radiation for 10 weeks, CHOS effectively inhibited the undesirable changes on the skin induced by UV. Furthermore, CHOS reduced the macroscopic and histopathological damages of mice skin. Kong et al (2017) demonstrated that CHOS help mitigating the disrupted collagenous fibers, as well as improving the relative content of type I collagen and the amount of total collagen. CHOS is believed to effectively inhibits the levels of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, and markedly improved the activities of antioxidant enzymes (SOD, GSH-Px, CAT), as well as the content of skin hydroxyproline and moisture (Kong et al., 2017; Muanprasat et al., 2017).

2.3.3 Anti-microbial

Chitosan as well as its hydrolysates has been found to have antibacterial activity and the inhibitory levels have been shown to be significantly dependent on the DP (degree of polymerization) molecular weight according to the finding of (Y.-J. Jeon et al., 2001). The same group of researchers has proved that 0.1% of chitosan as well as high molecular weight, medium molecular weight and low molecular weight chitosan oligosaccharides could inhibit microbial growth of gram positive, gram negative and lactic acid bacteria.

In the upcoming report of (X. Liu et al., 2018), CHOS has been investigated its anti-microbial activity in a form of chitosan oligosaccharide-N-chlorokojic acid

mannich base polymer as a potential antibacterial material. Furthermore, (Lu et al., 2014) reported that, Nitric oxide-releasing chitosan oligosaccharides could be beneficially applied in bio application as antibacterial agents. The water solubility of chitosan oligosaccharides allows for both beneficial diffusion into biofilms and subsequent association with bacteria, resulting in efficient eradication of biofilm bacteria at concentrations eliciting minimal toxicity to L929 mouse fibroblast cells. Experiments are underway to evaluate the biocidal efficacy of these materials against a broad spectrum of bacteria strains including methicillin-resistant *Staphylococcus aureus* and cystic fibrosis-related *P. aeruginosa* strains (e.g., mucoid/alginate producing strains) (Lu et al., 2014).

A study was conducted to understand the influence of Maillard reaction (MR) on bioactivity of chitosan oligosaccharide (CHOS). The antibacterial activity against *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus* and preservative effect of two kinds of CHOS MR products (MRPs) on *Penaeus vannamei* were evaluated (Sun et al., 2017).

2.3.4 Anti-cancer and other disease

Antitumor activity of CHOS have been examined against Sarcoma 180 solid (S180) or Uterine cervix carcinoma No. 14 (U14) tumor cell-bearing mice. Among variety of CHOS, medium molecular weight COS with molecular weight range from 1.5 to 5.5 kDa effectively inhibited the growth of both tumor cells in the mice. In addition, the administration of MMWCHOS resulted in increased thymus weight among lymphoid organs. The mice treated with , medium molecular weight CHOS was found to improved survival rate and larger number of survivors after 40 days of feeding

(Y. J. Jeon et al., 2002). The same group of scientists has stated that the most effective of medium molecular weight CHOS for antitumor activity in the S180- or U14-bearing mice was 20 mg/kg/day or more.

Amyloid precursor protein (APP) proteolysis is essential for the production of β-amyloid peptides (Aβ) that form senile plaques in Alzheimer's disease (AD) brains. The β-site amyloid protein precursor cleaving enzyme 1 (BACE1) is the rate limiting enzyme in the generation of Aβ from APP, inhibition of BACE1 is thereby considered as an attractive strategy for anti-AD drug discovery. (Dai et al., 2017) investigated the potential inhibitory effect of CHOS on both BACE1 expression in HEK293 APPswe cells and BACE1 enzymatic activity in vitro. Their results showed that COS (100–500 μg/ml) dose-dependently decreased the cell apoptosis, and potently repressed the secretion of both Aβ40 and Aβ42 as determined by ELISA. Moreover, treatment with CHOS resulted in a dramatic reduction in BACE1 mRNA and protein expression level, eIF2α phosphorylation as well as BACE1 enzymatic activity. They indicated that COS can ameliorate Aβ-associated neurotoxicity, which may be, at least in part, attributable to reductions in BACE1 enzymatic activity and expression.

HCT116, a human colon carcinoma cell line, were treated with different concentrations of CHOS (0, 100, 500 and 1000 ug/mL, respectively). These chitosan oligosaccharides with degree of polymerization 2-6 was found to induced apoptosis in this human colon cancer cells (Zou et al., 2017). Moreover, it also promoted mitosis and S cell cycle arrest in the cancer cells.

Chitosan oligosaccharides has also been found to process the biological ability to protect nucleus pulposus cells from hydrogen peroxide-induced apoptosis in

a rat experimental model (Jia et al., 2017). CHOS have been shown to help protect nucleus pulposus (NP) cells against intervertebral disc degeneration(IDD), however, its high molecular weight limits its clinical application.

2.3.5 Anti-obesity

Similar to chitosan, CHOS has been demonstrated to have effective mechanism in reducing body weight gain, reducing serum levels of triglyceride and cholesterol (total cholesterol and low-density lipoprotein), and alleviating lipid accumulation in the liver and adipose tissues in rodent models of obesity (Choi et al., 2012; Huang et al., 2015; Kang et al., 2012; Kumar et al., 2009; Sumiyoshi et al., 2006). As CHOS is water solubility and lower viscosity and is more easily absorbed through the intestine then chitosan, there is increasing interest in developing CHOS for anti-obesity and anti-dyslipidemia applications (Muanprasat et al., 2017). Multiple mechanisms have been proposed to mediate the anti-obesity and lipid-lowering effects of CHOS. It is reported that CHOS inhibits the pancreatic lipase activity and bind with bile acids, resulting in reduced intestinal fat absorption and increased fecal fat excretion (Kang et al., 2012; Sumiyoshi et al., 2006). There is also report on inhibition of CHOS on cell differentiation, triglyceride accumulation and the expression of adipogenic markers in 3T3-L1 cells, an adipocyte cell line (Cho et al., 2008). Furthermore, many researchers have been demonstrated that COS has biological activity to suppress in vivo adipogenesis in both rat and mouse models of obesity (Choi et al., 2012; Huang et al., 2015; Kumar et al., 2009).

2.3.6 Anti-hypertension

Hypertension is a worldwide health issue which is also a major risk factor for the development of cardiovascular disease, stroke and renal disease (Y. Zhang et al., 2006). Kidney fluid and salt balances, the renin–angiotensin–aldosterone system (RAAS), the sympathetic nervous system activity, and the blood vessel structure and function as well as genetic factors are factors involve in controlling blood pressure regulation. Any changes done to these factors will result in hypertension development. The renin angiotensin aldosterone system processes an enzyme called angiotensin converting enzyme (ACE) which act potentially as blood pressure regulator where Increasing ACE activity/function is regarded as a cause of hypertension. CHOS and its derivatives are believed to be a group of promising natural compounds acting as ACE inhibitors. According to (Michaud et al., 1997), the active obligatory site of ACE is positively charged and contains hydrogen bond acceptors and zinc as a cofactor, ACE inhibitors usually contain one or more functional groups such as carboxyl groups, hydrogen bond donors, and zinc binding moieties. So, in order to improve the potency of ACE inhibitors, ACE inhibitors negative charge density and numbers of hydrogen bond donors and/or zinc-binding moieties are main points to be focusing on.

There is a report reported that, CHOS exhibited an inhibitory effect on ACE activity, with an IC50 ranging from 0.9 μM to N100 μM depending on its MW and DD. The study also compared the ACE inhibitory activities of COS with varying degrees of polymerization (DP) from 1 to 10 indicated that the chitosan trimer (DP = 3) was the most potent derivative (Hong et al., 1998).

2.3.7 Other application

Chitosan oligosaccharides was recently undergone study on its application as drug carrier and release materials in a form of microcapsules (Lian et al., 2016). The researchers showed that the chitosan microcapsules as release materials have excellent antibacterial effects on Vibrio in the farming of *Penaeus vannamei* Boone while their controlled release could obviously reduce dosage of antibiotics and delivery times, and effectively improve the utilization rate of norfloxacin drugs for shrimps. There is also report on transport mechanism of chitosan-N-acetylcysteine, chitosan oligosaccharides or carboxymethyl chitosan decorated coumarin-6 loaded nanostructured lipid carriers across the rabbit ocular (Li et al., 2017).

Chitosan oligosaccharides was treated as dietary supplements for weaned pig at different levels to help the pig's growth performance, nutrient digestibility and small intestinal functions. The result was interestingly promising as diet supplement with 150 mg CHOS per kg was shown to promote nutrients absorption and digestibility efficiency (Suthongsa et al., 2017).

2.3.8 Current industrial application of CHOS

Chitosan oligosaccharides is applied in many industrial applications and at the same time, it is registered as intellectual property in so many fields. In 2017, there were 2,436 patents application regarding chitosan oligosaccharides granted (Lens, 2018a). According to the "The Lens", patents application related to chitosan oligosaccharides keep increasing since 1984 with only 1 patent application to 2,436 patents in 2017 (Figure 3). In total, there are 2,3749 patent applications of chitosan oligosaccharides up to now where United States owns 52.6% of the entire entries. The

top application of the patents of chitosan oligosaccharides are pharmaceuticals, nutraceuticals, preparation procedures, CHOS derivatives, application methods and the chitosan oligosaccharides itself.

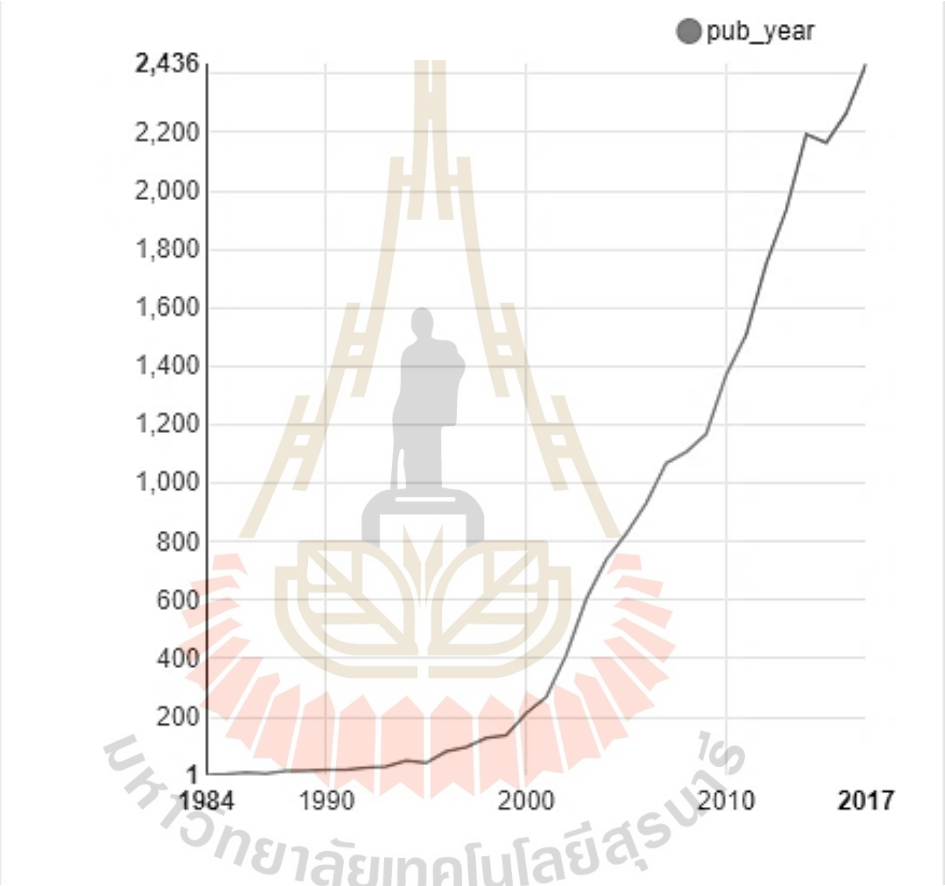


Figure 3. Patents application of chitosan oligosaccharides from 1984 to 2017 (Lens, 2018b)

Table 1. Biological activities of CHOS.

Application	CHOS preparation	DD	Molecular weight	Biological activities/activities mechanism	References
Anti-inflammation		90–95%	≤10000 Da	- have the anti-inflammatory effect via the stimuli of TNF-a in the LPS-stimulated inflammation in RAW 264.7 cells.	(Yoon et al., 2007)
	CHOS mixture in saline solution (50, 250 and 1,000 mg/kg of mice weight)	80-85%	1.2 & 5.3 kDa	- COS possess anti-inflammatory activity, which is dependent on dose and, at higher doses, also on the molecular weight.	(Fernandes et al., 2010)
	Dissolved in water	90%	5000–10,000 Da	- CHOS inhibited NF-κB signaling - CHOS activated apoptosis of intestinal epithelial cells - CHOS protected against mortality and intestinal inflammation in a mouse model of acute colitis induced by 5% dextran sulfate sodium (DSS).	(Yousef et al., 2012)
Anti-microbial	CHOS solution		≥10 kDa ≤10 kDa ≤1 kDa	- Inhibited growth of various bacteria	(Y.-J. Jeon et al., 2001)
	Maillard reacted CHOS	98%	8190 Da	- enhanced antibacterial activity and preservative effect on Maillard reaction products	(Sun et al., 2017)
Anti-photoaging	Dissolve in water (200, 100 & 50mg/ml)	≥90 %	≤1000 Da	- Inhibited UV-induced macroscopic appearance in mice skin - Prevented UV-induced reduction in skin hydration - Ameliorated UV-induced skin histochemical damages and epidermal hyperplasia - Mitigated UV-induced skin collagen degradation - Restored the reduced activities of skin antioxidant enzymes caused by UV - Suppressed the over production of pro-inflammatory cytokines triggered by UV	(Kong et al., 2017)

Table 2. **(Continued)** Biological activities of CHOS.

Application	CHOS preparation	DD	Molecular weight	Biological activities/activities mechanism	References
Antioxidant	Dissolve in ethanol		High molecular weight (90, 75 and 50 HMWCHOS), medium molecular weights (90, 75 and 50-MMWCHOS), and lower molecular weights (90, 75 and 50-LMWCHOS)	- CHOS has radical scavenging activity against all tested radicals	(Je et al., 2004)
Anti-obese	CHOS mixture with lard and soybean		≤4 kDa	- ameliorate HF diet-induced weight gain and improve serum and liver lipid profile abnormalities, which are associated, at least in part, with altered adipose tissue gene expression involved in adipogenesis and inflammation.	(Choi et al., 2012)
	Dissolved in water COS1-H, 1000 mg/kg/day; COS1-M, 500 mg/kg/day; CHOS1-L, 250 mg/kg/day; CHOS2-H, 1000 mg/kg/day; CHOS2-M, 500 mg/kg/day; and CHOS2-L, 250 mg/kg/day		≤1000 and ≤3000	- CHOS1, and CHOS2 improved dyslipidemia and prevented body weight gains by inhibiting the adipocyte differentiation in obese rats induced by a high-fat diet	(Huang et al., 2015)
Anti-tumor/anti-cancer	Dissolved in water		High mw, medium mw and low mw	- MMWCHOS with molecular weight range from 1.5 to 5.5 kDa effectively inhibited the growth of both tumor cells in the mice	(Y. J. Jeon et al., 2002)

Table 3. **(Continued)** Biological activities of CHOS.

Application	CHOS preparation	DD	Molecular weight	Biological activities/activities mechanism	References
Anti-aging	Dissolved in water	≥90 %	average molecular weight ≤1000 Da, degree of polymerization of 3–7, water soluble	- COS has anti-aging activity in D-gal-induced subacute aging mice, associated with enhancing the antioxidant defenses, reducing oxidative stress, and improving the immune function	(Kong et al., 2018)

2.4 Macrophage

Macrophages are a type of phagocyte, typically white blood cell that engulfs and digests cellular debris, foreign substances, microbes, cancer cells, and anything else that does not have the types of proteins specific to healthy body cells on its surface in a process called phagocytosis. Macrophages are differentiated monocytes, which turn into macrophages when they leave the blood. Macrophages are also responsible for alerting the immune system to the presence of invaders (Doebel et al., 2017). Besides phagocytosis, macrophages are innate immune cells that provide host defense and have tissue-specific roles in the maintenance of organ homeostasis and integrity. They play a critical role in nonspecific defense (innate immunity) and also help initiate specific defense mechanisms (adaptive immunity) by recruiting other immune cells such as lymphocytes. For example, they are important as antigen presenters to T cells. In humans, dysfunctional macrophages cause severe diseases such as chronic granulomatous disease that result in frequent infections (Park et al., 2017; Saradna et al., 2018). Macrophages also play important role in the initiation of inflammation against intruders. Along with other residing cells such as vascular endothelial cells, dendritic cells, and interstitial fibroblasts, macrophages are recruited whenever there is initial stimulus. Mature macrophages are the main expressers of pro-inflammatory cytokines such IL-1 and TNF-α, so macrophages play key roles in production, mobilization, activation, and regulation of inflammatory (Kobayashi, 2005). Despite all the important roles of inflammation such as healing, tissue damage rebuilds or fight against trauma or infection in the body, inflammation is found out to be a cause behind some serious health issues as well. Recent discovery over the last 10-20 years showed that inflammation involves in many diseases such as chronic diseases, cancer, diabetes,

obesity, Alzheimer's diseases and even Atherosclerosis (Couzin-Frankel, 2010). That is where anti-inflammation is becoming a crucial part of human health issue. There is a dozen of anti-inflammatory drugs developments in the purpose of reducing or inhibiting inflammation when it is over-inflamed, making sick people sicker or causing serious health issues (Agatonovic-Kustrin et al., 2018; Butler, 1984; Khalil et al., 2020; Laskin et al., 2010; Rehmat et al., 2020; Shabbir et al., 2020; X. Zhang et al., 2016).

2.5 Cell-based assay

There are a variety of assay technologies available that use standard plate readers to measure metabolic markers to estimate the number of viable cells in culture (Jitprasertwong et al., 2014). Cell viability assays are used to determine if the test molecules have effects on cell proliferation or show direct cytotoxic effects that lead to cell death (Pechsrichuang, 2017). Cell-based assays are also widely used for measuring receptor binding and a variety of signal transduction events that may involve the expression of genetic reporters, trafficking of cellular components, or monitoring organelle function (Kong et al., 2017). Regardless of the type of cell-based assay being used, it is important to know how many viable cells are remaining at the end of the experiment. There are a variety of assay methods that can be used to estimate the number of viable eukaryotic cells. MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay is a sensitive and reliable colorimetric assay to investigate the viability of any cell lines by measuring the cell metabolic activities (Chacon et al., 1997; Kuete et al., 2017; Mahajan et al., 2012). The basic principle of the assay is that the MTT reagent (yellow water-soluble tetrazolium dye) is primarily reduced by an enzyme called dehydrogenases of the cellular mitochondria. It is then

formed purple colored formazan crystals. In order to measure the optical density of the formazan, it is needed to be solubilized by DMSO-based solubilization reagent. Measuring the solubilized nanoparticles optical density, estimation of the extent of cellular cytotoxicity of any treatments to the target cell could be done. If the cells are not viable, they would not be able to dehydrogenate any MTT into formazan crystals and by comparing to the viable cells control, percent of viability of the treated and untreated groups could also be calculated and analyzed as simple as making viable cells control group as 100 percent viability (Kuete et al., 2017; Mahajan et al., 2012). Quantitative assay such as ELISA (enzyme linked immunosorbent assay) is another sensitive and reliable colorimetric assay for cell-based assay. It could be used to measure expression level of almost any intracellular and extracellular proteins such as pro-active cytokines involving with specific cellular mechanism, for instant, pro-inflammatory cytokines, inside any cells (Giblin et al., 2018).

2.6 Cosmeceutical innovation

Innovation is often also explained as the application of improvements or better solutions that keep up to new requirements or demands, unarticulated needs, or existing market needs. Innovation can also be defined as something original and more effective or as a consequence new break-through in the market or society (Frankelius, 2009). According to Organization for Economic Co-operation and Development (OECD), innovation is production or adoption, assimilation, and exploitation of a value-added novelty in economic and social spheres; renewal and enlargement of products, services, and markets; development of new methods of production; and establishment of new management systems. It is both a process and an outcome (Edison et al., 2013).

Innovation can be accomplished by creating better or new more effective prototypes, products, processes, technologies, systems as well as services which are already in the market and society (Tony Davila et al., 2006).

Cosmetics were originally created as a medium to express consumer-selves and enhance stature (Nozaki, 2017). Facial & body care and personal hygiene products are the two main categories of cosmetic. However, there are products such as sunscreen products and related products, for instance, sunbathing lotions, skin tanning products or skin whiteners, somehow is not categorized in both facial & body care and personal hygiene products (Salvador et al., 2007). Worldwide consumption of cosmetic products growth is unstoppable and its expanding from women to men, to small babies and children, is undeniable (Salvador et al., 2007). With this increasing pace, developing cosmetic products in order to keep up with marker demands, neither by new creation nor innovation, is crucially important.

2.7 Skin care product as Cosmetics innovation

Skin care products are products which are directly applied on skin. These products are commonly commercialized as lotion, cream, gel or moisturizer. Skin care products have multiple functions including skin protection, moisturizing, appearance changing, smoothing, restoring and also therapeutic effects (Costa et al., 2017). To meet the need of consumers, varieties of skincare products, containing several known active natural ingredients, are available in the market at the moment. Some popular natural ingredient seen in many soap brands, body scrubs and creams products are curcumin, coconut oil, olive oil as well as some others such as honey, avocado, aloe vera, tea tree

oil, sea salt, shea butter, lemon essential oil (Axe, 2015). Skin is the first line of defense against the outside world. While most women know to take care of their face, many neglects complete body skin care. 64% moisturize their face daily and only 42% do the same for the rest of their body. Huge skin care product producer like, Vaseline, put huge effort onto skin healing project. Innovation with just 100% petrolatum or as combination of petrolatum, Theobroma cacao seed butter and fragrance, Vaseline jelly is commercialized as skin protectant with ability to heal cracked and dry skin by locking moisture on the skin (Vaseline). Despite focusing on skin healing and protection, another skin care producer, Nivea, comes up with appearance changing products. With the power of silk innovation, Nivea products are commercialized to have special ability to make human skin soft, silky and smooth, but with the same goal, their products are intended to be used as moisturizer to keep skin intensive hydrated (Nivea). Sederma, a cosmetic company dedicated to the development of active ingredients and in the creation of innovative concepts for Cosmetics, based on the mastery of biotechnology, fine chemistry and vegetal extraction, has launched an active ingredient that is based on oligosaccharides to improve both barrier function and skin hydration. Subliskin, a Sederma skin care product with anti-ageing, moisturizing, protecting activity, contains specific oligosaccharides obtained by a symbiosis-like biotechnological process. The oligosaccharides in the ingredient are said to act on keratinocytes and induce chain reactions down to dermal fibroblasts. These reactions allow the synthesis of elements that are essential to cutaneous homeostasis and regeneration (Schaefer, 2009; Sederma).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

All chemical and reagents used in this research study were analytical grade and purchased from trustful supplier with clear and detail material data sheet as well as quality control certificate.

3.1.1 Chitosan

Food grade chitosan was supported by Marine Bioresource Co.ltd Thailand. Chitosan (Off-white yellow appearance) used in this study is highly purified chitosan with percentage of purity >90%, produced from chitin of dried shrimp shell from tropical ocean. The degree of deacetylation (DD) was higher than 90%, pH 6-8 with water insolubility and solubility in acid solutions (99%). Chitosan particle size was smaller than 1.5 mm with less than 1% ash content.

3.1.2 Bacteria

Lactobacillus plantarum TLG02: (Nguyen et al., 2011; Sak-Ubol et al., 2016)

E.coli TOP10: Cloning strain and expressing strain, Invitrogen (Life Technologies, Darmstadt, Germany)

3.1.3 Cell line

Human Acute Monocytic Leukemia cell line: CLS Cell Lines Service GmbH

3.1.3 Instruments

Autoclave:	Hiclave HA-3000MIV, Hiryama, Japan
Balance:	Precisa 205A, Precisa Instruments, Switzerland
	Precisa 3000C, Precisa Instruments, Switzerland
Centrifuge machine:	Eppendorf centrifuge 5810 R, Eppendorf, USA
Deep freezer -80 ºC:	Heto, Ultra Freeze, Denmark
ELISA reader:	Sunrise, TECAN, Austria
Electroporator:	Eppendorf 2510, Eppendorf, USA
Freezer -20 ºC:	Heto, HLLF 370, Denmark
Gel electrophoresis apparatus:	Mini Protein® 3 cell, BioRad, USA
Heat Box:	HB1, Wealtee Corp., USA
Incubator shaker:	C24 Incubator shaker, New Brunswick Scientific, USA

Incubator:	Memmert, BE 500, WTB Binder BD 115, Shel-Lab 2020 Low Temperature Incubator, Sheidon, USA
Laminar hood:	Holten LaminAir HBB 2448, Denmark
	BH2000 Series Class II Biological Safety Cabinet,
	BHA120 & BHA180, Cycle -Apac
Microcentrifuge:	Mini spin plus, Eppendorf, USA
	Eppendorf 54154, Eppendorf, Germany
pH meter:	Ultra Basic pH meter, Denver Instruments, Germany
Rotator:	Certomat TCC, B. Braun Biotech International, Germany
Shaker:	Innova 2300 platform shaker, New Brunswick Scientific, UK
	Certomat TC2, B. Braun Biotech International, Germany
Spectrometer:	Ultraspec 2000, Pharmacia biotech, UK
	UltraflexTM TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany)

	Size exclusive chromatography (Pharmacia Biotech, Uppsala, Sweden)
Stirrer:	Variomag Electronicrührer Poly 15, Germany
	Magnetic stirrer MSH300, USA
Thermomixer:	Thermomixer compact, Eppendorf, USA
Vortex:	Vortex-Genie 2 G506, Scientific Industries, USA

3.2 Methods

3.2.1 Expression of Chitosanase using Food Grade Expression system

Production of Chitosanase was carried out as according to (Sak-Ubol et al., 2016). In short, batch fermentation with pH control was carried out in 3-L MRS medium using a bioreactor. Recombinant *L. plantarum* strains was taken from a glycerol stock stored at -80 °C, re-streaked on appropriate MRS plates and grown overnight at 37 °C. Ten colonies were picked and grown in 5 mL MRS broth overnight, then sub-culture into two flasks of 100 mL of MRS and cultivate at 37 °C without shaking for 18–24 h. The two overnight cultures were pooled together, mixed well and after measuring the cell density at 600 nm, they were used to inoculate 3 L of MRS medium to an OD 600 of ~0.1. After incubation at 30 °C with 100 rpm agitation under anaerobic condition to an OD600 of ~0.3, the cultures were induced with 12.5 ng/mL of IP-673 (amino acid sequence of IP-673 is Met-Ala-Gly-Asn-Ser–Ser-Asn-Phe-Ile-

His-Lys-Ile-Lys-Gln-Ile-Phe-Tr-His-Arg). During further cultivation (30 °C with 100 rpm), the pH was controlled at pH 6.5 using 3.0 M sodium hydroxide. Chitosanase enzyme was in the fermentation broth.

3.2.2 Expression of Chitosanase using E.coli TOP10

The methodology was adapted from previous work of (Pechsrichuang et al., 2016; Pechsrichuang et al., 2013). pMY202-OmpA-BscCsn46A was transformed into *E.coli* TOP10: Cloning strain and expressing strain, Invitrogen (Life Technologies, Darmstadt, Germany). 10 colonies were picked after overnight growing on LB-agar plate and each colony were grown in 5mL of LB broth media with 250rpm shaking at 37ºC overnight. Then, 2% of overnight culture was inoculated into 1000 mL of TB broth and grown using air-lifted bottle system in a water bath maintaining temperature at 37˚C until optical density (OD) at 600 nm reach 1. After that, the temperature was cooled down to room temperature (25-28 ˚C) for 30 mins. Then, 0.1mM of IPTG was added to induce the expression, and further expressed at room temperature for 20 hours. The recombinant Csn was expressed and secreted outside of the *E.coli* TOP10 into the culture broad. These recombinant Csn were tagged with 10x histidine tag for further purification as downstream processing. After bio-separation, which were mainly centrifugation and filtration, the culture broth was concentrated using cross-flow concentrator (Viva Flow 200, Satorious) after filtered with 0.2 microns filter and one-step purification technique with HisPur Ni-NTA Resin (Thermofisher) was used to purify secreted enzymes.

3.2.3 Enzyme activity assay

Chitosanase activity assay was carried out according to (Pechsrichuang et al., 2013; Sak-Ubol et al., 2016). In short, reaction mixture consisted of 40 µL of appropriately diluted sample and 160 µL of 0.5 % chitosan (w/v) (in 200 mM sodium acetate buffer, pH 5.5, and preincubated at 50 °C for 30 min). The reaction was incubated in a Thermomixer Comfort (Eppendorf AG, Hamburg, Germany) at 50 °C for 5 min, with mixing at 900 rpm. The reaction was stopped by adding 200 µL of DNS solution, and the mixture was centrifuged at 12,000g for 5 min to remove the remaining chitosan that was precipitated. The color in the supernatant was developed by heating at 100 °C for 20 min and cooling on ice. The reducing sugar in the supernatant was determined by measuring OD at 540 nm, using 1–5 µmol/mL of d-glucosamine as standards. The reactions were done in triplicate and we report mean values with standard deviations.

3.2.4 SDS-PAGE

As described in (Sak-Ubol et al., 2016), denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of Laemmli with 12 % (w/v) polyacrylamide gels. The protein samples were briefly heated (10 min) in the loading buffer at 100 °C using a heat block (Eppendorf), and then cooled on ice before loading. Protein bands were visualized by staining with Coomassie brilliant blue R-250.

3.2.5 Protein determination

Protein concentrations were determined using the BCA method with bovine serum albumin as standard. Analysis were operated according to company instruction.

3.2.6 Production of Chitosan oligosaccharides

3.2.6.1 Production of Chitosan oligosaccharides lactic acid using food grade enzyme

Chitosan oligosaccharides was produced using enzyme hydrolysis reaction method where enzyme usage conditions were 0.05 and 0.1 µg per 1 mg of chitosan to be hydrolyzed, respectively. 1% Chitosan was dissolved using 2% lactic acid solution from 85% lactic acid and then pH was adjusted to 5.5 using sodium hydroxide (1N). Chitosan substrate solution was pre-incubated for 30 min at 37 ^{o}C before enzyme is added. The reaction mixture was carried on for 48 hours where enzymes were added twice at the start of the reaction and after 24 hours of reaction. The reaction mixture was then heated to inactivate enzyme activity at 100 ^{o}C for 20 min, then cool down before being centrifuged and filtered to remove all the precipitate in the solution. Chitosan oligosaccharides was lyophilized using freeze-dryer where the product mixture was totally frozen before the operation.

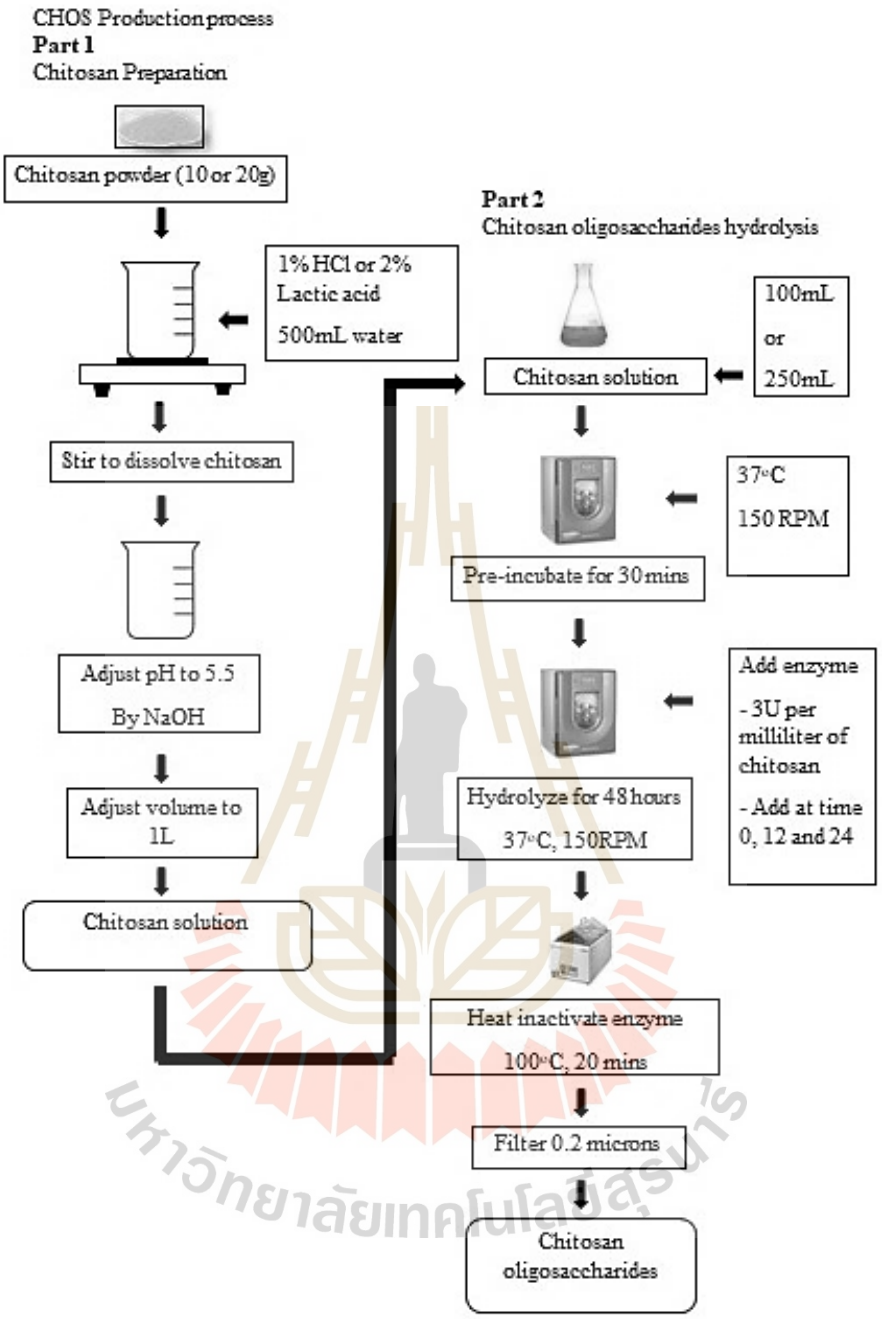


Figure 4. CHOS production process diagram. Overall production process of CHOS is separated into 2 part. Part 1: preparation of chitosan solution, chitosan powder was dissolved in HCl or Lactic acid depending of different study conditions at different concentration (10g or 20g per liter), pH 5.5. Part 2: Hydrolysis reaction, 100mL or 250mL of chitosan solution in HCl or lactic acid were used for the degradation reaction at 37 ºC.

3.2.6.2 Production of Chitosan oligosaccharides in lactic acid using enzyme from *E.coli* TOP10

Chitosan oligosaccharides was produced using enzyme hydrolysis reaction method where enzyme concentration conditions were 0.1, 0.5 and 1 unit per milliliter of chitosan solution to be hydrolyzed, respectively. 2% chitosan was dissolved using 2% lactic acid solution from 85% lactic acid and then pH was adjusted to 5.5 using sodium hydroxide (1N). Chitosan substrate solution was pre-incubated for 30 min at 37 ^{o}C before enzyme is added. The reaction mixture was carried on for 24 hours . The reaction mixture was taken at different time points then heated to inactivate enzyme activity at 100 ^{o}C for 20 min, then cool down and stored for further analysis such as TLC. Sample to be obtained as died powder was centrifuged and filtered to remove all the precipitate in the solution. Chitosan oligosaccharides was obtained as final dried powder product after undergone lyophilization using freeze-dryer where the product mixture was totally frozen before the operation.

3.2.6.3 Production of chitosan oligosaccharides in lactic acid using enzyme from *E.coli* TOP10 – higher volume

Chitosan oligosaccharides was produced using enzyme hydrolysis reaction method where enzyme concentration was 3 units per 1mL of 2% chitosan solution to be hydrolyzed. 1% and 2% of chitosan was dissolved using 2% lactic acid solution from 85% lactic acid and then pH was adjusted to 5.5 using sodium hydroxide (1N), respectively. Chitosan substrate solution was pre-incubated for 30 min at 37 ^{o}C before enzyme is added. The reaction mixture was carried on for 48 hours where enzymes were added 3 times, at the start of the reaction, at 12 hours and after 24 hours of reaction. The reaction mixture was then heated to inactivate enzyme activity at 100

^{o}C for 20 min, then cool down before being centrifuged and filtered to remove all the precipitate in the solution. Chitosan oligosaccharides was obtained as final dried powder product after undergone lyophilization using freeze-dryer where the product mixture was totally frozen before the operation.

3.2.6.4 Production of chitosan oligosaccharides in hydrochloric acid using enzyme from *E.coli* TOP10

Chitosan oligosaccharides was produced using enzyme hydrolysis reaction method where enzyme concentration was 3 units per 1mL of 2% chitosan solution to be hydrolyzed. 1% of chitosan was dissolved using 1% hydrochloric acid solution from 37% hydrochloric acid stock and then pH was adjusted to 5.5 using sodium hydroxide (1N). Chitosan substrate solution was pre-incubated for 30 min at 37 ^{o}C before enzyme is added. The reaction mixture was carried on for 48 hours where enzymes were added 3 times at the start of the reaction, at 12 hours and after 24 hours of reaction. The reaction mixture was then heated to inactivate enzyme activity at 100 ^{o}C for 20 min, then cool down before being centrifuged and filtered to remove all the precipitate in the solution. Chitosan oligosaccharides was obtained as final dried powder product after undergone lyophilization using freeze-dryer where the product mixture was totally frozen before the operation.

3.2.6.5 Production of Chitosan oligosaccharides using chitosanase in broth media from *E.coli* TOP10 expression system

250mL of 2 chitosan was preincubated at 37^{o}C with 150rpm shaking for 30 mins. After preincubation, approximately 3U of chitosanase per milliliter of chitosan was added into the reaction flask and left incubate for 48 hours. The same

amount of enzyme was added at 12 hours and 24 hours of reaction. The total volume of broth media containing enzyme was 220 mL. The reaction was stopped by heating at 95ºC for 20 mins to inactivate the enzyme. Chitosan oligosaccharides was obtained as final dried powder product after undergone lyophilization using freeze-dryer where the product mixture was totally frozen before the operation.

3.2.7 Analysis of Chitosan Oligosaccharides

3.2.7.1 Chitosan precipitation test

To investigate whether there is chitosan left over in the hydrolysis reaction, base precipitation technique was used since chitosan is not soluble in high pH solution. Basically, after hydrolysis reaction, base solution (10M sodium hydroxide) was added into hydrolyzed chitosan solution to adjust pH to more than 7 to precipitate chitosan which left over from the reaction.

3.2.7.2 Glucosamine concentration measurement

The assay was carried out according to chitosanase activity assay of (Pechsrichuang et al., 2013; Sak-Ubol et al., 2016) with slightly modification. In short, reaction mixture was collected from the hydrolysis reaction at designed time points and the reaction was stopped by adding 200 µL of DNS solution, and the mixture was centrifuged at 12,000g for 5 min to remove the remaining chitosan that was precipitated. The color in the supernatant was developed by heating at 100 °C for 20 min and cooling on ice. The reducing sugar in the supernatant was determined by measuring OD at 540 nm, using 1–5 µmol/mL of d-glucosamine as standards.

3.2.7.3 Thin Layer Chromatography

CHOS samples powder was weighted and dissolved in distilled water and a solution of 100mg/mL was prepared. 2 and 3 µl was the solution was spotted onto the TLC silica plate with the distance from each sample of 0.5 cm. Hot air blower was used to dry to the sample after each 1µl of spotting. CHOS prepared in Thailand and commercial CHOS was used as control. The prepared plate was then left stand in a mobile-phase prepared beaker (14ml isopropanol, 4ml water and 2ml ammonia) for about 2 hours until the visible mobile phase wet through to the top of the plate, then the plate was dried, and the same chromatography was repeated. After that, the plate was dried, and soaked into 10% sulfuric acid in ethanol rapidly for about 1-2 mins and then the visualization was done by heating the plate at 150^{o}C using a plate heater until the sample on the plate became visible.

3.2.7.4 NMR spectroscopy

In order to analyze the degree of polymerization of the hydrolyzed products as well as degree of scission throughout the whole hydrolysis reaction process, samples were subjected to ^{1}H NMR. Hydrolyzed CHOS samples was collected and freeze-dried using a freeze-dryer. Dried powder samples were then dissolved in deuterium water (D_2O), and then samples pD were adjusted to 4.5 (±2) using deuterium chloride (DCl) or sodium deuterium oxide (NaOD). The pD adjusted samples were lyophilized overnight and then once again, dissolved in deuterium water, pD was as well checked and adjusted. Following previous work done by (Petsrichuang et al., 2018; Sorbotten et al., 2005). ^{1}H NMR spectra were generated at 400 MHz with the temperature of 85 ^{o}C. Aα, Aβ,Dα and Dβ are the integrals of the reducing end signals of α and β anomers of GlcNAc and GlcN, respectively, while A and D equal the

integrals of the peaks representing sugars in internal positions and at the non-reducing end. So, DPn formula was DPn = (Aα+Aβ+Dα+Dβ+A+D)/(Aα+Aβ+Dα+Dβ).

3.2.7.5 Size exclusive chromatography

SEC was used for baseline separation of CHOS as previously described (Sorbotten et al., 2005). Three XK 26 columns were connected in series and packed with Superdex™ 30 (Pharmacia Biotech, Uppsala, Sweden), with an overall dimension of 2.60×180 cm. The mobile phase, (0.15 M ammonium acetate, pH 4.5) was pumped through the system using an LC-10ADvp pump (Shimadzu GmbH, Duisburg, Germany) at a flow rate of 0.8 ml/min. Products were detected using a refractive index (RI) detector (Shodex RI-101, Shodex Denko GmbH, Dusseldorf, Germany) coupled to a CR 510 Basic Data logger (Campbell Scientific Inc., Logan, UT). Before being injected into the column, CHOS samples were frozen and freeze-dried before they were dissolved in the mobile phase and filtered through 0.22μm filter.

3.2.7.6 Analysis of CHOS by mass spectrometry (MS)

Identification of CHOS samples was carried out using a Matrix-Assisted Laser Desorption Ionization mass spectrometry (MALDI TOF/TOF MS). An UltraflexTM TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) with gridless ion optics under the control of Flexcontrol was used to generate MS spectra. For CHOS samples crystallization, 1 μl of sample was mixed with 1 μl of 10% (w/v) 2,5dihydroxybenzoic acid (DHB) in 30% acetonitrile and spotted onto a MALDI target plate and dried using a hot-air blower (Bahrke et al., 2002; Petsrichuang et al., 2018). The MS experiments were conducted in reflectron mode with a 20-kV accelerating potential.

3.2.8 Biological activity assay

3.2.8.1 Macrophage assay

Proinflammatory cytokine Interleukin 1 beta (IL1β) in macrophage was investigated. The human macrophage cell line, THP-1, was cultured according to instruction. The chitosan oligosaccharides pre-treatment and the Lipopolysaccharide (LPS) exposure was performed. Expression of MAPK and changes of Tumor Necrosis Factor alpha (TNF-α), cytokine, IL-6 and IL1β in macrophages cell was observed using commercial kits and the analysis was carried out according to company protocol with some modification if needed.

3.2.8.2. Differentiation of THP-1 monocytes

THP-1 monocytes cell line was used as host cell for biological activities assay of the hydrolyzed chitosan oligosaccharides. However, THP-1 monocytes are not mature macrophages, so differentiation is needed to induce immature monocytes to become mature monocytes or macrophages. Vitamin D3 was used as inducer for the differentiation at a concentration of 0.2 µm. The differentiation process was carried out for 48 hours. Differentiation confirmation was carried using cell morphology as criteria such shape, size and suspension/attachment to cell culture flasks characteristic of the differentiated cell. THP-1 suspension cells were cultured in complete RPMI media (Roswell Park Memorial Institute media) until reaching 90 to 95% confluency. Cells were harvested and 5.10^5 of THP-1 cells were then seeded into a 24-wells plate with combination of 0.2 µm of vitamin D3 in the complete media. Seeded cells were cultured for 2 days in order to induce the differentiation.

3.2.8.3 Anti-inflammation activity of CHOS on differentiated monocytes

Anti-inflammation assay of CHOS on THP-1 cell line was carried in 24-wells plates with number of cells needed for the assay per well was 5.10^5 cells. 0.2 µm of vitamin D3 as final concentration was prepared for differentiation of all seeded cells in each well. The cells were left growing for 48 hours. Chitosan oligosaccharides, dissolved in water solutions were prepared at 100 mg/mL with pH was adjusted to 7 using sodium hydroxide and filtered at 0.22 micron, was added into differentiated THP-1 culture as pretreatment for 24 hours at various concentration ranging from 50 µg/mL to 200 µg/mL. Then, the cells were exposed to lipopolysaccharides for 6 hours at a concentration of 100 ng/mL of the cell culture volume. Cell culture supernatant of each well was collected, centrifuged and stored for proinflammatory cytokines detection.

IL-1βeta, was targeted cytokines for proinflammatory cytokines detection using ELISA based technique. The detection assay was carried out according the company standard protocol. First of all, the capture antibodies were immobilized into the 96-wells plate overnight the night before assay. Then the immobilized antibodies were washed away with PBST as washing buffer. Immobilized plate was blocked with reagent diluent for an hour at room temperature. After washing out blocking reagent, 200 µl of samples and standards was added to the plate and incubated for 2 hours in room temperature. Washing steps were then repeated before addition of detection antibodies. The plate was incubated for another 2 hours in the room temperature. Washing steps were repeated before streptavidin-HRP was added and the plate was incubated in the dark for 20 mins. After another washing, substrate solution was added to the plate and incubated in the dark for another 20 mins. After incubation,

the reaction was stopped by adding stop solution. Optical density was determined using a microplate reader with wavelength set to 450 nm with correction set up at 540 nm. Data was analyzed using Microsoft excel 2016 and GraphPad Prism 6 for windows.

3.2.8.4 Cell cytotoxicity analysis

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Invitrogen) was used for cellular cytoxicity analysis method for investigating CHOS cytotoxicity on THP-1 cell viability. 1 × 105 cells THP-1 cells in 100 µl containing 0.2 µM of Vitamin D3 were seeded into 96 wells plate and incubated at 37 ℃ and 5% CO2 for 48 hours. The cells were then treated with 25, 50 and 100 µg/ml of CHOS for 24h. After incubation, culture media was removed and 100 µl of 0.5 mg/mL MTT solution was added and the cells were further incubated for 3 h. Then, the MTT solution was discarded and purple formazan crystal was dissolved in 150 µl of solubilize reagent (Invitrogen). The absorbance was measured at 570 nm, using a microplate reader (BMG Labtech, Ortenberg, Germany). The absorbance of untreated cells was taken as 100% viability. All experiments were performed in triplicate.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Chitosanase Production

4.1.1 Expression and Purification of Chitosanase

Enzymatic hydrolysis was selected for production of chitosan oligosaccharides due to its advantages over chemical and physical methods such as environmental friendly, production control and more. *Bacillus subtillis* Chitosanase 46A (EC. 3.321.312), which was previously engineered by molecular biotechnology group of Suranaree university of technology (Pechsrichuang et al., 2016; Pechsrichuang et al., 2013; Petsrichuang et al., 2018), was over-expressed using IPTG. With *tac* promoter and OmpA signal peptides (Pechsrichuang et al., 2016), recombinant Csn was expressed and secreted outside of the *E.coli* TOP10 into the culture broad. These recombinant Csn were tagged with 10x histidine tag for further purification as downstream processing. After bio-separation, which were mainly centrifugation and crossflow filtration, the culture broth was concentrated using cross-flow concentrator (Viva Flow 200, Satorious) after filtered with 0.2 microns filter.

One-step purification technique was used to purify secreted enzymes as according to (Pechsrichuang et al., 2016). SDS-PAGE analysis of purified enzymes was carried out (Figure 5) as confirmation. Chitosanase was successfully expressed, secreted and purified as according to SDS-PAGE analysis which could be confirmed

with previous work (Pechsrichuang et al., 2016; Pechsrichuang et al., 2013). The enzymes were expressed and secreted heterogeneously for 20 hours after induction with IPTG with protein molecular weight of 28 kDa (Pechsrichuang et al., 2016; Sak-Ubol et al., 2016)

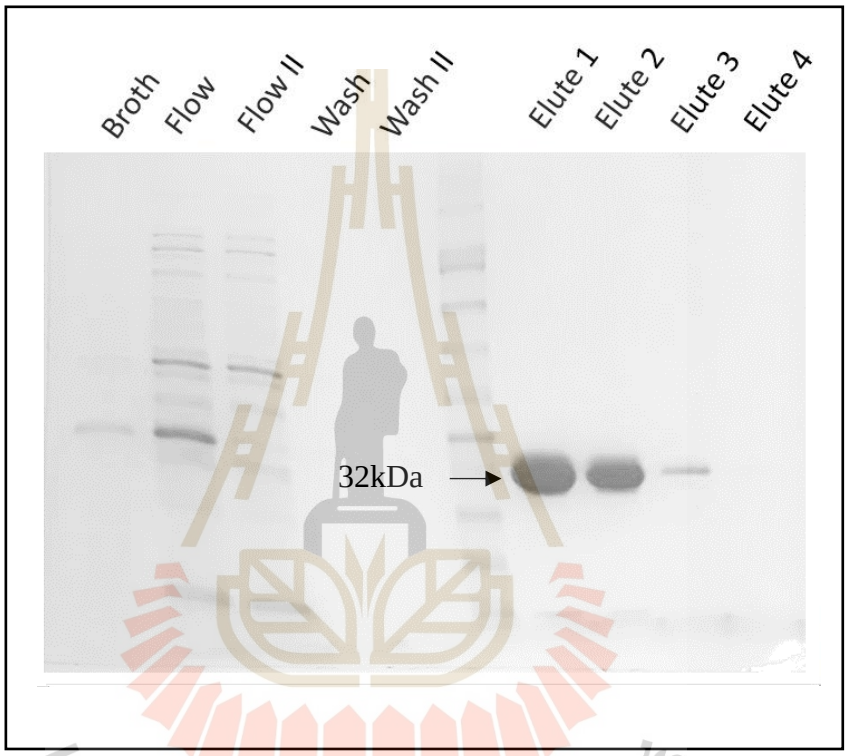


Figure 5. SDS-PAGE analysis of expressed, secreted and purified BsCsn46A. Equal volumes (15µL/lane) of samples were loaded into each lane. Purified protein was obtained from culture broth after 20 h of induction with 0.1 mM IPTG and were separated by SDS-PAGE on 15% gels.

4.1.2 Expression of Chitosanase using food grade expression system

Lactobacillus plantarum TGL02 was used as expression host for the production of food grade chitosanase. According to (Sak-Ubol et al., 2016), the major

proportion of the expressed enzyme is found in the bacterial culture supernatant which was reproducible in this experiment. After the expression for 20 hours, the culture supernatant was collected by centrifugation, filtered and then subjected to concentration process using a cross flow concentration system with molecular weight cut off filter membrane at 10,000 Da. The concentrated culture broth was next undergone purification process using gravity-flow column technique. With resin affinity beads which has high affinity specific binding to histidine tag and well-engineered histidine tag on the recombinant enzyme, the enzyme was well purified. Bases on previous study, the enzyme molecular weight was 32 kDa as displayed on SDS-PAGE result (Figure 5) as expected (Pechsrichuang et al., 2016; Sak-Ubol et al., 2016). The purified enzyme was then subjected concentration process, using centrifugation technique with molecular weight cut-off filter membrane centrifugation tube, in order to reduce the sample volume and increase enzyme concentration. As expected, enzyme concentration is higher after concentration (Figure 5).

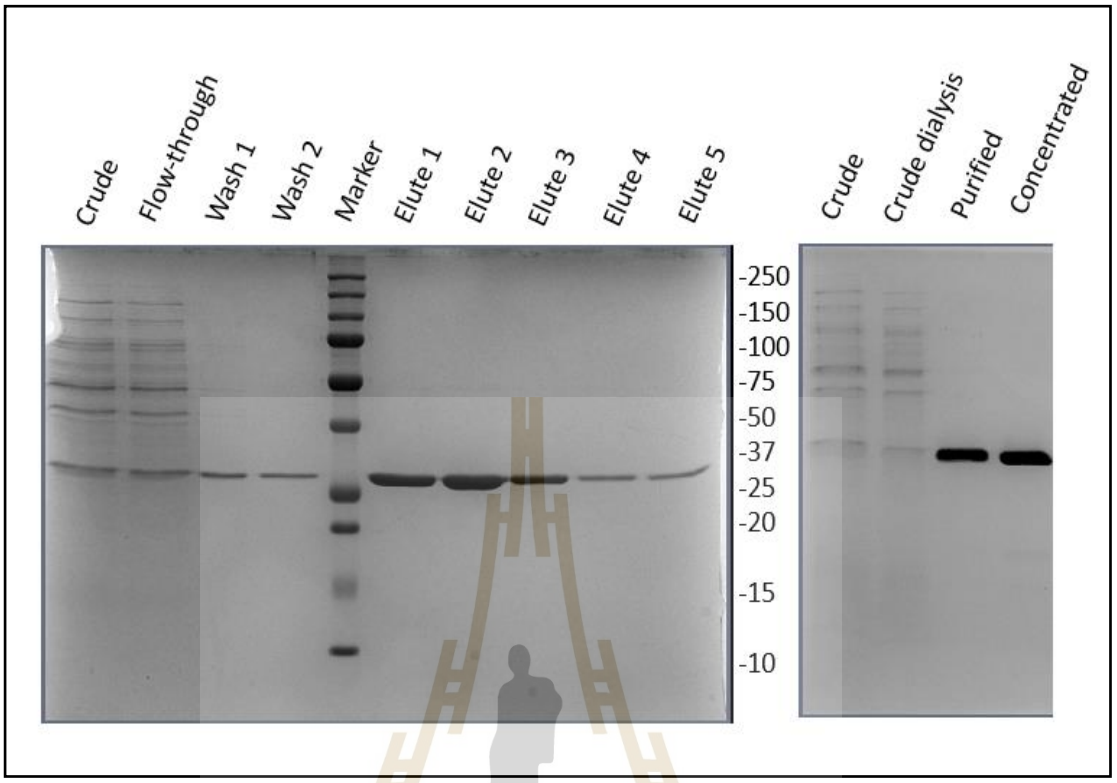


Figure 6. SDS-PAGE of chitosanase using food grade expression system. Equal volumes (15µL/lane) of samples were loaded into each lane. Purified protein was obtained from culture broth after 20 h of induction with 0.1 mM IPTG and were separated by SDS-PAGE on 15% gels.

Table 4. Enzymatic activity BsCsn46A

Samples	Induction time (h)	Enzymatic activity (Total units[a])
		OmpA Signal Peptides
Broth (TOP10)	20	80.0±1
Purified (TOP10)	20	1200.0±1
Purified Concentrated (TOP10)	20	1325.0±1
Broth (Food grade)	20	16.0±1
Purified (Food grade)	20	162.0±1
Concentrated (Food grade)	20	413.0±1

4.2 Chitosan Oligosaccharides Production

4.2.1 Production of Chitosan oligosaccharides using Food Grade enzyme

At different time points of hydrolysis reaction, CHOS samples were collected and were subjected to TLC (Figure 8). 2 different enzyme concentration were used, 0.05 and 0.1 µg, respectively. Hydrolyzed chitosan oligosaccharides samples were collected from the reaction at different time from 5mins, 10 mins, 30 mins, 60 mins, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours and enzyme were added again at 24 hours and samples were collected at 6 hours, 12 hours and 24 hours after second enzyme addition. Enzyme chitosanase was found to hydrolyze chitosan with time depending manner. According the TLC result, chitosan was cleaved shorter and shorter throughout reaction time, however, as shortest as dimers, the enzyme never cleaves chitosan to become chitosan oligosaccharides to monomer level. This phenomena was previously described by (Petsrichuang et al., 2018). The reaction solution was then subjected to lyophilization using a freeze dryer. The dried CHOS powder was then dissolved in water at a concentration of 100 mg/mL for TLC analysis. The sample was

fully water soluble which expressed that the water-insoluble chitosan was totally depolymerized by the enzyme during hydrolysis reaction. However, the sample of early stage of hydrolysis, specially at time points, 5 mins, 10 mins, 30 mins, 1 hours, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 30 hours and 36 hours, respectively, were not subjected to lyophilization and solubility testing. Judging from the TLC result, there were high molecular weight chitosan in the reaction inside the reaction solution until 24 hours of the reaction. To these time points, dimers and trimers CHOS molecules were merely visualized on the silica gel plate while larger hydrolyzed molecules such as tetramers and pentamers were seen more. After second enzyme addition at 24-hour time point, shorter chain of hydrolyzed product was seen more while larger and longer molecules were fading out of the TLC plate. Still, no monomer sample was visualized even at 48 hours of reaction. Unfortunately, production of chitosan oligosaccharides using food grade enzyme was discontinued due to technical difficulty such as low yield of food grade enzyme production.

4.2.2 Production of chitosan oligosaccharides using *E.coli* TOP10 expression system enzyme

Different concentrations 0.1, 0.5, and 1 U/mL, respectively, of enzyme from E.coli TOP10 were used to hydrolyzed chitosan. 2 different reaction temperature 37 ^{o}C and 50 ^{o}C was also studied. CHOS sample were collected at different time points. As shown in Figure 9 , TLC results of the hydrolyzed CHOS at different conditions shows that enzyme concentration, time and temperature do have effects on the final hydrolyzed products. At early time of hydrolysis reaction, longer CHOS were displayed on the TLC plate while shorter CHOS such DP2 and DP3 could not be seen. However, after continuous incubation, shorter and shorter CHOS such as DP2, DP3 and DP4

started to be seen on the TLC plate. However, from the start to the end of the hydrolysis reaction, monomer (DP1) could not be seen with the TLC analysis. This result confirmed previous study which reported that BsCsn46A never cleave chitosan to a monomer level due to its subsite binding reference (Petsrichuang et al., 2018). The TLC results also dedicated that there was possibility that with enzyme concentration of 0.1U/mL, 0.5U/mL and 1U/mL, we could not totally degrade chitosan. Precipitation test of the final hydrolyzed solution of the 3 reactions also proved that, using 0.1 U/mL of enzyme for the reaction, all chitosan was not degraded totally (Figure 7). Glucosamine concentration released during the reaction was also investigated using DNS methods. According the glucosamine concentration of each reaction, there is significant different between those reaction with different enzyme concentration as well as reaction temperature. As expected, higher enzyme concentration reaction, produced higher concentration of glucosamine, except for 0.5 U/mL and 0.1 U/mL reaction at 50 ^{o}C reaction which were not significant different. Comparing between temperature, at 37 ^{o}C with 0.1 U/mL of enzyme, glucosamine concentration at the end of 24 hours reactions was 47 µMol/mL, higher than 0.1 U/mL enzyme reaction at 50 ^{o}C, 17 µMol/mL. At 37 ^{o}C with 0.5 U/mL of enzyme, glucosamine concentration at the end of 24 hours reactions was 67.8 µMol/mL, higher than 0.5 U/mL enzyme reaction at 50 ^{o}C, 59 µMol/mL. At 37 ^{o}C with 1 U/mL of enzyme, glucosamine concentration at the end of 24 hours reactions was 69.8 µMol/mL, higher than 1 U/mL enzyme reaction at 50 ^{o}C, 59 µMol/mL (Figure 10). Interestingly, at 37 ^{o}C, glucosamine was produced more than at 50 ^{o}C, even using the same concentration of enzyme, however, higher concentration of enzyme produced higher concentration of glucosamine within the same reaction setup. This proved that enzyme concentration has a crucial role on the

hydrolysis reaction. Basically, to maximize the degree of scission (α), other way, means to totally hydrolyze all of the chitosan substrate, more enzyme is needed for the process to be done which was also demonstrated in previous studies on the subject (Pechsrichuang, 2017; Petsrichuang et al., 2018; Sorbotten et al., 2005).

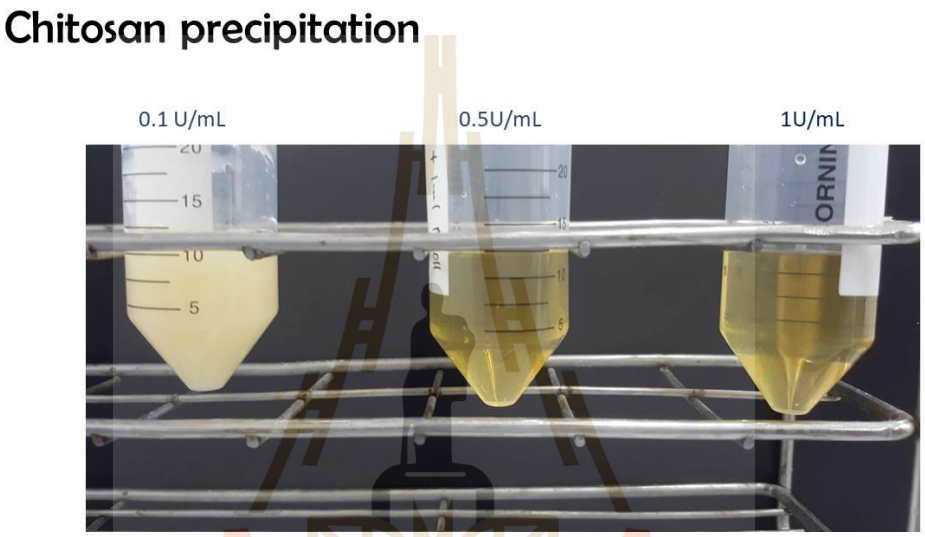


Figure 7. Precipitation test of chitosan hydrolyzed using different concentration of *Bs*Csn46A enzyme (0.1, 0.5 & 1 U/mL) at 37 ^{o}C. Visible turbidity of the solution indicated precipitated chitosan which left over from the reaction.

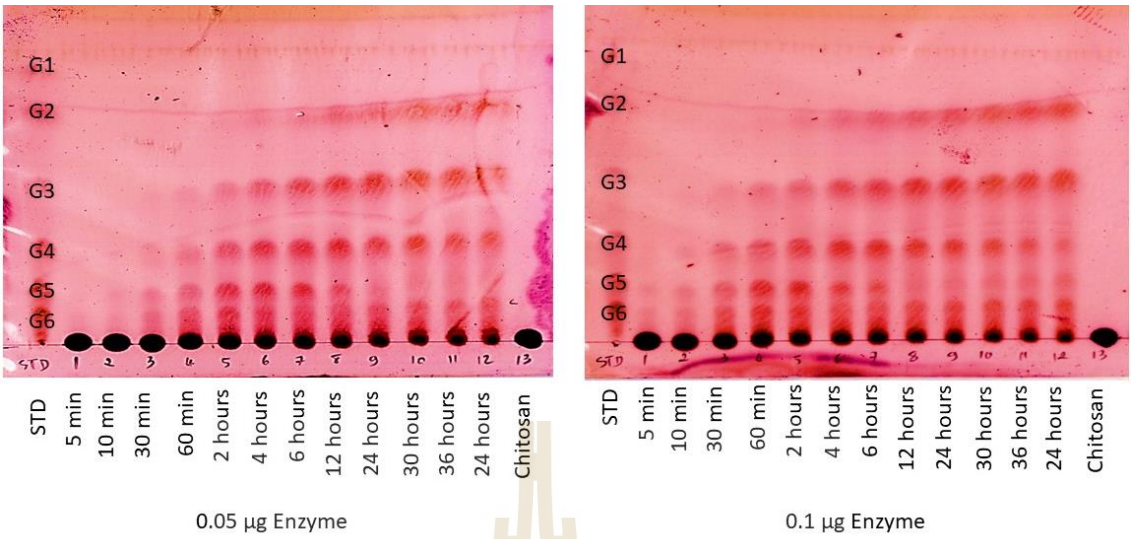


Figure 8. Thin layer chromatography of chitosan oligosaccharides produced by food grade enzyme. 2 different enzyme concentration were used, 0.05 and 0.1 µg, respectively. Hydrolyzed chitosan oligosaccharides samples were collected from the reaction at different time from 5mins, 10 mins, 30 mins, 60 mins, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours and, more enzyme was added again at 24 hours and samples were collected at 6 hours, 12 hours and 24 hours after second enzyme addition.

Table 5. CHOS samples produced in this study.

CHOS sample		Conditions					
	Reaction time	**Enzyme concentration**	**Enzyme usage**	**Enzyme**	**Chitosan concentration**	**Solvent condition**	**Reaction volume**
CHOS 1	48 hours	3U/mL	x3 (Time: 0,12,24)	Pure	10g/L	1% HCL	100 mL
CHOS 2	48 hours	3U/mL	x3 (Time: 0,12,24)	Pure	10g/L	2% LAC	100 mL
CHOS 3	48 hours	3U/mL	x3 (Time: 0,12,24)	Pure	10g/L	2% LAC	250 mL
CHOS 4	48 hours	3U/mL	x3 (Time: 0,12,24)	Pure	20g/L	2% LAC	100 mL
CHOS 5	48 hours	3U/mL	x3 (Time: 0,12,24)	Pure	20g/L	2% LAC	250 mL
CHOS 24(1/2)	24 hours	3U/mL	x2 (Time: 0,12,)	Pure	10g/L	2% LAC	100 mL
CHOS 24(2/2)	24 hours	3U/mL	x2 (Time: 0,12,)	Pure	20g/L	2% LAC	100 mL
CHOS Broth	48 hours	3U/mL	x3 (Time: 0,12,24)	Broth	20g/L	2% LAC	100 mL
CHOS 6	6 hours	0.5U/mL	x1	Pure	20g/L	2% LAC	100 mL
CHOS 12	12 hours	0.5U/mL	x1	Pure	20g/L	2% LAC	100 mL
CHOS 24	24 hours	0.5U/mL	x1	Pure	20g/L	2% LAC	100 mL

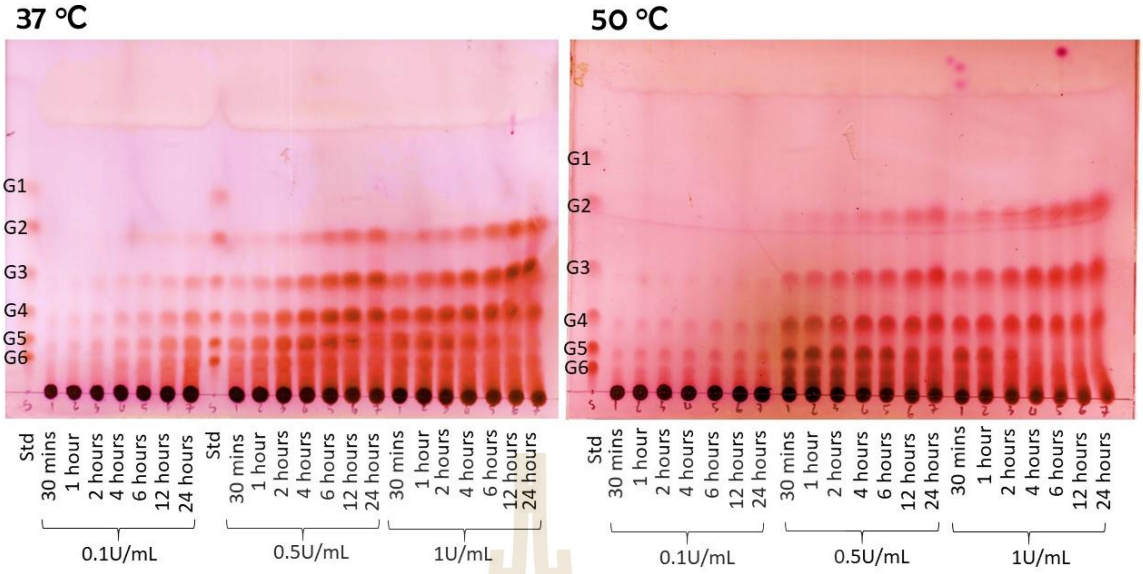


Figure 9. Thin layer chromatography of hydrolyzed CHOS at different conditions. At 2 different temperature 37°C and 50°C, chitosan was hydrolyzed using different concentration of BsCsn46A enzyme (0.1, 0.5 & 1 U/mL). The TLC figure displayed appearance of CHOS samples after hydrolyzed at various time points.

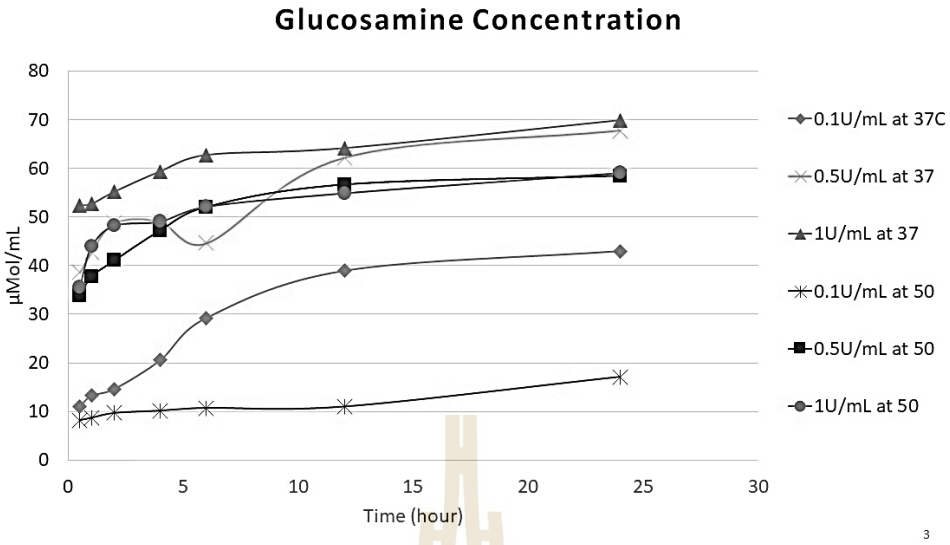


Figure 10. Glucosamine concentration released during hydrolysis reaction of Chitosan at different conditions. At 2 different temperature 37ºC and 50ºC, chitosan was hydrolyzed using different concentration of BsCsn46A enzyme (0.1, 0.5 & 1 U/mL). The figure also displayed concentration of glucosamine at various time points.

Another approach to production of CHOS using BsCsn46A was carried out where higher concentration of enzyme was used. 3U of enzyme per milliliter of chitosan solution were used for the hydrolysis reaction. Different preparations of chitosan solution were also prepared as shown in table 3. The same amount of enzyme was added to the reaction 3 times at the start of the hydrolysis, after 12 hours of the reaction and after 24 hours of the reaction. Final hydrolyzed product was collected at 48 hours after hydrolysis reaction. Samples were freeze-dried and then dissolved in water to determine that there was no insoluble chitosan left-over.

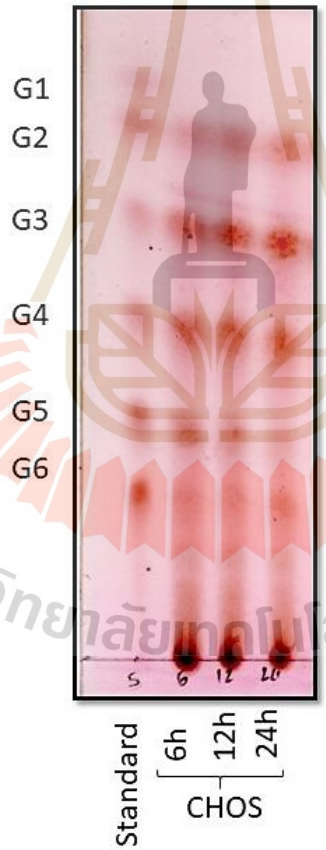


Figure 11. TLC of CHOS hydrolyzed by BsCsn46A at different incubation time points. CHOS was produced using chitosanase at different time of hydrolysis reaction at 6 hours, 12 hours and 24 hours

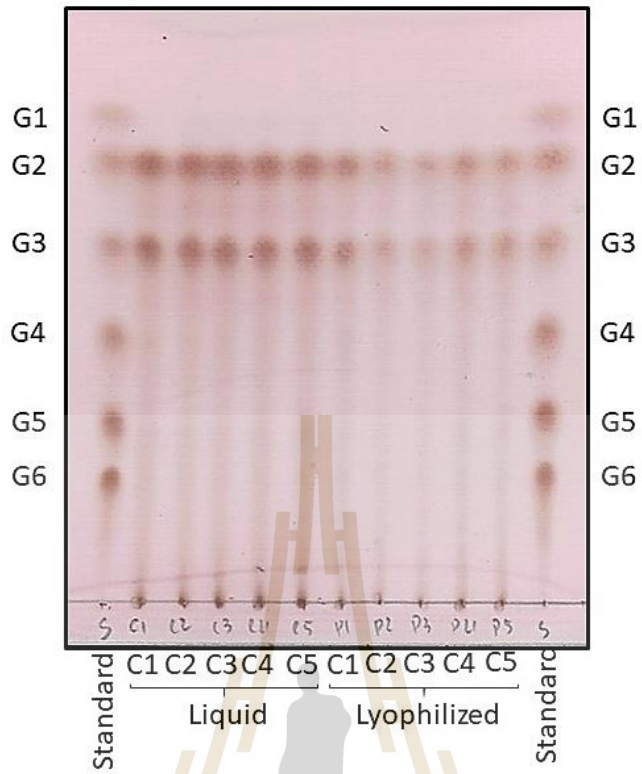


Figure 12. TLC analysis of CHOS hydrolyzed by BsCsn46A enzyme for 48 hours, stored as liquid vs lyophilized powder. CHOS hydrolyzed for 48 hours with enzyme addition at time 0, 12 and 24, final CHOS solution after hydrolysis reaction was separated into 2 parts, 1 was kept at solution and 1 was lyophilized using a freeze dryer.

2ul of each sample were loaded onto TLC plate in order to separate CHOS sample based on oligomers size from the mixture. As could be seen on the TLC plate (Figure 11 - 12), dominant molecules of the hydrolyzed CHOS were dimers and trimers (Petsrichuang et al., 2018). These CHOS samples were further taken into deeper characteristics analysis.

4.3 Characteristics of Chitosan oligosaccharides

4.3.1 Composition analysis of CHOS using Maldi-TOF MS

The chemical compositions of CHOS mixture obtained after 48 hours degradation of chitosan were analyzed by MALDI-TOF-MS and the results are summarized in Table 4. As expected, the majority components of the mixture are CHOS with degree of polymerization DP2 and DP. Longer degree of polymerization of CHOS such as DP4, DP5 and DP6 are also co-existed inside the mixture of the final degradation product, however, they were not as dominant as those with DP2 and DP3. Corresponding the previous study, monomer molecules were not found in the mixture of CHOS after the degradation since in this study we used the same enzyme from previous work (Pechsrichuang, 2017; Pechsrichuang et al., 2013; Petsrichuang et al., 2018). Figure 13.14 & 15 visualizes the mass spectroscopy spectra of analyzed chitosan oligosaccharides labelled with mass dedicated chemical compositions where to be noted that order of the D (D-glucosamine) and A (Acetyl-D-glucosamine) are their sequence dedication. CHOS hydrolyzed using broth enzyme also found to maintain their cleaving characteristics as the final product after hydrolysis reaction are dimers and trimers, however, there were more than just the oligomers itself since the broth

enzyme was in the form of media supernatant. There were some oligomers which were could not be identified as their molecular weight was not corresponding to those known one. That could be a part of the broth LB media or longer CHOS which are left over from the hydrolysis, however, there were not chitosan left over from the reaction as after hydrolysis, samples were freeze-dried and dissolved in water. All samples were totally water soluble as expected. We also identified chemical composition of market available commercial CHOS. The material contains some familiar components such as D2, D3 and D4, but there were also some unidentified molecular mass peaks which showed longer length oligomers. The NMR analysis showed that the average degree of polymerization of this CHOS was DP10 which is considerably long comparing to our produced CHOS in this study. This commercial CHOS also subjected to biological activity investigation.

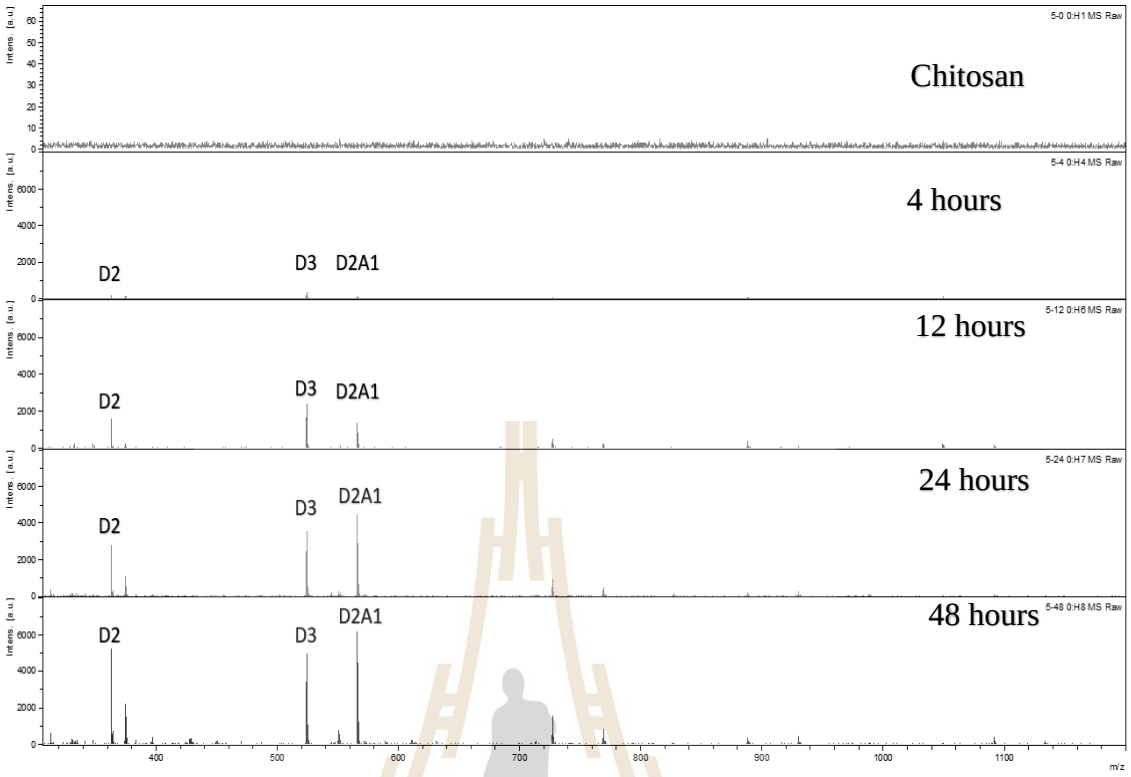


Figure 13. Mass spectrometry spectra of chemical composition analysis of degraded CHOS at different time points. Chitosan and CHOS samples were collected during degradation at different time points and subjected to MALDI-TOF analysis.

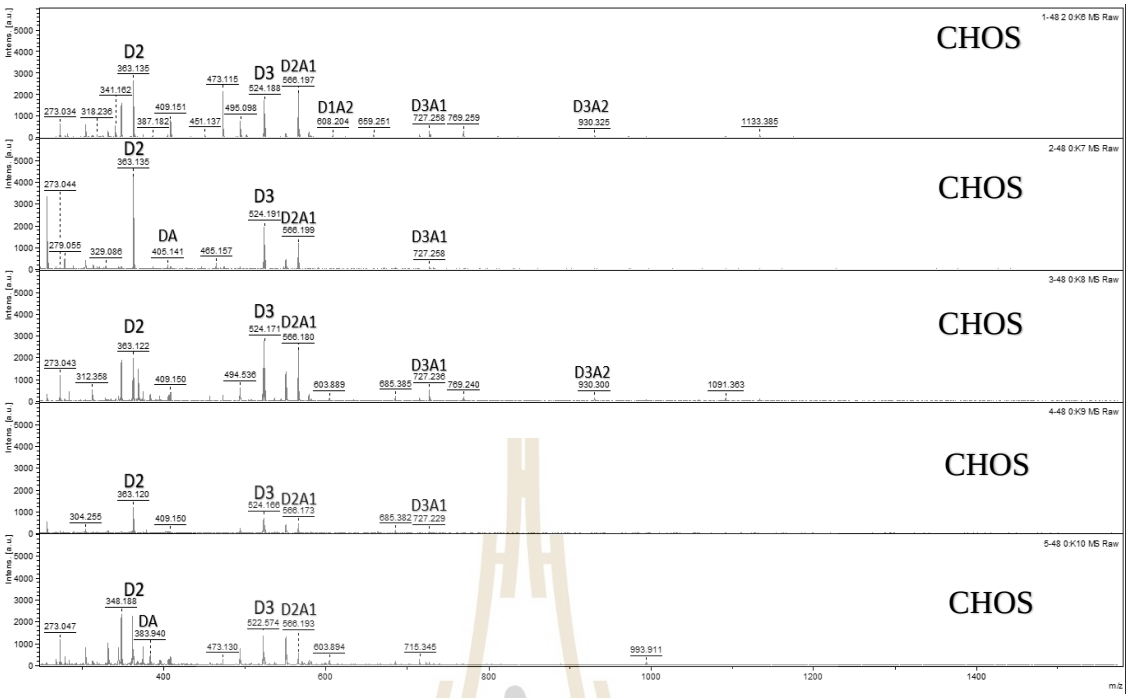


Figure 14. Maldi-TOF MS spectra of degraded CHOS samples C1-C5 using *Bs*Csn46A for 48 hours at 37 ºC. All CHOS samples were freeze-dried, then dissolved in water and crystallized by DHB reagent before subjected to mass spectrometry analysis

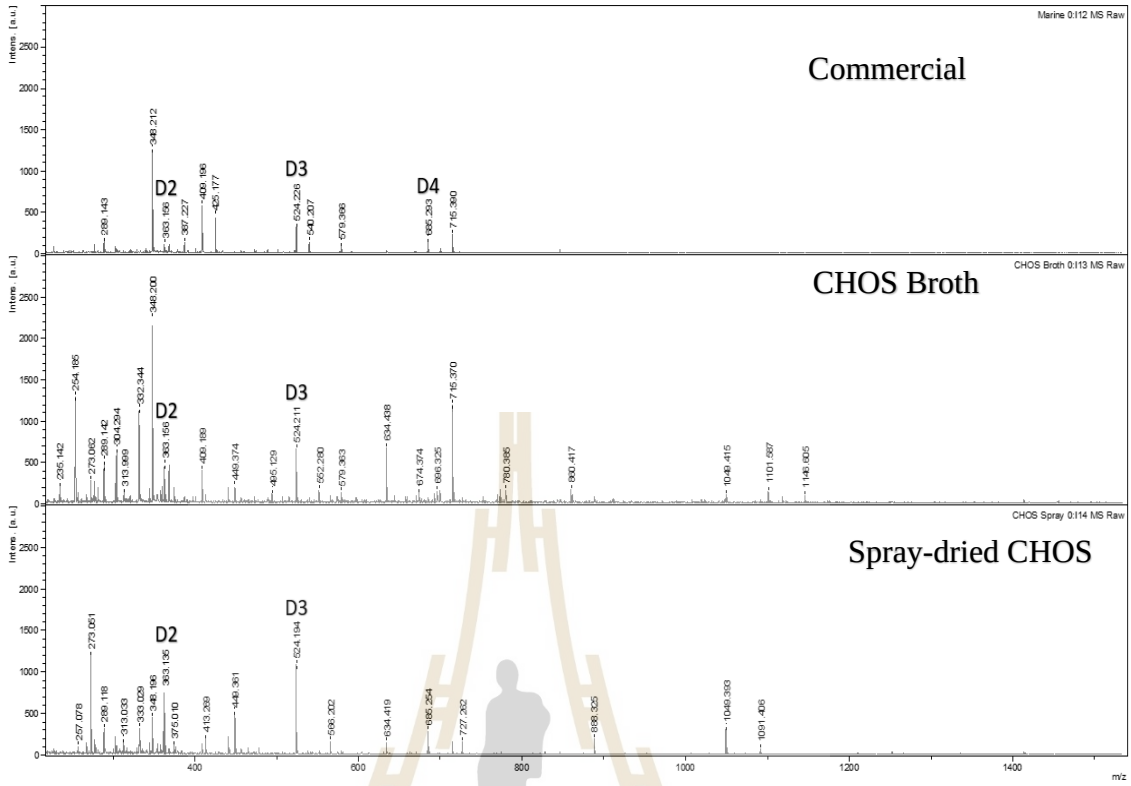


Figure 15. Maldi-TOF MS spectra of degraded CHOS samples. All samples were dry powder, then dissolved in water and crystallized by DHB reagent before subjected to mass spectrometry analysis

Table 6. Chemical compositions of CHOS.

CHOS Sample	Monomer	Dimers	Trimers	Tetramers	Pentamers	Hexamers
1	-	D2, D1A1	D3, D2A1	D3A1, D2A2	D3A2	D3A3
2	-	D2, A2	D3, D2A1	D3A1	-	-
3	-	D2	D3, D2A1	D4, D3A1, D2A2	D3A2	A3D3, A2D4
4	-	D2	D3, D2A1	D4, D3A1	-	-
5	-	D2, D1A1	D3	D4	D1A4	-
CS	-	D2	D3	-	-	-
CM	-	D2, A2	D3	D4	-	-
CB	-	D2	D3	-	-	-

4.3.2 Size exclusive chromatography

After enzymatic hydrolysis of chitosan with BsCsn46A, final products of the reaction were freeze-dried and then were dissolved in 0.15M ammonium acetate for separation analysis using size exclusive chromatography. The separation was based on degree of polymerization of the oligomers in the mixture. As shown in Figure 16, the dominant component oligomers in the final product mixture were DP2 and DP3. There were also longer degree of polymerization CHOS such as DP4, DP5 and DP6 peaks which were getting lower and lower after time to time of the degradation reaction. The SEC results corresponding the mass spectrometry analysis results but eventually visualize better picture of the CHOS properties. This results also confirmed previous

study but notably, the starting substrate chitosan was a mixture of long polymers and some short dimers with degree of deacetylation of 90% as displayed on SEC chromatograms. SEC results also proved that lactic acid did not have any effects on chitosan during substrate preparation.

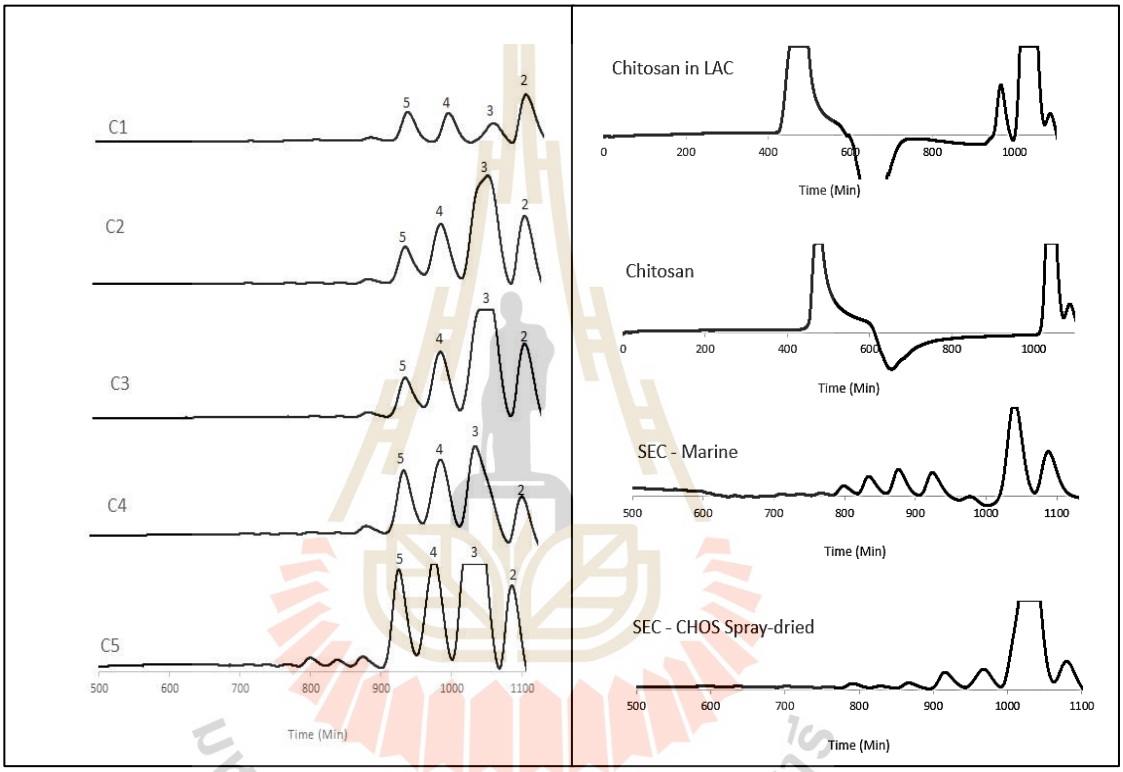


Figure 16. Analysis of CHOS samples after hydrolysis reaction by size exclusion chromatography (SEC). All CHOS (C1-C5) samples hydrolyzed by *Bs*Csn46A for 48 hours at 37 ^{o}C (Left panel) and Chitosan dissolved in lactic acid solution, Chitosan dissolved in SEC mobile phase, commercial CHOS and CHOS dry powder using spray drying (Right panel).

Time-course degradation experiment was also carried out and injected into SEC columns (Figure 17). After an hour of reaction until 12 hours of reaction, longer polymers were still seen on the chromatograms which were later on almost disappeared at the end of the reaction for 48 hours. The dominant component of the final product mixture were clearly dimers, trimers and tetramers as well as pentamers. According to this SEC results plus NMR results, there was no monomer in the final reaction mixture since BsCsn46A never cleave chitosan into shorter length than dimers due to its natural binding preference, subsite specificity, acetylated sugar units (Petsrichuang et al., 2018).

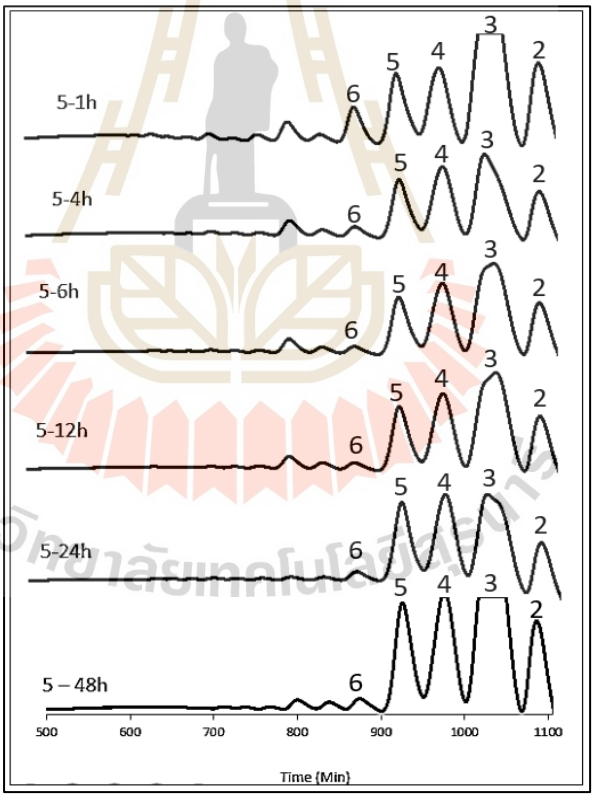


Figure 17. Analysis of CHOS during time-course degradation of hydrolysis reaction by size exclusion chromatography (SEC). Size distribution of CHOS after degradation at different time points at 37 ^{o}C using *Bs*Csn46A. The peaks are labeled with indicating DP.

4.3.3 NMR Spectroscopy

To determine degree of polymerization (DP) of chitosan oligosaccharides produced using enzyme technology, simply described as length or number repeated molecule of the oligomers as well as degree of scission (α) of the hydrolysis reaction, ^{1}H NMR was employed. Time-course degradation curve showed rapid increasing of degree of scission while degree of polymerization of the starting material, which is chitosan, also decreased rapidly at the start of the reaction (Figure 18). According to previous study of the characteristics of the chitosanase used in the reaction, these results were well defined relatable to the enzyme behavior (Petsrichuang et al., 2018). At 100 mL volume scale of the reaction, the degree of scission of C-4 was 0.5 which is higher than C-5, 0.4 but with higher reaction volume of 250 mL. Despite the different degree of scission, both reactions have reached their maximum degradation since the degree of polymerization for both C-4 and C-5 were 2 and 2.3, respectively. However, starting chitosan was a low degree of acetylation (DA) chitosan since its degree of deacetylation (DD) was 90%, thus, may affect the performance of the enzyme due to its binding reference and subsite specificity (Petsrichuang et al., 2018).

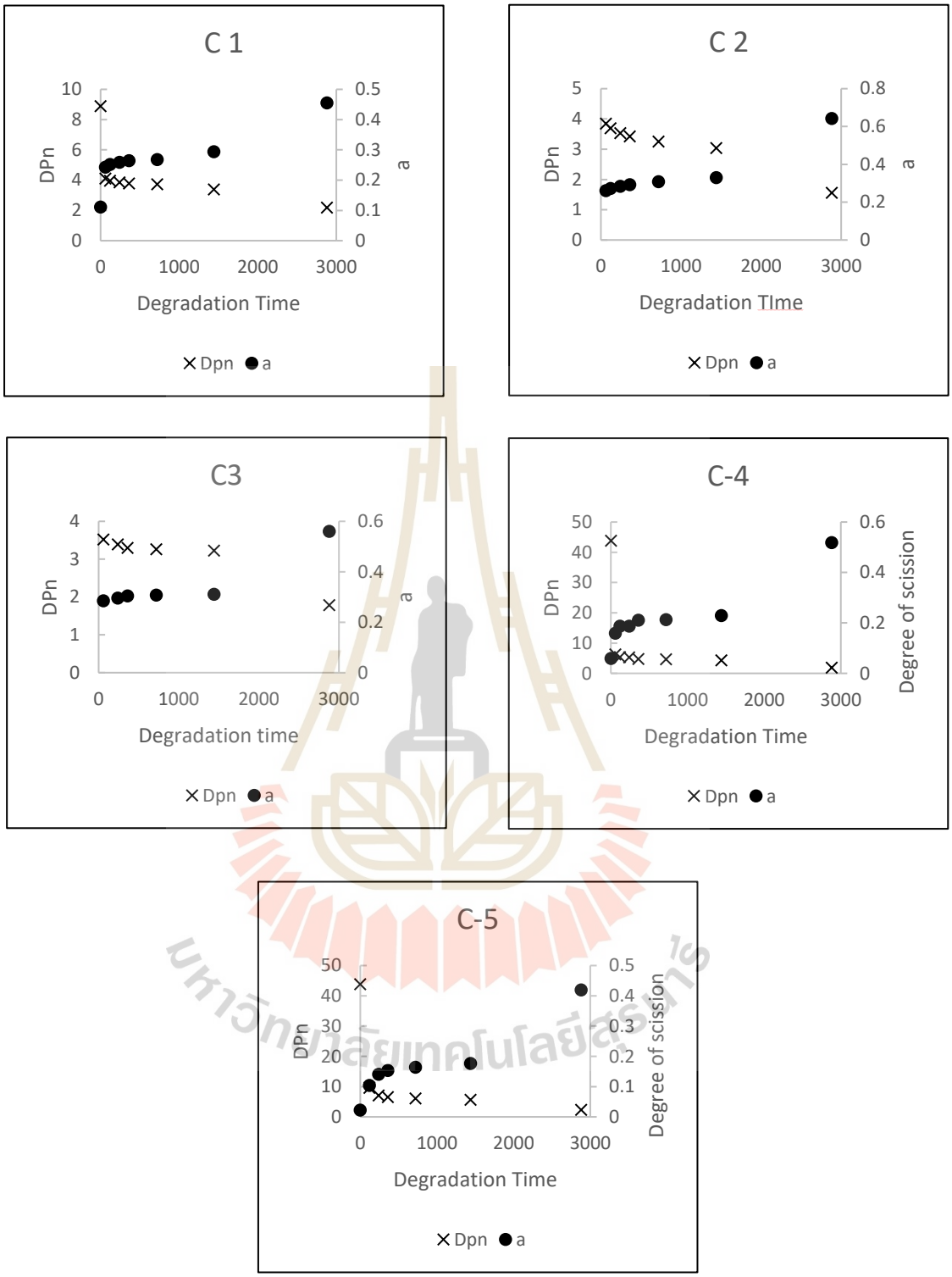


Figure 18. Time-course degradation of chitosan showing increasing of degree of scission (α) and decreasing of degree of polymerization (DP) during the hydrolysis reactions

4.4 Biological activity of chitosan oligosaccharides

As an approach toward cosmeceutical innovation, hydrolyzed product of chitosan was subjected to biological properties assay such anti-inflammation activity testing on human cell line, since CHOS was previously hypothesized and found to be an active biological compound with promising properties (more detail in literature review).

4.4.1 Cellular Viability

The bioactive chitosan oligosaccharides did not possess any cytotoxicity properties toward the human cells at concentration ranging from 25ug/mL, 50ug/mL, and 100ug/mL (Appendix 8).

4.4.2 Anti-inflammation property of chitosan oligosaccharides against LPS

Chitosan oligosaccharides was used as pretreatment for human differentiated monocytes against bacterial lipopolysaccharides challenge. Mature monocytes after differentiation by vitamin D3 for 48 hours was pretreated with chitosan oligosaccharides at solution different concentration from 25, 50 and 100µg/mL, respectively for 24 hours before LPS was exposed to the pretreated cells for 6 hours. The cell culture supernatant was then collected by centrifugation and proinflammatory cytokines was subjected to detection using ELISA based assay. Interleukin 1 beta cytokines was detected in order to investigate anti-inflammation effect of CHOS on the LPS-challenged human differentiated monocytes. Effect of Different CHOS samples was studied (Figure 19-26). Expression of IL-1beta cytokine was expressed as picogram per milliliter of culture supernatant. Average value of duplication was reported.

Different CHOS samples hydrolyzed at different time were shown to be possessing different effects on the cells. CHOS samples with smaller molecules, for instant, CHOS C1-C5 (CHOS hydrolyzed for 48 hours with 3U/mL enzyme concentration and 3x enzyme addition) with major component of dimers and trimers, were found to have highest IL-1β secretion inhibition level compared to CHOS hydrolyzed for 6, 12 and 24 hours with less enzyme concentration (Figure 19, 25-26). Among the CHOS samples, only small molecules CHOS, hydrolyzed for 48 hours, were found to inhibit pro-inflammatory cytokines secretion. Inflammatory response to bacterial LPS usually occur on the plasma membrane of the cell where toll-like receptor 4 is main receptor act as pattern recognition receptors to this LPS ligand (Takeuchi et al., 2010). This could lead to possibility of CHOS binding to the toll-like receptor 4, blocking LPS from interacting with the receptors, since CHOS molecules are small and were pretreated. Furthermore, pretreatments with CHOS alone without any exposure to LPS, were found to induce noticeable expression of pro-inflammatory cytokines.

Anti-inflammation effect of CHOS was previously reported on intestinal epithelial cells including T84, HT-29 and Caco-2 (Muanprasat et al., 2015). The molecular mechanism of the anti-inflammatory effect was found to be CHOS promoting tight junction inside the cells through AMPK-dependent pathway.

This result expressed opposite finding against other studies including (Kong et al., 2017; Kong et al., 2018; Muanprasat et al., 2015), where higher molecular weight of CHOS were found to possess better biological activities.

Figure 27 displays anti-inflammation activities of all CHOS samples (liquid form and powder form at different concentrations) as in percent of inhibition against

LPS-challenged macrophages. Using cell only without any treatments as 100% of inhibition and no pretreatment as 0% of inhibition, it is shown that concentrations had significant effects on the inhibition of pro-inflammatory cytokines activity by CHOS.

However, the same CHOS samples after undergone lyophilization, were found to be losing some of its anti-inflammatory properties, even though none significant biochemical changes were found between the none lyophilized and lyophilized group. According to literature study on freeze drying, this operation could be causing structural changes to the saccharides during the process, therefore the reason behind this quality loss remains unknown.

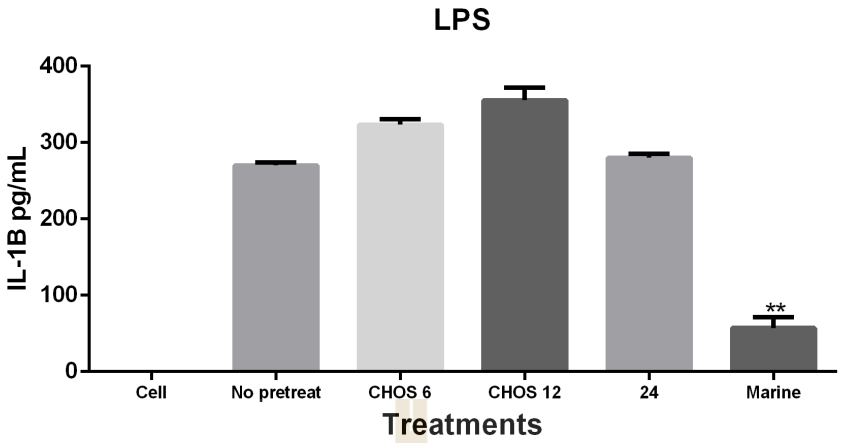


Figure 19. IL-1β secretion level by CHOS-pretreated (50µg/mL) macrophages after LPS-challenge for 6 hours. Samples (Left to right) Cell: differentiated macrophage, no pretreat (cell exposed to LPS without CHOS pretreatment), CHOS 6, CHOS 12, CHOS 24 and commercial CHOS

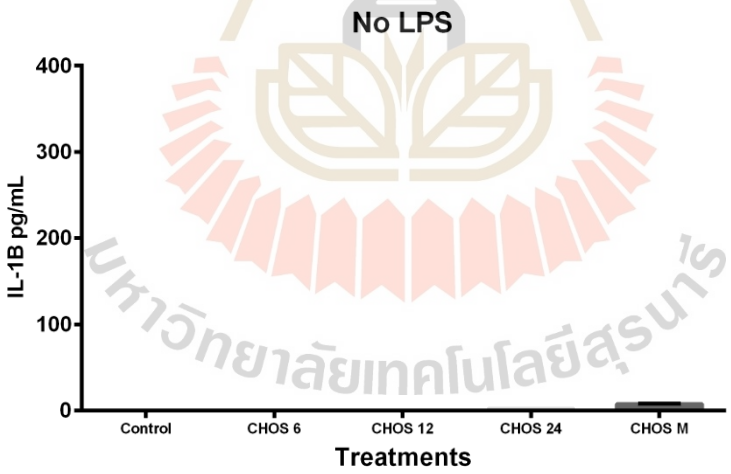


Figure 20. IL-1β secretion level by liquid CHOS-pretreated (50µg/mL) macrophages without LPS-challenge for 6 hours. Samples (Left to right) Cell: differentiated macrophage, no pretreat (cell exposed to LPS without CHOS pretreatment), C1, C2, C3, C4, C5 and commercial CHOS

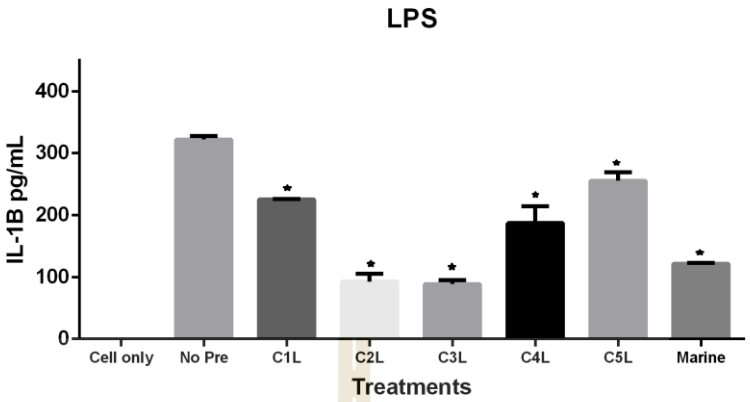


Figure 21. IL-1β secretion level by liquid CHOS-pretreated (50µg/mL) macrophages after LPS-challenge for 6 hours. Samples (Left to right) Cell: differentiated macrophage, no pretreat (cell exposed to LPS without CHOS pretreatment), C1, C2, C3, C4, C5 and commercial CHOS

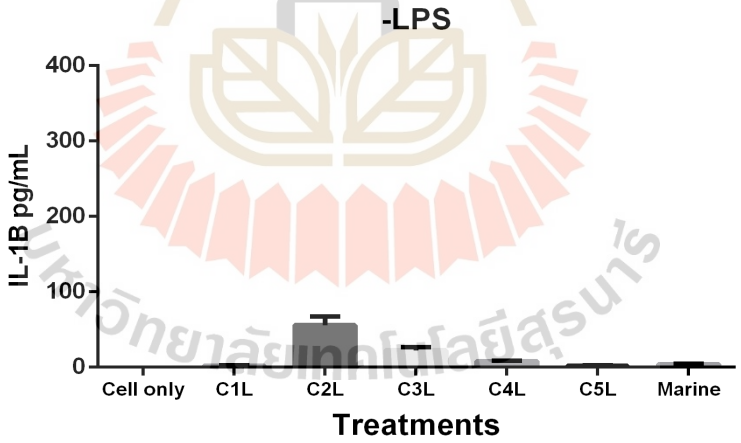


Figure 22. IL-1β secretion level by liquid CHOS-pretreated (50µg/mL) macrophages after LPS-challenge for 6 hours. Samples (Left to right) Cell: differentiated macrophage, no pretreat (cell exposed to LPS without CHOS pretreatment), C1, C2, C3, C4, C5 and commercial CHOS

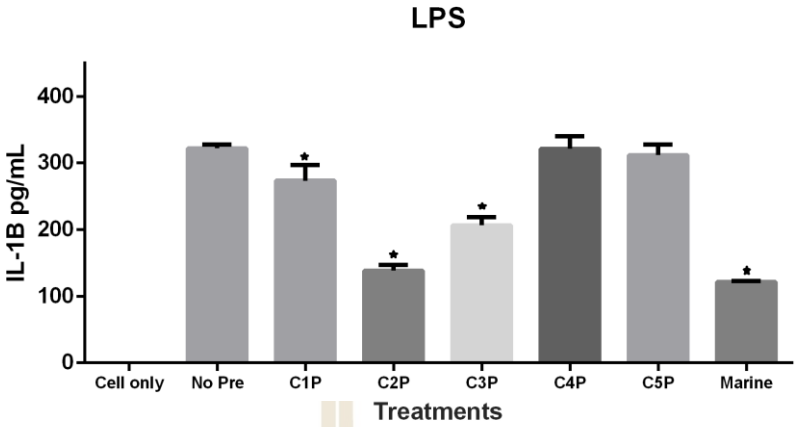


Figure 23. IL-1β secretion level by lyophilized CHOS-pretreated (50µg/mL) macrophages after LPS-challenge for 6 hours. Samples (Left to right) Cell: differentiated macrophage, no pretreat (cell exposed to LPS without CHOS pretreatment), C1, C2, C3, C4, C5 and commercial CHOS

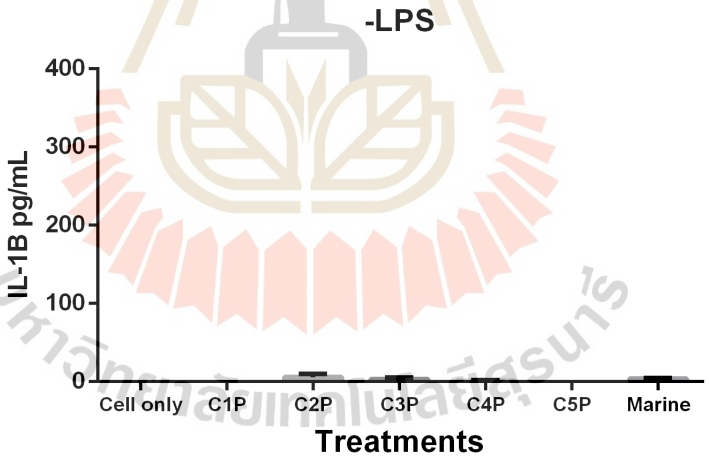


Figure 24. IL-1β secretion level by lyophilized CHOS-pretreated (50µg/mL) macrophages without LPS-challenge for 6 hours. Samples (Left to right) Cell: differentiated macrophage, no pretreat (cell exposed to LPS without CHOS pretreatment), C1, C2, C3, C4, C5 and commercial

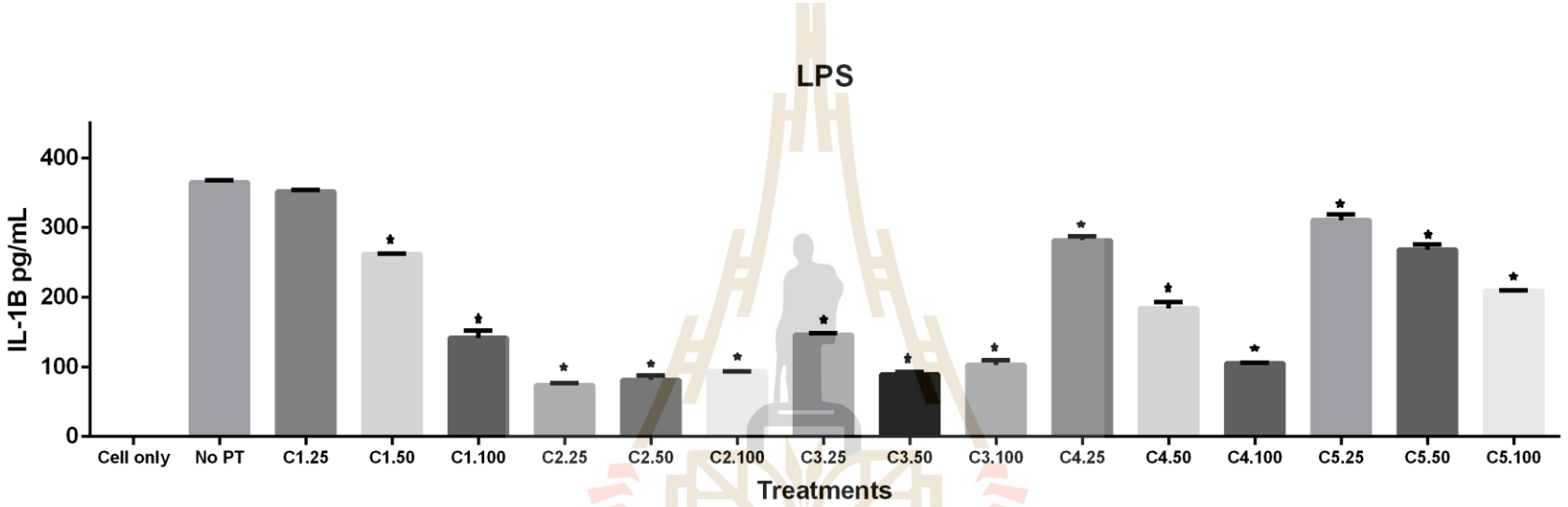


Figure 25. IL-1β secretion level in differentiated macrophages pretreated with liquid CHOS at different concentration from 25, 50 and 100ug/mL for 24 hours and induced inflammation by LPS for 6 hours.

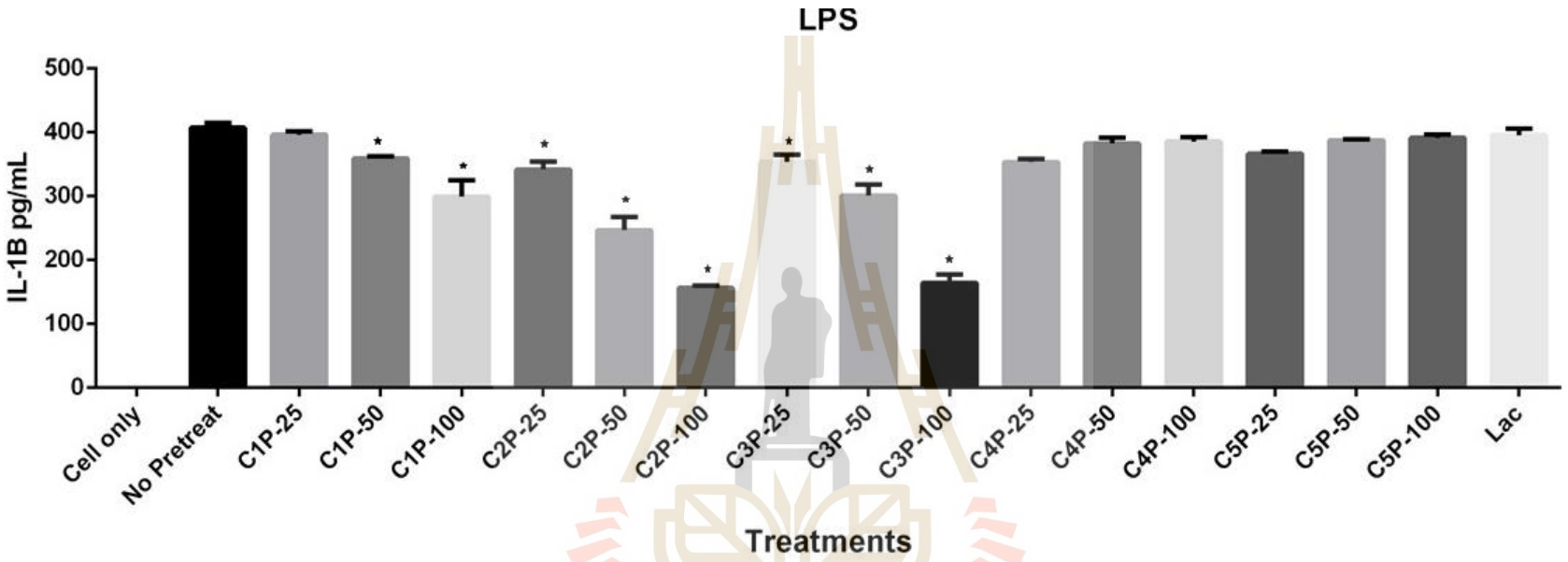


Figure 26. IL-1β secretion level in differentiated macrophages pretreated with lyophilized CHOS at different concentration from 25, 50 and 100ug/mL for 24 hours and induced inflammation by LPS for 6 hours. Lactate was also treated onto the cells as control.

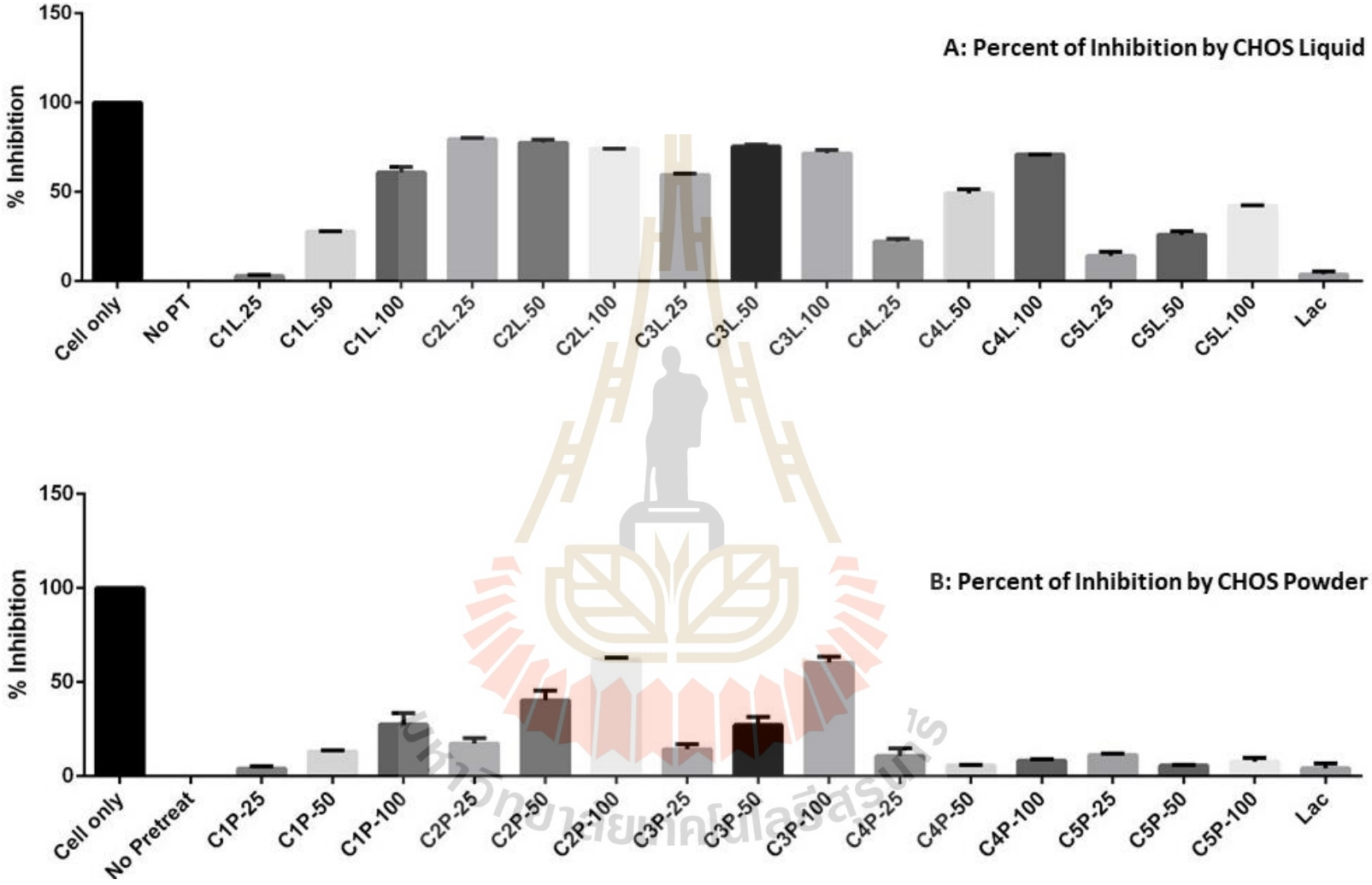


Figure 27. Percent of inhibition of CHOS samples (liquid and powder form at different concentrations, 25, 50 & 100 µg/ML) against LPS-challenged macrophages. A: CHOS in liquid form, B: CHOS in powder form

CHAPTER V

CONCLUSION

According to our previous studies and this study, we proved that *Bs*Csn46A can be used to efficiently converse chitosan into a mixture of variety of soluble chitosan oligosaccharides with various degree of polymerization from shortest DP2 to longest DP6 as expected from the enzyme natural characteristics (Petsrichuang et al., 2018). Concentration of enzyme, temperature and reaction volume do have effects on the final product of the degradation. Eventually, this enzyme is potentially an industrial prosperity. Chitosan oligosaccharides produced using this enzyme as well as other methodologies also could be an important source for industrial application since the material holds such promising biological activities, anti-inflammation (from this study), anti-oxidants (Kong et al., 2017; Pechsrichuang, 2017; Petsrichuang et al., 2018), Apoptosis (Zou et al., 2017) and more.

In summary, CHOS, produced from enzyme technology using BsCsn46A chitosanase, could protect human macrophage cells from inflammation induced by bacterial lipopolysaccharides when it is pretreated as pretreatment before exposing the cells to LPS. The anti-inflammation effects of chitosan oligosaccharides effected the macrophage at concentration depending manners. Finally, this enzymatic hydrolyzed CHOS could be a beneficial material for formulation of applicable products. However, further studies are needed. The bioconversion of chitosan into chitosan

oligosaccharides could yield up to 100% after 48 h of enzymatic reaction and the majority of the final products were dimers and trimers. None of the CHOS products showed toxicity to human cell lines. However, analysis of CHOS protection against LPS-induced inflammation of human macrophage indicated that CHOS generated from different bioconversion processes showed different degree of anti-inflammatory activity, despite the similarity of the product composition. These results indicated that batch-to-batch variation can occur and careful analysis of functional property of CHOS is essential. More biological activities investigations (other pro-inflammatory cytokines and its related mechanisms, in vivo assays) should be carried out with the produced CHOS in order to uncover its actual potentials for further development, especially towards cosmeceutical innovations.

REFERENCES

Aam, B. B., Heggset, E. B., Norberg, A. L., Sorlie, M., Varum, K. M., & Eijsink, V. G. (2010). Production of chitooligosaccharides and their potential applications in medicine. **Mar Drugs**, *8*(5), 1482-1517. doi:10.3390/md8051482

Agatonovic-Kustrin, S., & Morton, D. W. (2018). Chapter 3 - The Current and Potential Therapeutic Uses of Parthenolide. In R. Atta ur (Ed.), *Studies in Natural Products Chemistry* (Vol. 58, pp. 61-91): Elsevier.

Axe, J. (2015). 13 Best Ingredients for Your Natural Skin Care Ritual. *Food is medicine*. Retrieved from https://draxe.com/about-dr-josh-axe/

Bahrke, S., Einarsson, J. M., Gislason, J., Haebel, S., Letzel, M. C., Peter-Katalinić, J., & Peter, M. G. (2002). Sequence analysis of chitooligosaccharides by matrix-assisted laser desorption ionization postsource decay mass spectrometry. **Biomacromolecules**, *3*(4), 696-704. doi:10.1021/bm020010n

Barroso Aranda, J., Contreras, F., Bagchi, D., & Preuss, H. G. (2002). Efficacy of a novel chitosan formulation on fecal fat excretion: A double-blind, crossover, placebo-controlled study. **Journal of Medicine**, *33*(1-4), 209-225.

Berezina, N. (2016). Production and application of chitin. In *Physical Sciences Reviews* (Vol. 1).

Butler, R. N. (1984). 4.13 - Tetrazoles. In A. R. Katritzky & C. W. Rees (Eds.), *Comprehensive Heterocyclic Chemistry* (pp. 791-838). Oxford: Pergamon.

Chacon, E., Acosta, D., & Lemasters, J. J. (1997). 9 - Primary Cultures of Cardiac Myocytes as In Vitro Models for Pharmacological and Toxicological Assessments. In J. V. Castell & M. J. Gómez-Lechón (Eds.), *In Vitro Methods in Pharmaceutical Research* (pp. 209-223). San Diego: Academic Press.

Chang, S.-H., Wu, C.-H., & Tsai, G.-J. (2017). Effects of chitosan molecular weight on its antioxidant and antimutagenic properties. **Carbohydrate Polymers**. doi:10.1016/j.carbpol.2017.11.047

Cho, E. J., Rahman, A., Kim, S. W., Baek, Y. M., Hwang, H. J., Oh, J. Y., . . . Yun, J. W. (2008). Chitosan oligosaccharides inhibit adipogenesis in 3T3-L1 adipocytes. **Journal of Microbiology and Biotechnology**, *18*(1), 80-87.

Choi, E. H., Yang, H. P., & Chun, H. S. (2012). Chitooligosaccharide ameliorates diet-induced obesity in mice and affects adipose gene expression involved in adipogenesis and inflammation. **Nutr Res**, *32*(3), 218-228. doi:10.1016/j.nutres.2012.02.004

Cnubben, N. H., Tel, S. L., Hemmes, M. A., Langenkamp-Brand, A., Grossouw, D., & Jansen, H. T. (2016). A single oral dose of a polyglucosamine influences the bioavailability of [9-(14)C]-oleic acid in adult female Gottingen minipigs. **BMC Obes**, *3*, 18.

Costa, R., & Santos, L. (2017). Delivery systems for cosmetics - From manufacturing to the skin of natural antioxidants. **Powder Technology**, *322*, 402-416. doi:10.1016/j.powtec.2017.07.086

Couzin-Frankel, J. (2010). Inflammation Bares a Dark Side. **Science**, *330*(6011), 1621. doi:10.1126/science.330.6011.1621

Dai, X., Chang, P., Li, X., Gao, Z., & Sun, Y. (2017). The inhibitory effect of chitosan oligosaccharides on beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) in HEK293 APPswe cells. **Neurosci Lett**, *665*, 80-85. doi:10.1016/j.neulet.2017.11.052

Doebel, T., Voisin, B., & Nagao, K. (2017). Langerhans Cells - The Macrophage in Dendritic Cell Clothing. **Trends Immunol**, *38*(11), 817-828. doi:10.1016/j.it.2017.06.008

Dubuisson, P., Picard, C., Grisel, M., & Savary, G. (2018). How does composition influence the texture of cosmetic emulsions? **Colloids and Surfaces A: Physicochemical and Engineering Aspects**, *536*, 38-46. doi:10.1016/j.colsurfa.2017.08.001

Edison, H., bin Ali, N., & Torkar, R. (2013). Towards innovation measurement in the software industry. **Journal of Systems and Software**, *86*(5), 1390-1407. doi:10.1016/j.jss.2013.01.013

Egan, A. M., O'Doherty, J. V., Vigors, S., & Sweeney, T. (2016). Prawn Shell Chitosan Exhibits Anti-Obesogenic Potential through Alterations to Appetite, Affecting Feeding Behaviour and Satiety Signals In Vivo. **PLoS One**, *11*(2), e0149820. doi:10.1371/journal.pone.0149820

Egan, A. M., Sweeney, T., Hayes, M., & O'Doherty, J. V. (2015). Prawn Shell Chitosan Has Anti-Obesogenic Properties, Influencing Both Nutrient Digestibility and Microbial Populations in a Pig Model. **PLoS One**, *10*(12), e0144127. doi:10.1371/journal.pone.0144127

Elieh-Ali-Komi, D., & Hamblin, M. R. (2016). Chitin and Chitosan: Production and Application of Versatile Biomedical Nanomaterials. **Int J Adv Res (Indore)**, *4*(3), 411–427.

Fernandes, J. C., Spindola, H., de Sousa, V., Santos-Silva, A., Pintado, M. E., Malcata, F. X., & Carvalho, J. E. (2010). Anti-inflammatory activity of chitooligosaccharides in vivo. **Mar Drugs**, *8*(6), 1763-1768. doi:10.3390/md8061763

Frankelius, P. (2009). Questioning two myths in innovation literature. **The Journal of High Technology Management Research**, *20*(1), 40-51. doi:10.1016/j.hitech.2009.02.002

Ge, J., Yue, P., Chi, J., Liang, J., & Gao, X. (2018). Formation and stability of anthocyanins-loaded nanocomplexes prepared with chitosan hydrochloride and carboxymethyl chitosan. **Food Hydrocolloids**, *74*, 23-31. doi:10.1016/j.foodhyd.2017.07.029

Giblin, S. P., Murdamoothoo, D., Deligne, C., Schwenzer, A., Orend, G., & Midwood, K. S. (2018). Chapter 21 - How to detect and purify tenascin-C. In R. P. Mecham (Ed.), *Methods in Cell Biology* (Vol. 143, pp. 371-400): Academic Press.

Hong, S. P., Kim, M. H., Oh, S. W., Han, C. H., & Kim, Y. H. (1998). ACE inhibitory and antihypertensive effect of chitosan oligosaccharides in SHR. **Korean J. Food Sci. Technol.**, *30*, 1476-1479.

Huang, L., Chen, J., Cao, P., Pan, H., Ding, C., Xiao, T., . . . Su, Z. (2015). Anti-obese effect of glucosamine and chitosan oligosaccharide in high-fat diet-induced obese rats. **Mar Drugs**, *13*(5), 2732-2756. doi:10.3390/md13052732

Je, J. Y., Park, P. J., & Kim, S. K. (2004). Free radical scavenging properties of hetero-chitooligosaccharides using an ESR spectroscopy. **Food Chem Toxicol**, *42*(3), 381-387. doi:10.1016/j.fct.2003.10.001

Jeon, Y.-J., Park, P.-J., & Kim, S.-K. (2001). Antimicrobial effect of chitooligosaccharides produced by bioreactor. **Carbohydrate Polymers**, *44*(1), 71-76. doi:https://doi.org/10.1016/S0144-8617(00)00200-9

Jeon, Y. J., & Kim, S. K. (2002). Antitumor activity of chitosan oligosaccharides produced in ultrafiltration membrance reactor system. **Journal of Microbiology and Biotechnology**, *12*(3), 503-507.

Jia, P., Yu, L., Tao, C., Dai, G., Zhang, Z., & Liu, S. (2017). Chitosan oligosaccharides protect nucleus pulposus cells from hydrogen peroxide-induced apoptosis in a rat experimental model. **Biomed Pharmacother**, *93*, 807-815. doi:10.1016/j.biopha.2017.06.101

Jitprasertwong, P., Jaedicke, K. M., Nile, C. J., Preshaw, P. M., & Taylor, J. J. (2014). Leptin enhances the secretion of interleukin (IL)-18, but not IL-1beta, from human monocytes via activation of caspase-1. **Cytokine**, *65*(2), 222-230. doi:10.1016/j.cyto.2013.10.008

Kang, N. H., Lee, W. K., Yi, B. R., Park, M. A., Lee, H. R., Park, S. K., . . . Choi, K. C. (2012). Modulation of lipid metabolism by mixtures of protamine and chitooligosaccharide through pancreatic lipase inhibitory activity in a rat model. **Lab Anim Res**, *28*(1), 31-38. doi:10.5625/lar.2012.28.1.31

Karin, M., & Clevers, H. (2016). Reparative inflammation takes charge of tissue regeneration. **Nature**, *529*(7586), 307-315. doi:10.1038/nature17039

Khalil, A., Nawaz, H., Hanif, M. A., & Rehman, R. (2020). Chapter 24 - Ginkgo biloba. In M. A. Hanif, H. Nawaz, M. M. Khan, & H. J. Byrne (Eds.), *Medicinal Plants of South Asia* (pp. 317-329): Elsevier.

Khoushab, F., & Yamabhai, M. (2010). Chitin research revisited. **Mar Drugs**, *8*(7), 1988-2012. doi:10.3390/md8071988

Kobayashi, N. F. a. K. (2005). Macrophage in inflammation. **Current Drug Targets - Inflammation & Allergy**, *4*, 8. doi:10.2174/1568010054022024

Kong, S. Z., Li, D. D., Luo, H., Li, W. J., Huang, Y. M., Li, J. C., . . . Li, S. D. (2017). Anti-photoaging effects of chitosan oligosaccharide in ultraviolet-irradiated hairless mouse skin. **Exp Gerontol**, *103*, 27-34. doi:10.1016/j.exger.2017.12.018

Kong, S. Z., Li, J. C., Li, S. D., Liao, M. N., Li, C. P., Zheng, P. J., . . . Hu, Z. (2018). Anti-Aging Effect of Chitosan Oligosaccharide on d-Galactose-Induced Subacute Aging in Mice. **Mar Drugs**, *16*(6). doi:10.3390/md16060181

Kuete, V., Karaosmanoğlu, O., & Sivas, H. (2017). Chapter 10 - Anticancer Activities of African Medicinal Spices and Vegetables. In V. Kuete (Ed.), *Medicinal Spices and Vegetables from Africa* (pp. 271-297): Academic Press.

Kumar, S. G., Rahman, M. A., Lee, S. H., Hwang, H. S., Kim, H. A., & Yun, J. W. (2009). Plasma proteome analysis for anti-obesity and anti-diabetic potentials of chitosan oligosaccharides in ob/ob mice. **Proteomics**, *9*(8), 2149-2162. doi:10.1002/pmic.200800571

Laskin, D. L., Gardner, C. R., & Laskin, J. D. (2010). 5.06 - Phagocytes. In C. A. McQueen (Ed.), *Comprehensive Toxicology (Second Edition)* (pp. 133-153). Oxford: Elsevier.

Lee, S. H., Senevirathne, M., Ahn, C. B., Kim, S. K., & Je, J. Y. (2009). Factors affecting anti-inflammatory effect of chitooligosaccharides in lipopolysaccharides-induced RAW264.7 macrophage cells. **Bioorg Med Chem Lett**, *19*(23), 6655-6658. doi:10.1016/j.bmcl.2009.10.007

Lens. (2018a). Patent Results for Chitosan Oligosaccharides. Retrieved from https://www.lens.org/lens/search?formAction=%2Flens%2Fsearch&q=Chitosan+Oligosaccharides+

Lens. (2018b). Patent Results for Chitosan Oligosaccharides. Retrieved from https://www.lens.org/lens/search?p=0&q=Chitosan+Oligosaccharides+&v=analysis&formAction=%2Flens%2Fsearch

Li, J., Tan, G., Cheng, B., Liu, D., & Pan, W. (2017). Transport mechanism of chitosan-N-acetylcysteine, chitosan oligosaccharides or carboxymethyl chitosan decorated coumarin-6 loaded nanostructured lipid carriers across the rabbit ocular. **Eur J Pharm Biopharm**, *120*, 89-97. doi:10.1016/j.ejpb.2017.08.013

Lian, Z., Pan, R., & Wang, J. (2016). Microencapsulation of norfloxacin in chitosan/chitosan oligosaccharides and its application in shrimp culture. **Int J Biol Macromol**, *92*, 587-592. doi:10.1016/j.ijbiomac.2016.07.074

Lin, T. J. (2010). Evolution of Cosmetics: Increased Need for Experimental Clinical Medicine. **Journal of Experimental & Clinical Medicine**, *2*(2), 49-52. doi:https://doi.org/10.1016/S1878-3317(10)60009-5

Liu, G. *Chitin, Chitosan and Chitin-Oligosaccharide Assessment and Health Benefits*.

Liu, X., Xia, W., Jiang, Q., Yu, P., & Yue, L. (2018). Chitosan oligosaccharide-N-chlorokojic acid mannich base polymer as a potential antibacterial material. **Carbohydrate Polymers**, *182*, 225-234. doi:10.1016/j.carbpol.2017.11.019

Lodhi, G., Kim, Y. S., Hwang, J. W., Kim, S. K., Jeon, Y. J., Je, J. Y., . . . Park, P. J. (2014). Chitooligosaccharide and its derivatives: preparation and biological applications. **Biomed Res Int**, *2014*, 654913. doi:10.1155/2014/654913

Lu, Y., Slomberg, D. L., & Schoenfisch, M. H. (2014). Nitric oxide-releasing chitosan oligosaccharides as antibacterial agents. **Biomaterials**, *35*(5), 1716-1724. doi:10.1016/j.biomaterials.2013.11.015

Lyu, Q., Shi, Y., Wang, S., Yang, Y., Han, B., Liu, W., . . . Liu, W. (2015). Structural and biochemical insights into the degradation mechanism of chitosan by chitosanase OU01. **Biochim Biophys Acta**, *1850*(9), 1953-1961. doi:10.1016/j.bbagen.2015.06.011

Mahajan, S. D., Law, W.-C., Aalinkeel, R., Reynolds, J., Nair, B. B., Yong, K.-T., . . . Schwartz, S. A. (2012). Chapter three - Nanoparticle-Mediated Targeted Delivery of Antiretrovirals to the Brain. In N. Düzgüneş (Ed.), *Methods in Enzymology* (Vol. 509, pp. 41-60): Academic Press.

Michaud, A., Williams, T. A., Chauvet, M. T., & Corvol, P. (1997). Substrate dependence of angiotensin I-converting enzyme inhibition: Captopril displays a partial selectivity for inhibition of N-acetyl-seryl- aspartyl-lysyl-proline hydrolysis compared with that of angiotensin I. **Molecular Pharmacology**, *51*(6), 1070-1076.

Molteni, M., Gemma, S., & Rossetti, C. (2016). The Role of Toll-Like Receptor 4 in Infectious and Noninfectious Inflammation. **Mediators Inflamm**, *2016*, 6978936. doi:10.1155/2016/6978936

Muanprasat, C., & Chatsudthipong, V. (2017). Chitosan oligosaccharide: Biological activities and potential therapeutic applications. **Pharmacol Ther**, *170*, 80-97. doi:10.1016/j.pharmthera.2016.10.013

Muanprasat, C., Wongkrasant, P., Satitsri, S., Moonwiriyakit, A., Pongkorpsakol, P., Mattaveewong, T., . . . Chatsudthipong, V. (2015). Activation of AMPK by chitosan oligosaccharide in intestinal epithelial cells: Mechanism of action and potential applications in intestinal disorders. **Biochem Pharmacol**, *96*(3), 225-236. doi:10.1016/j.bcp.2015.05.016

Muxika, A., Etxabide, A., Uranga, J., Guerrero, P., & de la Caba, K. (2017). Chitosan as a bioactive polymer: Processing, properties and applications. **Int J Biol Macromol**, *105*(Pt 2), 1358-1368. doi:10.1016/j.ijbiomac.2017.07.087

Nguyen, T. T., Mathiesen, G., Fredriksen, L., Kittl, R., Nguyen, T. H., Eijsink, V. G., . . . Peterbauer, C. K. (2011). A food-grade system for inducible gene expression in Lactobacillus plantarum using an alanine racemase-encoding selection marker. **J Agric Food Chem**, *59*(10), 5617-5624. doi:10.1021/jf104755r

Nivea. Soft, silky skin: The power of silk. Retrieved from https://www.niveausa.com/advice/nice-skin/silky-skin-80

Nohynek, G. J., Antignac, E., Re, T., & Toutain, H. (2010). Safety assessment of personal care products/cosmetics and their ingredients. **Toxicol Appl Pharmacol**, *243*(2), 239-259. doi:10.1016/j.taap.2009.12.001

Nozaki, F. (2017). Chapter 1 - General Aspects of Cosmetics in Relation to Science and Society: Social, Cultural, Science, and Marketing Aspects∗. In *Cosmetic Science and Technology* (pp. 3-14). Amsterdam: Elsevier.

Park, I., Kassiteridi, C., & Monaco, C. (2017). Functional diversity of macrophages in vascular biology and disease. **Vascul Pharmacol**, *99*, 13-22. doi:10.1016/j.vph.2017.10.005

Paul-Clark, M. J., George, P. M., Gatheral, T., Parzych, K., Wright, W. R., Crawford, D., . . . Mitchell, J. A. (2012). Pharmacology and therapeutic potential of pattern recognition receptors. **Pharmacol Ther**, *135*(2), 200-215. doi:10.1016/j.pharmthera.2012.05.007

Pechsrichuang, P. (2017). *Production and Biological Activity of Chito-oligosaccharide Generated by Enzyme Technology*. (Doctor of Philosophia), Suranaree University of Technology, Suranaree University of Technology

Pechsrichuang, P., Songsiriritthigul, C., Haltrich, D., Roytrakul, S., Namvijtr, P., Bonaparte, N., & Yamabhai, M. (2016). OmpA signal peptide leads to heterogenous secretion of B. subtilis chitosanase enzyme from E. coli expression system. **Springerplus**, *5*(1), 1200. doi:10.1186/s40064-016-2893-y

Pechsrichuang, P., Yoohat, K., & Yamabhai, M. (2013). Production of recombinant Bacillus subtilis chitosanase, suitable for biosynthesis of chitosan-oligosaccharides. **Bioresour Technol**, *127*, 407-414. doi:10.1016/j.biortech.2012.09.130

Petsrichuang, P., Lorentzen, S. B., Aam, B. B., Tuveng, T. R., Hamre, A. G., Eijsink, V. G. H., & Yamabhai, M. (2018). *Bioconversion of chitosan into chito-oligosaccharides (CHOS) using family 46 chitosanase from Bacillus subtilis (BsCsn46A)* (Vol. 186).

Pokhis, K., Bitterlich, N., Cornelli, U., & Cassano, G. (2015). Efficacy of polyglucosamine for weight loss-confirmed in a randomized double-blind,

placebo-controlled clinical investigation. **BMC Obes**, *2*, 25. doi:10.1186/s40608-015-0053-5

Primex. History of chitin Retrieved from http://www.primex.is/quality-and-environment/history-of-chitin/

Rehmat, S., Khera, R. A., Hanif, M. A., Ayub, M. A., & Hussain, A. I. (2020). Chapter 27 - Henna. In M. A. Hanif, H. Nawaz, M. M. Khan, & H. J. Byrne (Eds.), *Medicinal Plants of South Asia* (pp. 355-368): Elsevier.

Rong Huei Chen, J. R. C., Ju Shii Shyur. (1997). Effects of ultrasonic conditions and storage in acidic solutions on changes in molecular weight and polydispersity of treated chitosan. **Carbohydrate Research**, *299*, 287-294.

Sak-Ubol, S., Namvijitr, P., Pechsrichuang, P., Haltrich, D., Nguyen, T. H., Mathiesen, G., . . . Yamabhai, M. (2016). Secretory production of a beta-mannanase and a chitosanase using a Lactobacillus plantarum expression system. **Microb Cell Fact**, *15*, 81. doi:10.1186/s12934-016-0481-z

Salvador, A., & Chisvert, A. (2007). *Analysis of Cosmetic Products* (1st ed.). Radarweg 29, PO Box 211, 1000 AE Amsterdam, The Netherlands, The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, UK: Elsevier

Saradna, A., Do, D. C., Kumar, S., Fu, Q. L., & Gao, P. (2018). Macrophage polarization and allergic asthma. **Transl Res**, *191*, 1-14. doi:10.1016/j.trsl.2017.09.002

Schaefer, K. (2009). Oligosaccharides for Barrier Function and Hydration. Retrieved from http://www.cosmeticsandtoiletries.com/formulating/function/active/79303682.html

Sederma. Subliskin Retrieved from http://www.sederma.com/home.aspx?view=dtl&d=content&s=111 &r=178&p=1138&prodID=108

Shabbir, F., Hanif, M. A., Ayub, M. A., Jilani, M. I., & Rahman, S. (2020). Chapter 17 - Damask Rose. In M. A. Hanif, H. Nawaz, M. M. Khan, & H. J. Byrne (Eds.), *Medicinal Plants of South Asia* (pp. 217-230): Elsevier.

Sorbotten, A., Horn, S. J., Eijsink, V. G., & Varum, K. M. (2005). Degradation of chitosans with chitinase B from Serratia marcescens. Production of chito-oligosaccharides and insight into enzyme processivity. **FEBS J**, *272*(2), 538-549. doi:10.1111/j.1742-4658.2004.04495.x

Sumiyoshi, M., & Kimura, Y. (2006). Low molecular weight chitosan inhibits obesity induced by feeding a high-fat diet long-term in mice. **Journal of Pharmacy and Pharmacology**, *58*(2), 201-207. doi:10.1211/jpp.58.2.0007

Sun, T., Qin, Y., Xu, H., Xie, J., Hu, D., Xue, B., & Hua, X. (2017). Antibacterial activities and preservative effect of chitosan oligosaccharide Maillard reaction products on Penaeus vannamei. **Int J Biol Macromol**, *105*(Pt 1), 764-768. doi:10.1016/j.ijbiomac.2017.07.100

Suthongsa, S., Pichyangkura, R., Kalandakanond-Thongsong, S., & Thongsong, B. (2017). Effects of dietary levels of chito-oligosaccharide on ileal digestibility of nutrients, small intestinal morphology and crypt cell proliferation in weaned pigs. **Livestock Science**, *198*, 37-44. doi:10.1016/j.livsci.2017.02.004

Synowiecki, J., & Al-Khateeb, N. A. (2003). Production, Properties, and Some New Applications of Chitin and Its Derivatives. **Critical Reviews in Food Science and Nutrition**, *43*(2), 145-171. doi:10.1080/10408690390826473

Takeuchi, O., & Akira, S. (2010). Pattern recognition receptors and inflammation. **Cell**, *140*(6), 805-820. doi:10.1016/j.cell.2010.01.022

Tony Davila, Epstein, M. J., & Shelton, R. (2006). *Making Innovation Work How to Manage It, Measure It, and Profit from It*. Pearson Education, Inc, Upper Saddle River, New Jersey 07458: Tim Moore.

Trivedi, V. R., Satia, M. C., Deschamps, A., Maquet, V., Shah, R. B., Zinzuwadia, P. H., & Trivedi, J. V. (2016). Single-blind, placebo controlled randomised clinical study of chitosan for body weight reduction. **Nutr J**, *15*, 3. doi:10.1186/s12937-016-0122-8

V.D., D. V. a. V. (1998). Pharmaceutical applications of chitosan **Pharm. Sci. Tech. Today**, *1*, 246-253.

Vaseline. Vaseline Jelly Original Retrieved from http://www.vaseline.us/product/petroleum-jelly/vaseline-jelly.html

Walsh, A. M., Sweeney, T., Bahar, B., & O'Doherty, J. V. (2013). Multi-functional roles of chitosan as a potential protective agent against obesity. **PLoS One**, *8*(1), e53828. doi:10.1371/journal.pone.0053828

Wenwei, Z., Xiaoguang, Z., Li, Y., Yuefang, Z., & Jiazhen, S. (1993). Some chemical changes in chitosan induced by γ-ray irradiation. **Polymer Degradation and Stability**, *41*(1), 83-84. doi:https://doi.org/10.1016/0141-3910(93)90065-Q

Xu, Q., Wang, W., Qu, C., Gu, J., Yin, H., Jia, Z., . . . Du, Y. (2017). Chitosan oligosaccharides inhibit epithelial cell migration through blockade of N-acetylglucosaminyltransferase V and branched GlcNAc structure. **Carbohydr Polym**, *170*, 241-246. doi:10.1016/j.carbpol.2017.04.075

Xu, Q., Wang, W., Yang, W., Du, Y., & Song, L. (2017). Chitosan oligosaccharide inhibits EGF-induced cell growth possibly through blockade of epidermal growth factor receptor/mitogen-activated protein kinase pathway. **Int J Biol Macromol**, *98*, 502-505. doi:10.1016/j.ijbiomac.2017.02.021

Yoon, H. J., Moon, M. E., Park, H. S., Im, S. Y., & Kim, Y. H. (2007). Chitosan oligosaccharide (COS) inhibits LPS-induced inflammatory effects in RAW 264.7 macrophage cells. **Biochem Biophys Res Commun**, *358*(3), 954-959. doi:10.1016/j.bbrc.2007.05.042

Yousef, M., Pichyangkura, R., Soodvilai, S., Chatsudthipong, V., & Muanprasat, C. (2012). Chitosan oligosaccharide as potential therapy of inflammatory bowel disease: therapeutic efficacy and possible mechanisms of action. **Pharmacol Res**, *66*(1), 66-79. doi:10.1016/j.phrs.2012.03.013

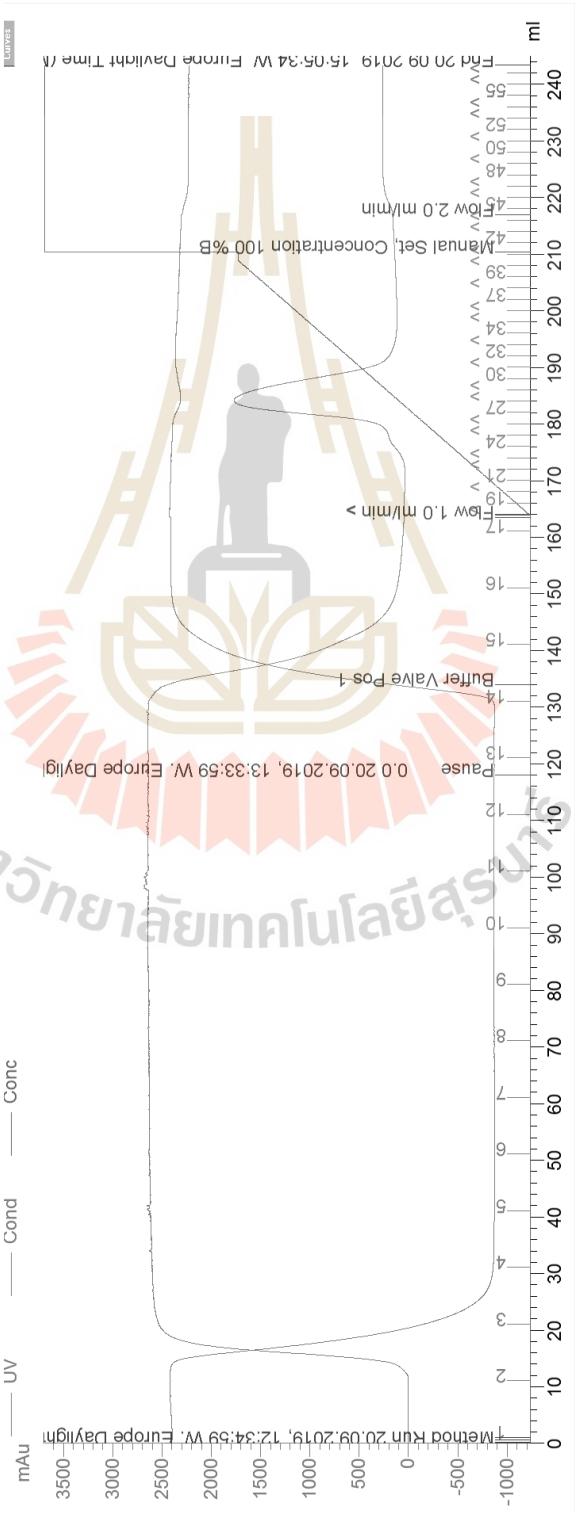
Zhang, X., Battiston, K. G., McBane, J. E., Matheson, L. A., Labow, R. S., & Santerre, J. P. (2016). 3 - Design of biodegradable polyurethanes and the interactions of the polymers and their degradation by-products within in vitro and in vivo environments. In S. L. Cooper & J. Guan (Eds.), *Advances in Polyurethane Biomaterials* (pp. 75-114): Woodhead Publishing.

Zhang, Y., Lee, E. T., Devereux, R. B., Yeh, J., Best, L. G., Fabsitz, R. R., & Howard, B. V. (2006). Prehypertension, diabetes, and cardiovascular disease risk in a population-based sample: the Strong Heart Study. **Hypertension**, *47*(3), 410-414. doi:10.1161/01.HYP.0000205119.19804.08

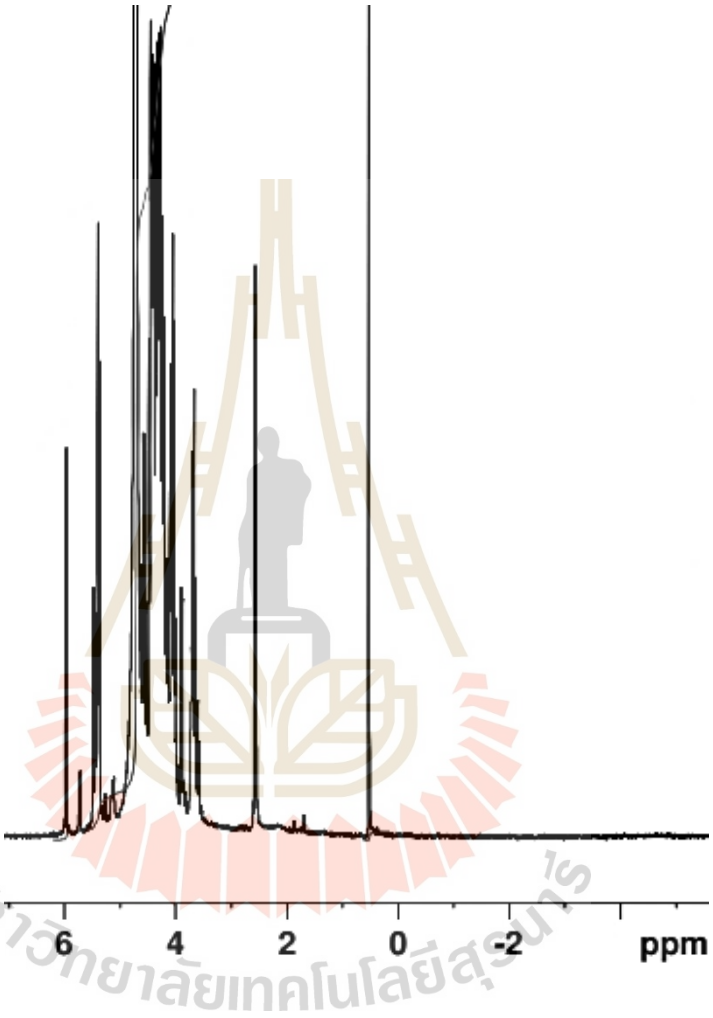
Zou, P., Yuan, S., Yang, X., Zhai, X., & Wang, J. (2017). Chitosan oligosaccharides with degree of polymerization 2-6 induces apoptosis in human colon carcinoma HCT116 cells. **Chem Biol Interact**, *279*, 129-135. doi:10.1016/j.cbi.2017.11.010

APPENDIX

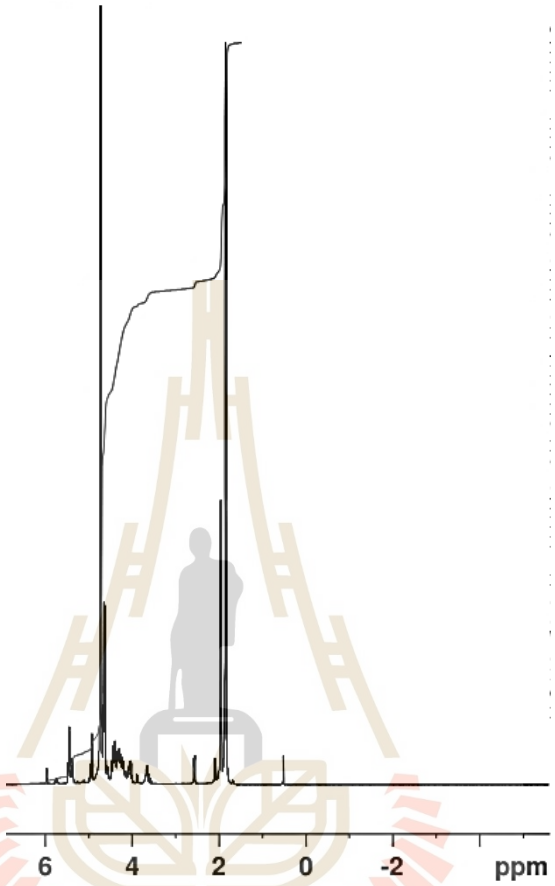
*Bs*Csn46A purification



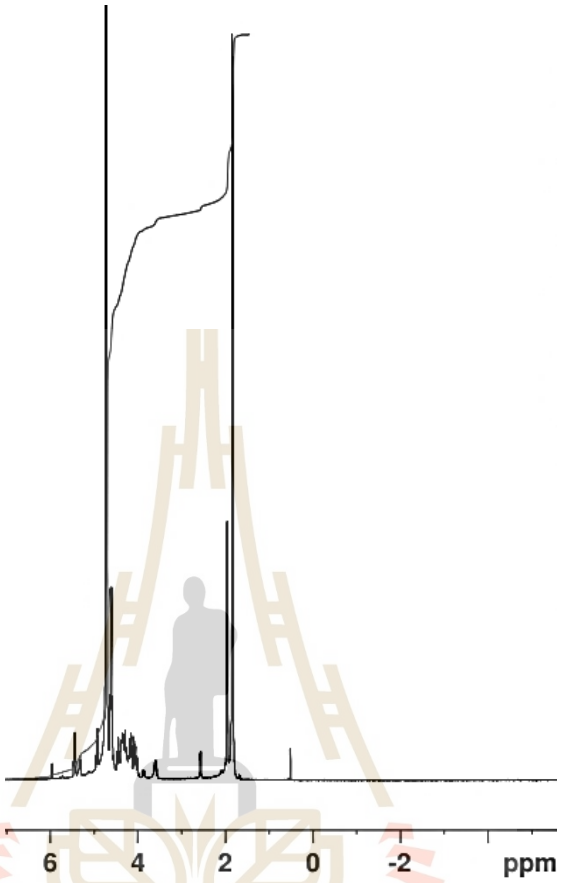
NMR Spectra C-1 : 1% HCL 1% Chitosan



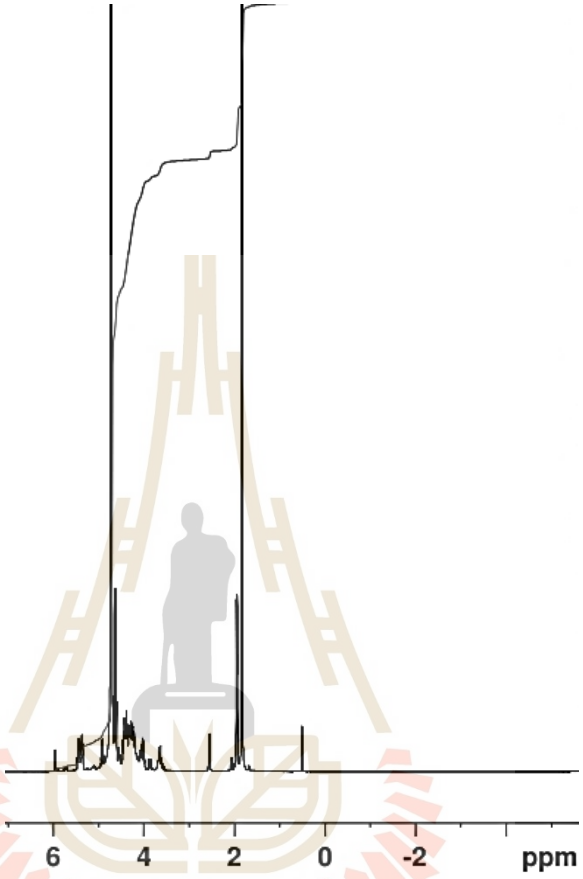
NMR Spectra C-2 : 2% LAC 1% Chitosan



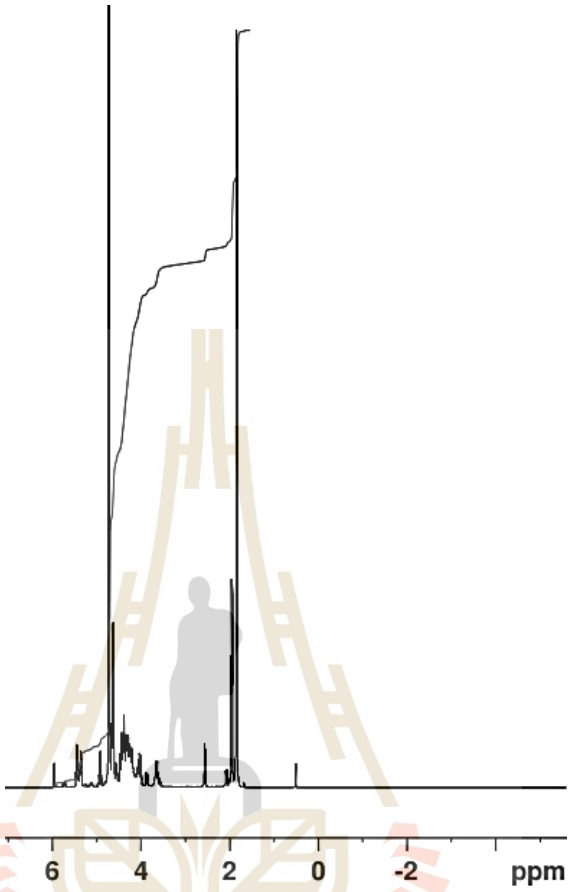
NMR Spectra C-3 : 2% LAC 1% Chitosan



NMR Spectra C-4: 2% LAC 2% Chitosan

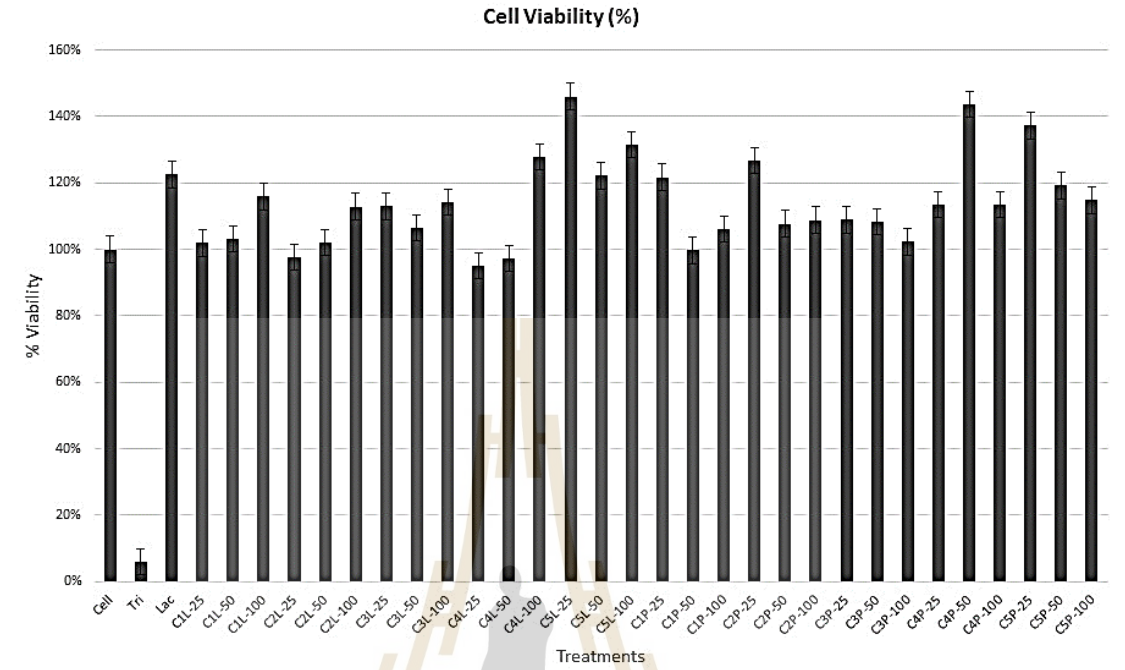


NMR Spectra C-5: 2% LAC 2% Chitosan



NMR Spectra commercial CHOS

Cell viability analysis by MTT assay



BIOGRAPHY

Mr. Chai Noy Soem was born on September 25th, 1993 in Battambang Province, Cambodia. He graduated with a Bachelor's Degree from Department of Food Processing, Faculty of Agriculture and Food Processing, University of Battambang in 2015. In 2016-2019, He had an opportunity to study Master degree in biotechnology in School of Biotechnology, Institute of Agricultural technology, Suranaree University of Technology. He received Erasmus+ KA107 scholarship from USAMV supporting on his study and research experience at Faculty of Chemistry, Biotechnology, Biotechnology and Food Science, Norwegian University of Life Science (NMBU), Romania for 3 months. His work and research interests are in bioconversion of chitosan into chitosan oligosaccharide (CHOSs) and biological activities of CHOS in medical application. He had presented research work in Merck Young Scientist Awards competition at Thailand Science Park Convention Center, Thailand (Poster presentation; Biological activity of Chitosan oligosaccharides toward Cosmeceutical innovation). The 8th School of Biotech International colloquium, August 2019, Suranaree University of Technology, Thailand (Poster presentation; Production of chitosan oligosaccharides using enzyme technology)