CONSTRUCTION OF A MALTOSE BINDING PROTEIN-CHITOSANASE FUSION PROTEIN AND ITS APPLICATION IN THE FORMATION OF ANTI-INFLAMMATORY CHITO-OLIGOSACCHARIDES

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CONSTRUCTION OF A MALTOSE BINDING PROTEIN-CHITOSANASE FUSION PROTEIN AND ITS APPLICATION IN THE FORMATION OF ANTI-INFLAMMATORY CHITO-OLIGOSACCHARIDES

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

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วาเฮนี ริซกี้ อาพริลเลีย : การสร้างโปรตีนที่เชื่อมต่อระหว่างโปรตีนที่จับกับมัลโตสกับเอนไซม์ไคโตซาเนส และการประยุกต์ใช้ในการผลิต ไคโตโอลิโกแซคคาไรด์ที่มีฤทธิ์ต้านการอักเสบ (CONSTRUCTION OF A MALTOSE BINDING PROTEIN-CHITOSANASE FUSION PROTEIN AND ITS APPLICATION IN THE FORMATION OF ANTI-INFLAMMATORY CHITO-OLIGOSACCHARIDES) อาจารย์ที่ปรึกษาวิทยานิพนธ์ : ศาสตราจารย์ ดร.มณฑารพ ยมาภัย, 46 หน้า.

คำสำคัญ: ไคโตซาน/ไคโตซาเนส/ไคโตซาน-โอลิโกแซ็กคาไรด์/โปรตีนจับมัลโตส

ไคโตซาเนสเป็นเอนไซม์ที่สลายพันธะ เบต้า-1,4 ไกลโคซิดิกในไคโตซาน เพื่อผลิตไคโตโอลิโกแซกคาไรด์ (คอซ) ที่มีมูลค่าทางอุตสาหกรรม การศึกษาก่อนหน้านี้ได้ทำการเปลี่ยนไคโตซานเป็นคอซ โดยใช้เอนไซม์ไคโตซาเนสที่ผลิตจากเชื้อ บาซิลัส ซับซิลิส ซึ่งเป็นเอนไซม์ที่มี กรดอะมิโน ฮิสติดีน 10 ตัว เชื่อมอยู่ที่ปลายคาร์บอกซิล (*Bs*CsnA-10xHis) ทำให้สามารถทำเอนไซม์นี้ให้บริสุทธิ์โดยวิธีโครมาโทกราฟีแบบ ไอแมค (IMAC) ส่วนในการศึกษานี้ เราใช้ไคโตซาเนสในรูปแบบ ที่เชื่อมต่อกับโปรตีนที่จับกับมัลโตส (MBP-*Bs*CsnA) สำหรับกระบวนการเปลี่ยนไคโตซานเป็น คอซ ในขนาดใหญ่ขึ้น ข้อดีของการใช้ MBP-*Bs*CsnA คือขั้นตอนการชะล้าง (Elution) นั้นจะใช้น้ำตาล มอลโตสแทนนิกเกิล จึงสามารถหลีกเลี่ยงการปนเปื้อนของนิกเกิลกับเอนไซม์ ในขั้นตอนการชะล้างได้ ผลการวิจัยพบว่าค่ากิจกรรมจำเพาะของเอนไซม์ ไคโตซาเนสที่ผลิตด้วย MBP-*Bs*CsnA และ *Bs*CsnA-10xHis นั้นคล้ายคลึงกัน อย่างไรก็ตามความทนทานต่ออุณหภูมิสูงของเอนไซม์ในรูปแบบ MBP-*Bs*CsnA นั้นดีกว่าแบบ *Bs*CsnA-10xHis ทั้งนี้สามารถผลิตเอนไซม์ออกมาน้ำเลี้ยงเชื้อ ได้เฉลี่ย 150,000 ยูนิต ต่อการเพาะเลี้ยงโดยการเขย่าในขวดรูปชมพู่ปริมาตร 1 ลิตร นอกจากนี้ ยังพบว่า คอซ ที่ผลิตทั้งจาก MBP-*Bs*CsnA และ *Bs*CsnA-10xHis มีฤทธิ์ต้านการอักเสบด้วย ดังนั้นผลการวิจัยนี้จึงแสดงให้เห็นว่า MBP-*Bs*CsnA นั้นมีความน่าสนใจในการนำไปใช้สำหรับการเพิ่มมูลค่าไคโตซานในอุตสาหกรรมต่อไป

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2567

ลายมือชื่อนักศึกษา .....
ลายมือชื่ออาจารย์ที่ปรึกษา .....

WAHENI RIZKI APRILIA : CONSTRUCTION OF A MALTOSE BINDING PROTEIN-CHITOSANASE FUSION PROTEIN AND ITS APPLICATION IN THE FORMATION OF ANTI-INFLAMMATORY CHITO-OLIGOSACCHARIDES. THESIS ADVISOR : PROF. MONTAROP YAMABHAI, Ph.D. 46 PP.

Keyword: CHITOSAN/CHITOSANASE/CHITOSAN-OLIGOSACCHARIDE/MALTOSE BINDING PROTEIN

Chitosanase catalyzes the hydrolysis of the β-1,4 glycosidic bonds of chitosan to produce value-added chito-oligosaccharides (CHOS). Previously, we used C-terminal-10xhistidine-tagged *Bacillus subtilis* chitosanase (*Bs*CsnA-10xHis), purified via Immobilized Metal-Ion Affinity Chromatography (IMAC), to bio convert chitosan to CHOS. In this study, we explored the possibility of using Maltose binding protein (MBP)-*Bs*CsnA fusion for large-scale bioconversion of chitosan. The advantage of using MBP fusion is the elution step, of which maltose is used instead of Nickel, thus metal ion leaching can be avoided. Analysis of the MBP-*Bs*CsnA enzyme fusion indicated that the specific activity of MBP-*Bs*CsnA and *Bs*CsnA-10xHis is comparable. However, the thermostability of MBP-*Bs*CsnA construct is superior to that of *Bs*CsnA-10xHis. Routinely, about 150000 U of crude enzyme can be obtained from a 1-L culture in a shake flask. The CHOS generated by both constructs possessed anti-inflammatory activity, suggesting that MBP-*Bs*CsnA is an attractive format for industrial valorization of chitosan.

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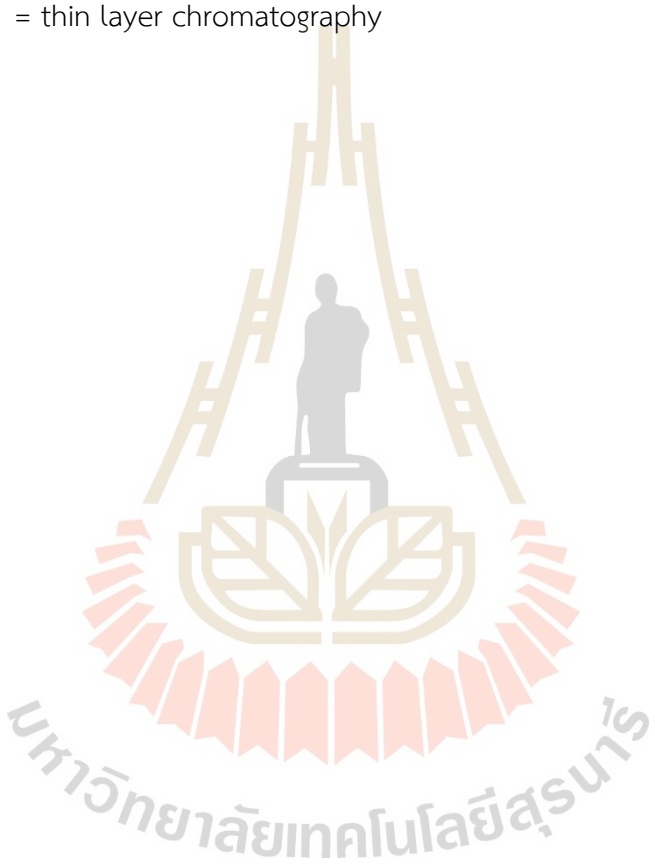
LIST OF ABBREVIATIONS

Amp	= ampicillin
BSA	= bovine serum albumin
*Bs*CsnA	= chitosanase from *Bacillus subtilis* 168
*Bs*CsnA-6xHis	= 6xhistidine-tagged *Bacillus subtilis* chitosanase
*Bs*CsnA-10xHis	= 10xhistidine-tagged *Bacillus subtilis* chitosanase
CsnA	= chitosanase
COS/CHOS	= chito-oligosaccharide
DDA	= degree of deacetylation
DNA	= deoxyribonucleic acid
DNS	= 3,5-dinitrosalicylic acid
DP	= degree of polymerization
EDTA	= ethylenediaminetetraacetic acid
GlcN	= D-glucosamine
IMAC	= Immobilized Metal-Ion Affinity Chromatography
IL-1β	= interleukin-1 beta
IPTG	= isopropyl-b-D-thiogalactopyranoside
MBP	= maltose-binding protein
OD	= optical density
PCR	= polymerase chain reaction

LIST OF ABBREVIATIONS (Continued)

pH	= log of the hydrogen in concentration
SDS-PAGE	= sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TB	= Terrific Broth
TLC	= thin layer chromatography



CHAPTER 1
INTRODUCTION

1.1 Significance of this study

Chitosanase (CsnA) or chitosan N-acetylglucosaminohydrolase (EC 3.2.1.132) is an enzyme which catalyzes the hydrolysis of β-1,4 glycosidic bond of chitosan, a recalcitrant waste from the seafood industry, to chito-oligosaccharides (COS/CHOS) (Aam et al., 2010; Dahiya, Tewari, & Hoondal, 2006; Khoushab & Yamabhai, 2010; Liu et al., 2009; Pechsrichuang, Yoohat, & Yamabhai, 2013; Thadathil & Velappan, 2014) which have a diverse range of biological activities such as inhibition of fungi and bacteria growth (Aam, 2010), anti-tumor and immunity-enhancing effects (Naveed et al., 2019), enhancement of phytoalexin production in higher plants (Zhou et al., 2015), and food additive properties (Fang, Cong, Zhou, Zhang, & Wang, 2024). Enzymatic bioconversion of chitosan to CHOS is superior to chemical or physical methods by its low cost, environmental compatibility, reproducibility, and production of well-defined CHOS (Jitprasertwong et al., 2021; Pechsrichuang et al., 2013; Sak-Ubol et al., 2016; Zhou et al., 2015) and so, chitosanase has a high demand across agricultural, food, medical, pharmaceutical, and cosmeceutical industries.

Recently, chitosanase production has been improved by recombinant DNA technology in which microbial chitosanase gene is cloned and expressed in a production host, leading to improvement in purity and yield of chitosanase (Sinha, Chand, & Tripathi, 2016). This approach leads to enhanced enzyme activity per unit as well as high specific activity (Sinha et al., 2016). We used recombinant 10xhistidine-tagged *Bacillus subtilis* chitosanase (*Bs*CsnA-10xHis) for both small scale and larger scale CHOS production and the anti-inflammatory activity of obtained CHOS mixtures were well-documented (Jitprasertwong et al., 2021; Yamabhai et al., 2024). Studies

have been done to improve the production of chitosanase in terms of expression, and purification (Table 1.1). For example, the chitosanase gene (*Bs*CsnA) from *B. subtilis* 168 has been expressed in *Escherichia coli* (Pechsrichuang et al., 2016; Pechsrichuang et al., 2013) as well as in *Lactobaciulus plantarum* (Sak-Ubol et al., 2016).

Table 1.1 Recombinant protein engineering for chitosanase production

Microorganism	Expression system	Secretion system by signal peptide	Selection marker	Epitope tag/ Fusion protein	Yield (mg/L)	Enzyme activity (U/mg)	Reference
Bacillus subtilis 168	*E. coli* TOP10	OmpA	amp^R	10x His tag	18.5	650	(Pechsrichuang et al., 2016)
Bacillus subtilis 168	*E. coli* TOP10	Native *Bacillus*	amp^R	10x His tag	0.4	650	(Pechsrichuang et al., 2016)
Bacillus subtilis 168	*E. coli* TOP10	OmpA	amp^R	10x His tag	14	904.7	(Pechsrichuang et al., 2013)
Bacillus subtilis 168	*Lactobacillus plantarum* WCFS1	Native *Bacillus*	amp^R	6x His tag	25	195	(Sak-Ubol et al., 2016)
Bacillus subtilis 168	*Lactobacillus plantarum* WCFS1	OmpA	amp^R	6x His tag	12	90	(Sak-Ubol et al., 2016)
Bacillus subtilis 168	*Lactobacillus plantarum* WCFS1	Native *Bacillus*	emr^R	6x His tag	79	800	(Sak-Ubol et al., 2016)
Bacillus subtilis 168	*Lactobacillus plantarum* TGL02	Native *Bacillus*	*alr*	6x His tag	39	800	(Sak-Ubol et al., 2016)

Commonly, recombinant chitosanase in expression medium is purified through IMAC (Immobilized Metal Affinity Chromatography) via 10xHis-tag (Pechsrichuang et al., 2016; Pechsrichuang et al., 2013; Sak-Ubol et al., 2016) which can be affected by the presence of strong reducing and chelating agents such as EDTA in the buffer system (Costa, Almeida, Castro, & Domingues, 2014). Alternatively, fusion of protein-of-interest to maltose-binding protein (MBP) followed by one-step purification using amylose beads is a safer method that may increase enzyme productivity (Lebendiker, 2011; Riggs, 2000). Maltose-binding protein (MBP) is a large (43 kDa) periplasmic protein of *E. coli* that can be used as a solubility enhancer tag (Fox, Kapust, & Waugh, 2001). In the present study, we engineered MBP-*Bs*CsnA, expressed it in *E. coli*, and used it to produce CHOS from chitosan.

1.2 Research objectives

1.2.1 Main objective

The main objective is to investigate the efficiency of recombinant *Bacillus subtilis* chitosanase-maltose binding protein fusion (MBP-*Bs*CsnA), which produce pure chitosanase as bioconversion of chitosan into chito-oligosaccharide (CHOS).

1.2.2 Specific objective

The main objective can be divided into 4 specific objectives as follows.

1.1. To investigate the recombinant MBP-*Bs*CsnA expression by *E. coli* TOP10

1.2. To obtain pure chitosanase by fusion of protein-of-interest to maltose-binding protein (MBP) followed by one-step purification using amylose beads

1.3. To characterize the property of recombinant MBP-*Bs*CsnA fusion which was expressed from *E. coli* TOP10

1.4. To investigate its potential application for the bioconversion of chitosan into CHOS

1.3 Scope of this study

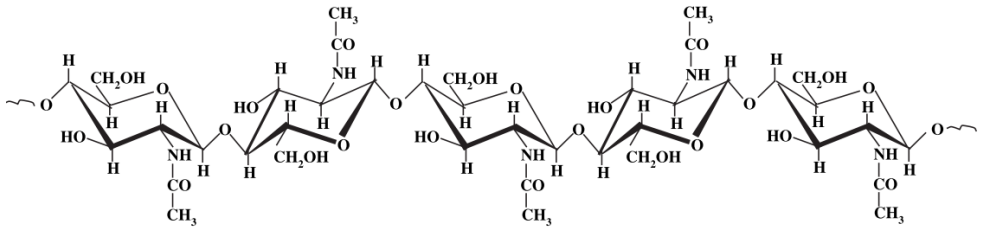
This study focuses on the development of an efficient recombinant *Bacillus subtilis* chitosanase-maltose binding protein fusion (MBP-*Bs*CsnA), which is used for the bioconversion of chitosan into chito-oligosaccharide (COS). The recombinant chitosanase was fused with Maltose Binding Protein (MBP) to avoid using 6xHis or 10xHis, followed by one-step purification using amylose beads. In addition, chitosan from 2 companies, Morena and Marine Bioresource, Thailand, were used as a substrate to produce CHOS. Finally, the biological assay of CHOS was done in vitro.

CHAPTER 2
LITERATURE REVIEWS

2.1 Chitin and Chitosan

Chitin is the second abundant biopolymer after cellulose, with a production of approximately 10^{10}–10^{12} tons annually (Zainol Abidin, Kormin, Zainol Abidin, Mohamed Anuar, & Abu Bakar, 2020). It is a glycan of β(1 ⟶ 4)-linked N-acetylglucosamine units, and it is widely distributed in crustaceans and insects as the protective exoskeleton and cell walls of most fungi (Ngo & Kim, 2014). Chitin is insoluble in water because of the highly extended hydrogen bonded semi-crystalline structure (Pillai, Paul, & Sharma, 2009). Chitin is arranged in three different microcrystalline structures; antiparallel (↑↓↑) sheets (α-chitin), parallele (↑↑↑) sheets (β-chitin) and a combination of both (γ-chitin), consist of two parallel strands which alternate with a single parallel strand (↑↑↓) (Rudall, 1963). The α-chitin is found in exoskeleton of arthropods, insects and fungal and yeast cell walls, while the β-form is mainly obtained from squid pen. The molecular arrangement of α-chitin is strongly packed with both inter- and intra-molecular hydrogen bonding, and it is the most stable form of the three crystalline variations, while β-chitin has weak intramolecular hydrogen bonding (Hackman & Goldberg, 1965). The degree of acetylation of chitin is >90%, the degree of polymerization is about 5,000–10,000, and its molecular weight can be 1,000–2,500 kDa (Kaczmarek, Struszczyk-Swita, Li, Szczęsna-Antczak, & Daroch, 2019).

a



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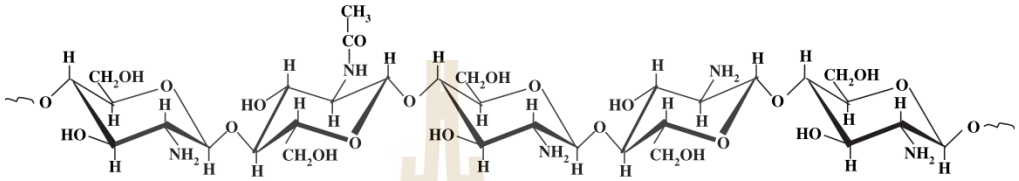

Figure 2.1 Primary structure of (a) chitin (b) chitosan (Harish Prashanth & Tharanathan, 2007).

Chitosan, a heteropolymer of D-glucosamine polymer (GlcN; D) and N-acetylD-glucosamine polymer, is a completely or partially deacetylated derivative of chitin (Harish Prashanth & Tharanathan, 2007). Chitosan can be classified according to degree of N-acetylation (DA) or fraction of N-acetylated residues (FA), the degree of polymerization (DP) or molecular weight (MW), the molecular weight distribution (PD) and the pattern of N-acetylation (PA) (Aam et al., 2010). The molecular weight of chitosan can be 10–1000 kDa (Moura, Moura, Soares, & Pinto, 2011). Chitosan is soluble in acid solutions such as acetic, formic, lactic, citric acids, and solvents such as dimethyl sulfoxide by structural modification; therefore, chitosan is a more suitable substrate for enzymatic bioconversion into chito-oligosaccharide (chitosan oligomers, COS or CHOS) than chitin, which must be dissolved in harsh acidic condition (Jagadish, Fabien, Stéphane, & Ada, 2017).

2.2 Chitosanase

Chitosanase (EC 3.2.1.132) is glycosyl hydrolases that catalyze the endohydrolytic of β-1,4-glycosidic bonds of partially acetylated chitosan to release chito-oligosaccharides (COS) (Thadathil & Velappan, 2014). Chitosan is a poly cationic natural polymer, an unbranched copolymer consisting of β-(1⟶4)-2-acetamido-D-glucose (N-acetyl-D-glucosamine, GlcNAc) and β-(1⟶4)-2-amino-D-glucose (D-glucosamine, GlcN), which can be found in nature as a structural component mostly in the cell wall of Zygomycetes fungi, Chlorophycean algae *Chlorella sp*., and in insect cuticle (Zitouni et al., 2013). Practical applications of chitosanase include the preparation of bioactive COS (Ming, Kuroiwa, Ichikawa, Sato, & Mukataka, 2006), preparation of fungal protoplasts mainly for Zygomycetes, a biocontrol agent to increase the resistance of plants against pathogenic fungi (Hsu, Chung, Chang, & Sung, 2012), chitosan mediated gene delivery and the bioconversion of marine crustacean chitinous bio waste (Wang, Tseng, & Liang, 2011). Enzymatic bioconversion of chitosan to COS is superior to chemical or physical methods by its low cost, environmental compatibility, reproducibility, and production of well-defined COS (Jitprasertwong et al., 2021; Pechsrichuang et al., 2013; Sak-Ubol et al., 2016), and so, chitosanase has a high demand across agricultural, food, medical, pharmaceutical, and cosmeceutical industries.

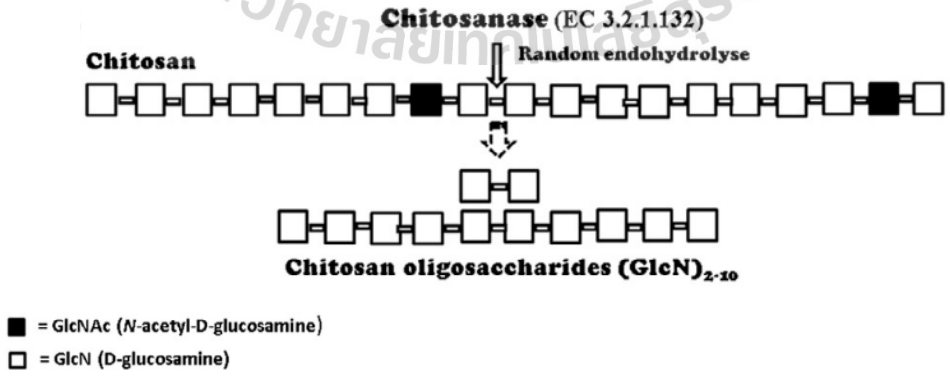

Figure 2.2 Chitosanase specificity (Thadathil & Velappan, 2014)

2.3 Chito-oligosaccharide

Chito-oligosaccharides (chitosan oligomers, COS or CHOS) are short chitosan polymers which has degree of polymerization varies from 2 to 20 units and the average molecular weight is <3.9 kDa (Liaqat & Eltem, 2018). In general, COS is fully soluble in water, partially soluble in methanol and dimethyl sulfoxide, and insoluble in acetone, butanol, ethanol, ethyl acetate, propanol, and pyridine (Phil, Naveed, Mohammad, Bo, & Bin, 2018). The properties of COS such as degree of deacetylation (DDA), degree of polymerization (PA), charge distribution, and nature of chemical modification are important factors influencing the biological activities of COS (Muzzarelli, 1996). COS have a diverse range of biological activities such as inhibition of fungi and bacteria growth (Aam et al., 2010), anti-tumor and immunity-enhancing effects (Naveed et al., 2019), enhancement of phytoalexin production in higher plants (Zhou et al., 2015), and food additive properties (Fang et al., 2024).

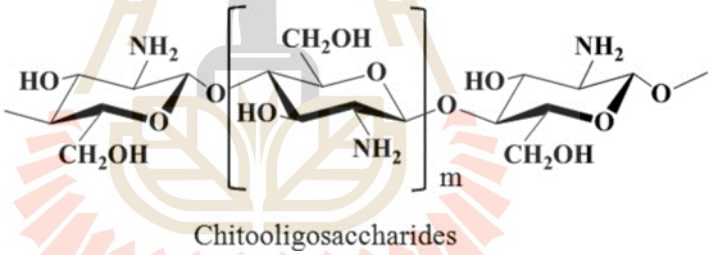

Figure 2.3 Chemical structure of chito-oligosaccharide (Vo, Ngo, Kang, Jung, & Kim, 2015)

2.4 Maltose Binding Protein

Maltose-binding protein (MBP) is a large (43 kDa) periplasmic and highly soluble protein of *E. coli* that acts as a solubility enhancer tag (Fox, Kapust, & Waugh, 2001; Kapust & Waugh, 2000). It has a native affinity property to handle target protein purification (Costa et al., 2014). MBP fusion vector, such as pMAL vectors by New England Biolabs, are available for cytoplasmic or periplasmic expression in all three reading frames, with factor Xa, enterokinase, or genenase I protease cleavage sequences (Kimple, Brill, & Pasker, 2013). In the study of MBP application, protein production can increase in comparison to commonly used tags e.g., the Fc, Glutathione

S-transferase (GST), SlyD, and serum albumin (ser alb) tag (Reuten et al., 2016). Moreover, the traditional antigen for immunological detection of deoxynivalenol in food and feed can be replaced by MBP fusion protein (Xu et al., 2018).

MBP allows one to use a simple capture affinity step on amylose-beads column, resulting in a protein that is often 70-90% pure (Lebendiker & Danieli, 2017). The MBP tag can enhance the solubility and expression of several difficult to express protein because of its large hydrophobic cleft that is able to alter its shape to accommodate different target proteins, promoting the latter's proper folding (Costa et al., 2014). The MBP is one of the most frequently used protein tags due to its capacity to stabilize, solubilize, and even crystallize recombinant proteins that are fused to it (Momin, Hameed, & Arold, 2019). Given that MBP is thought to be a highly stable monomeric proteins with known characteristics, fused passenger proteins are often studied without being cleaved from MBP (Momin et al., 2019).

2.5 Food Grade Expression System

Food grade expression systems are potential platforms for safety and efficient production of enzymes. There is some food grade expression system available based on FDA regulations, GRAS affirmation petitions, and GRAS notices (Z. Olempska-Beer, R. Merker, M. Ditto, & M. Dinovi, 2006). Those food grade expression systems are listed in Table 2.1. Nevertheless, these systems are still in a developmental process for the expression of enzyme from heterologous source in order to enable the reliable, efficient, and inexpensive production of high yields of enzymes (Wenzel, Müller, Siemann-Herzberg, & Altenbuchner, 2011).

Table 2.1. Different food-grade expression system available (based on FDA regulations, GRAS affirmation petitions, and GRAS notices) (Z. S. Olempska-Beer, R. I. Merker, M. D. Ditto, & M. J. DiNovi, 2006).

Source microorganism	Enzyme	Reference[a]
Aspergillus niger	Phytase	GRASP 2G0381
	Chymosin	21 CFR 184.1685
	Lipase	GRN 158
Aspergillus oryzae	Esterase–lipase	GRASP 7G0323
	Aspartic proteinase	GRN 34
	Glucose oxidase	GRN 106
	Laccase	GRN 122
	Lipase	GRN 43; GRN 75; GRN 103
	Pectin esterase	GRN 8
	Phospholipase A1	GRN 142
Bacillus licheniformis	α-amylase	GRASP 0G0363; GRN 22; GRN 24; GRN 79
	Pullulanase	GRN 72
Bacillus subtilis	α-acetolactate decarboxylase	21 CFR 173.115
	α-amylase	GRASP 4G0293; GRASP 7G0328
	Maltogenic amylase	GRASP 7G0326
	Pullulanase	GRN 20
Escherichia coli K-12	Chymosin	21 CFR 184.1685
Fusarium venenatum	Xylanase	GRN 54
Kluyveromyces marxianus var. *lactis*	Chymosin	21 CFR 184.1685
Pseudomonas fluorescens Biovar I	α-amylase	GRN 126
Trichoderma reesei	Pectin lyase	GRN 32

[a]GRASP, GRAS affirmation petition.

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Chitosan

The practical grade chitosan [product number 417963, 75% degree of deacetylation (DDA)] and low molecular weight chitosan [product number 448869 (75–85% DDA)] were purchased from Sigma-Aldrich. Chitosan solution was prepared by dissolving 1% chitosan (w/v) in distilled water and adding 1M acetic acid with stirring at 250-500 rpm. The chitosan solution was adjusted to pH 5.5 using 1M sodium acetate. The 500-700 kDa feed-grade chitosan (MORENA, Thailand) came as off-white flakes of 1-3 mm, having <1 % of insoluble content and ash. It was prepared from shrimp shells of the Pacific Ocean and the degree of deacetylation was higher than 90 %. It was water-insoluble but soluble in mild acidic solutions, such as 1-2 % hydrochloric acid, 1 % acetic acid, and 2 % lactic acid. The chitosan from Marine Bioresource (Thailand) was food-grade and presented as off-white yellow. It originated from the chitin of dried shrimp shells from the tropical ocean. The degree of deacetylation (DD) was over 90%. The size of the particles was less than 1.5 mm and contained less than 1% ash. The MW is about 2100 kDa. The purity exceeds food-grade standards, containing microbial, heavy metal, and other trace material levels lower than the regulatory limit.

3.2 Bacteria and cell line

E. coli TOP10 used as the cloning and expression host, was purchased from Invitrogen (Life Technologies, Darmstadt, Germany). The plasmid (pSIP409/*Bs*CsnA_nt) carrying chitosanase was used as the source of chitosanase gene for cloning. pMAL-p5x used as the vector, was purchased from New England Biolabs (USA). THP-1 (catalog no. 300356), a human monocyte cell line, was purchased from DSMZ Cell Line Services (Germany).

3.3 Cloning of chitosanase gene from *B. subtilis* strain 168 to pMAL-p5X

The gene encoding recombinant chitosanase from pSIP409/*Bs*CsnA_nt was cloned by a PCR-based method according to a previously published protocol (Pechsrichuang et al., 2013). The primers (Csn_Fw: 5' GCG GGA CTG AAT AAA GAT CAA AAG C 3' and Csn_Rv: 5' GCA CAG GGA TCC TCA TTT GAT TAC AAA ATT ACC GTA CTC GTT TGA AC 3') were designed based on the chitosanase gene from *B. subtilis* 168 (NCBI accession number: NC_000964 REGION: complement (2747984..2748817). By using restriction enzymes: *BamHI* and *XmnI*, the PCR product was ligated into pMAL-p5X. The recombinant construct was designated as pMAL-p5X-*Bs*CsnA and its integrity was confirmed by automated DNA sequencing (Macrogen, Korea).

3.4 Modelling the 3-dimensional structure of MBP-*Bs*CsnA

The 3-D structure of MBP-*Bs*CsnA was constructed by Phyre2 web portal of Imperial College, London (Kelley, Mezulis, Yates, Wass, & Sternberg, 2015) using 7 templates, namely, PDB ID: 7c6c, 8ax7, 6vls, 6x91, 3a3c, 8dei, and 4xai. About 96% of MBP-*Bs*CsnA residues could be modelled at >90% confidence and the obtained pdb format was visualized using the PyMOL Molecular Graphics System, Version 1.3 (Schrödinger Inc., U.S.A).

3.5 Analysis of MBP-*Bs*CsnA structure

The protein secondary structure (α-helix or β-sheet or coil) and relative solvent accessibility (exposed or buried) of MBP-*Bs*CsnA sequence was analysed using NetSurfP3 server of Department of Health Technology, Denmark (Hoie et al., 2022). The MBP-*Bs*CsnA recombinant protein is made up of 654 residues (72.5 kDa) in which maltose binding protein domain and chitosanase domain are connected with a spacer sequence enclosing a Factor Xa site.

3.6 Expression of purification of MBP-*Bs*CsnA

The expression of recombinant *B. subtilis* chitosanase was done according to a previously published protocol (Pechsrichuang et al., 2013) with modification and optimization. Briefly, a single colony of freshly transformed *E. coli* TOP10 harboring appropriate constructs was grown overnight in Terrific Broth (TB) medium containing 100 µg/ml ampicillin (TB-Amp) at 37 °C. Then 1% of the overnight culture was added into 0.1–0.2 L of TB-Amp broth and grown at 37 °C, 250 rpm until the OD_{600} reached 0.5. Subsequently, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and the incubation was continued at 27 °C with vigorous shaking (250 rpm) for 20 h. The cells were harvested by centrifugation at 4000 g for 30 min at 4 °C. The recombinant MBP-tagged chitosanase was purified by gravity column, using amylose resin (New England Biolabs, USA). Briefly, the crude enzyme in culture supernatant was incubated at 4 °C with rotation for 45 min with amylose resin, that had been pre-equilibrated with 5 column volume of Column Buffer (20 mM Tris–HCl, 200 mM NaCl and 1 mM EDTA). The mixture was then loaded into a gravity column followed by washing with 12 column volumes of Column Buffer. Next, MBP-tagged chitosanase was eluted with Column Buffer containing 10 mM maltose. The purified enzyme was stored at 4 °C.

3.7 SDS-PAGE

Denatured sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli (Laemmli, 1970). A protein ladder (10–250 kDa) was used as a protein standard and purchased from Precision Plus Protein™ Standards, All Blue, Catalog #161-0373, Bio-Rad. The samples were heated at 100 °C for 10 min in the loading buffer (Laemmli buffer) containing reducing agent (2-mercaptoethanol) and electrophoresed in a 12% SDS–PAGE gel. The gel was stained

with Coomassie Brilliant Blue R-250, followed by de-staining with methanol:glacial acetic acid:distilled water (3:1:6)(v/v).

3.8 Determination of MBP-*Bs*CsnA concentration

The concentration of MBP-tagged chitosanase was determined by Pierce™ BCA protein assay kit (Thermo Fisher Scientific Inc., USA), using bovine serum albumin (BSA) as the standard from Thermo Fisher Scientific Inc. (USA). The standard calibration curve was constructed from 20 to 2000 µg/ml of BSA.

3.9 MBP-*Bs*CsnA enzyme activity assay

Chitosanase activity was determined by the 3,5-dinitrosalicylic acid (DNS) method, as described previously (Pechsrichuang et al., 2013). The reaction mixer consisted of 40 µl of diluted enzyme (0.4 µg) and 160 µl of 0.5% chitosan (in 200 mM sodium acetate buffer, pH 5.5), which was 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 12000g for 5 min to remove the remaining chitosan. The color in the supernatant was developed by heating it 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 D-(+)-Glucosamine hydrochloride (G4875-100G, Sigma-Aldrich Co., Switzerland) as standards. The reactions were done in triplicate and their mean and standard deviation values were reported. One unit of chitosanase was defined as the amount of enzyme that released 1 µmol of D-glucosamine per min under standard assay conditions.

3.10 Effect of pH on MBP-*Bs*CsnA

To determine the pH stability of MBP-*Bs*CsnA, 280 µg of purified enzyme in 1 mL was incubated in various buffers without substrate at 30 °C, for 24 h. The pH stability of recombinant *Bs*CsnA-10xHis fusion was used as a control. The buffers used

were 100 mM glycine-HCl (pH 2-3), 100 mM sodium acetate (pH 4-7) and 100 mM Tris-HCl (pH 7-9). The reactions were 10-times diluted in a total volume of 200 µl. Then, 40 µl of the diluted samples were used to determine the remaining activity under standard assay conditions (Pechsrichuang et al., 2013).

The optimal pH of recombinant MBP-*Bs*CsnA fusion was determined under standard assay conditions (Pechsrichuang et al., 2013), using two buffer systems: 250 mM glycine–HCl (pH 2.4–4.4) and 250 mM sodium acetate buffer (pH 4–7.0). The reaction mixture consisted of 1.12 µg of purified enzyme and 0.5% chitosan (low molecular weight), (1:4) (v:v). The optimal pH of recombinant *Bs*CsnA-10xHis fusion was used as a control. It was not possible to determine the enzyme activity at pH > 7.0 because the chitosan substrates were insoluble at this condition.

3.11 Effect of temperature on MBP-*Bs*CsnA

Thermal stability of the enzyme without substrate was determined by incubating 15 µg of the purified enzyme in 50 mM sodium acetate buffer, pH 6.0, at various temperatures ranging from 4 to 80 °C for 30 min. The remaining enzyme activity was measured under standard assay conditions (Pechsrichuang et al., 2013). The thermal stability of recombinant *Bs*CsnA-10xHis fusion was used as a control.

The optimal temperature of MBP-*Bs*CsnA was measured by incubating 0.4 µg of the purified enzyme with 0.5% chitosan (practical grade) at temperatures ranging from 0 to 80 °C, for 5 min at pH 5.5. Meanwhile, to measure the thermal stability of MBP-*Bs*CsnA in the presence of substrates, 18 µg of the purified enzyme in a total volume of 50 µl was pre-incubated with 0.5% chitosan (low molecular weight) in 50 mM sodium acetate buffer pH 5.5, at 50 °C for 30 min. After incubation, the reactions were diluted 30 times in a total volume of 300 and 40 µl of the diluted samples were taken to determine the remaining activity under standard assay conditions. In addition, the thermal inactivation kinetics at 50 °C in the presence of chitosan were measured

by incubating 18 µg of the purified enzyme with 0.5% chitosan (low molecular weight) in 50 mM sodium acetate buffer pH 5.5, at 50 °C. After incubation at various time points (0, 0.5, 1, 6, 12 and 24 h), the reactions were diluted 10 times in a total volume of 300 and 40 µl of the diluted samples was taken to determine the remaining activity under standard assay conditions.

3.12 COS production

Chitosan powder (MORENA and Marine Bioresource, Thailand) was dissolved in 1 % HCl solution at 1 % (w/v) concentration by magnetic stirring, 250-500 rpm, at room temperature. The reaction pH was adjusted to 5.5 using NaOH (1 N). The substrate solution was pre-incubated for 30 min at 37 °C before 35 U/mL of MBP-*Bs*CsnA was added at 0, 6, and 24 h. The reaction mixture was incubated at 37 °C, with stirring at 200 rpm, for 48 h. After that, the reaction mixture was heated at 100°C for 20 min to inactivate the enzyme, cooled down, and centrifuged at 3000 rpm for 5 min. Some of the hydrolysis reaction mixture was stored as CHOS liquid –20 °C and the remaining COS was frozen at -80 °C overnight before lyophilization for 72 h using a SCANVAC COOLSAFE freeze-dryer (LaboGene™, Denmark). The obtained CHOS powder was stored at -20 °C until further analysis.

3.13 Analysis of hydrolytic products by thin layer chromatography (TLC)

Analysis of the hydrolytic products were done according to a previously published protocol with modification (Pechsrichuang et al., 2013). To be analyzed by TLC, CHOS samples were spotted and dried twice (1 µl each) on a Silica gel 60 F254 aluminum sheet (6.0 x 10.0 cm) purchased from Merck (Damsladt, Germany) and chromatographed for 1.5 hours in a mobile phase containing 30% ammonium solution:water:isopropanol (2:4:14)(v:v:v). The products were detected by dipping the TLC plate into Thymol-sulphuric acid in ethanol, followed by baking at 120 °C for 30

min. A mixture of 10 mM DP1-DP2 and 5 mM DP3-DP6 (Seikagaku Biobusiness Co., Japan) was used as a standard.

3.14 Anti-inflammatory assay

The ability of CHOS produced by MBP-*Bs*CsnA to inhibit the production of pro-inflammatory cytokine IL-1β in human macrophages was investigated. The human macrophage cell line, THP-1, was cultured as described previously (Jitprasertwong et al., 2021). The differentiation of THP-1 cells was carried out in a 24 wells plate for 48 h using Vitamin D3 (Merck, Germany) as an inducer of differentiation at a concentration of 0.2 μM. Cells were then pretreated for 24 h with CHOS samples, or with 0.2 μg/mL dexamethasone (Sigma-Aldrich, USA), a standard anti-inflammatory steroid drug, as a positive control. After pretreatment, cells were exposed to bacterial LPS 0.1 μg/mL (Invivogen, USA) for 6-7 h. The culture supernatant collected by centrifugation was used for analysis of IL-1β by an ELISA-based method (Human IL-1 beta/IL-1F2 DuoSet ELISA, Cat No. DY-201, R&D Systems, Inc., Minneapolis, USA). The ELISA procedure was carried out according to the manufacturer's instructions. Optical density measurements were done using a microplate reader (Tecan, Austria) at a measurement wavelength of 450 nm and a reference wavelength of 540 nm. Data was analyzed using Microsoft Excel 2016 and GraphPad Prism 8 software (GraphPad Software Inc., USA).

3.15 Statistical analysis

Statistical analysis was performed using One-way ANOVA, Dunnett's multiple comparisons test in GraphPad Prism 8 software (GraphPad Software Inc., USA).

3.16 Cream production

CHOS product was used as an active ingredient of cream. There were 3 parts ingredients to make cream. The cream ingredients were separately weighed for oil part, water part, and active ingredients. The oil part contains Butylhidroxy-toluene, Cetostearyl alcohol, cocoa butter, lipomulse luxe, and PEG-40 was incubated at 80°C

until completely melt, then Caprylic was added. The water part was dissolved using cycle hole stator of laboratory mixer (Silverson, USA) with various speed. Distilled water was added to a beaker and mixed at 3000rpm. Carbopol was continuously sprinkled into the beaker and mixed for 5-10 minutes. After the speed of mixer was increased to 5000rpm, Disodium EDTA was added and mixed for 2 minutes. Glycerin and PCA were added and mixed for 3 minutes. The speed of mixer was increased to 7000-8000rpm. TEA 10% was added and mixed for 3 minutes. The speed was gently reduced to 0rpm and switched the stator to square hole stator. The speed was increased to 8000rpm. The melted oil part was gently added and mixed for 3-5 minutes until completely homogenous, and the cream became smooth. After mixing water part and oil part, 100ug/mL of MB-CHOS was added to the cream with the additional ingredients such as Vit E, vanilla fragrance and curcumin.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Cloning of MBP-*Bs*CsnA

The entire *B. subtilis* CsnA gene was cloned into the pMAL-p5X vector, which had been previously digested with *BamHI* and *XmnI*. The PCR products (~834 bp) were digested with *BamHI-HF* and cloned into corresponding restriction sites on the pMAL-p5X plasmid (Fig. 4.1.a). In addition, the 3-dimensional structure of MBP-*Bs*CsnA was shown in Fig. 4.1.b. This result showed that in the fusion of Maltose Binding Protein (MBP) with the mature enzyme.

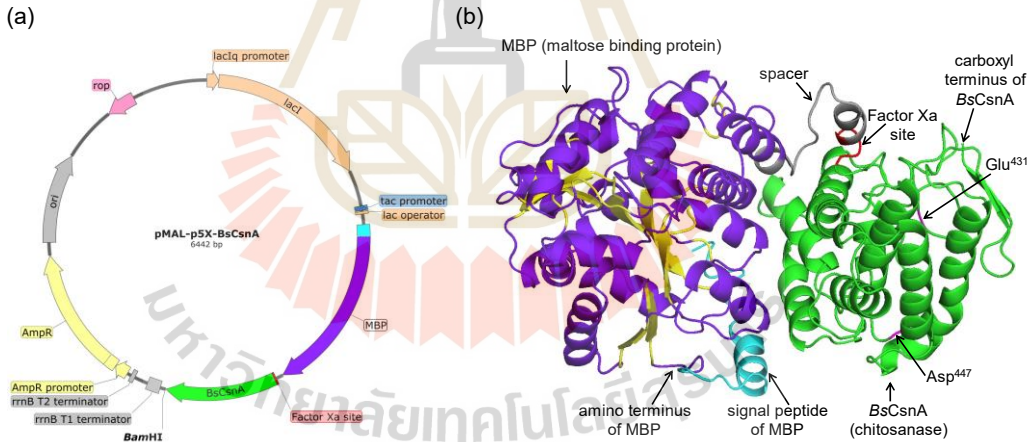

Figure 4.1 Maltose binding protein (MBP)-chitosanase (*Bs*CsnA) construct. (a) MBP-*Bs*CsnA construct in pMAL-p5X vector. (b) 3-dimensional structure of MBP-*Bs*CsnA. The signal peptide of MBP (cyan blue), MBP (violet), β-sheets of MBP (yellow), Factor Xa site (red) and *Bs*CsnA (green) were shown. The two catalytic residues of *Bs*CsnA (Glu^{431} and Asp^{447}) were magenta-coloured.

4.2 Expression of MBP-*Bs*CsnA in *E. coli* system

The recombinant MBP-*Bs*CsnA was expressed in the *E. coli* TOP10. The enzyme was collected at different times after induction and extracted from different compartments. Two hundred fifty milliliters of recombinant MBP-*Bs*CsnA were grown in Rich-Glucose-Amp medium until OD_{600} reached ~0.5 before IPTG was added to a final concentration of 0.3 mM, and incubation continued at room temperature (~ 27°C). Fifty ml of samples were taken at 0, 2, 4, and 20 h after induction with IPTG and extracted the enzyme from culture medium (broth), periplasm, and cell lysate.

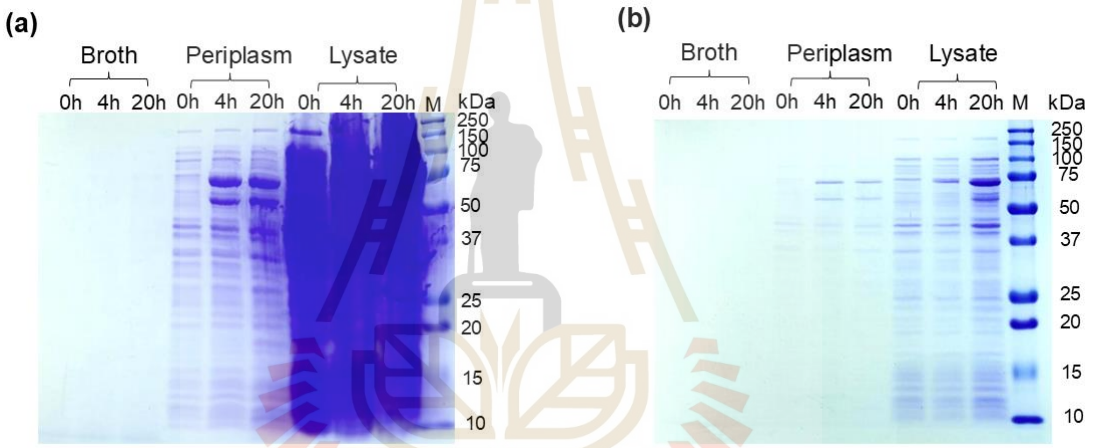

Figure 4.2 SDS-PAGE analysis of recombinant MBP-BsCsnA from different compartments of *E. coli* TOP10. The broth, periplasm, and lysate were loaded approximately 10 µl and 3 ug as indicated in panel (a) and (b), respectively. The gel was stained with Coomassie brilliant blue and All blue Prestained Protein Standards (BioRad #1610373, U.S.A.) was used as a marker.

The SDS-PAGE of the recombinant *Bs*CsnA in all three compartments was shown in Figure 4.2. The SDS-PAGE analysis of secreted enzyme into broth after inducing for different time was shown in Figure 4.2.1. The enzyme from MBP-*Bs*CsnA was secreted into the culture supernatant after inducing for 2h and getting increase for

20h. The molecular weight is approximately 73 kDa from SDS-PAGE analysis. The result demonstrated that the enzyme can be secreted into crude supernatant (broth).

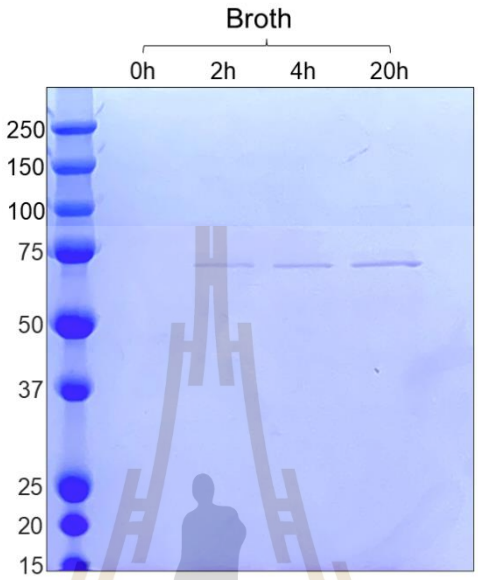

Figure 4.2.1 SDS-PAGE analysis of recombinant MBP-*Bs*CsnA from broth at different time after induction with 0.3 mM IPTG. Approximately 10 µl of broth were loaded into each lane. The gel was stained with Coomassie brilliant blue and All blue Prestained Protein Standards (BioRad #1610373, U.S.A.) was used as a marker.

4.3 Comparison of the bacteria cell growth in Terrific Broth (TB), Lurria Broth (LB), and Rich media

The condition of optimization was optimized by growing the recombinant MBP-*Bs*CsnA in different growth media. The single colony was picked and incubated on Terrific Broth (TB), Luria Broth (LB), and Rich media, respectively. After the incubation of bacteria cells on TB, LB, and Rich media at 37°C for 15 hours with 250rpm shaking, the appearance on TB media was turbid whereas on LB and Rich media was clear. The results showed that bacteria cells can grow on TB media and cannot grow neither LB

nor Rich media. TB media contains many chemical ingredients compared to LB and Rich media. KH_2PO_4 and K_2HPO_4 are additional chemicals on TB media.

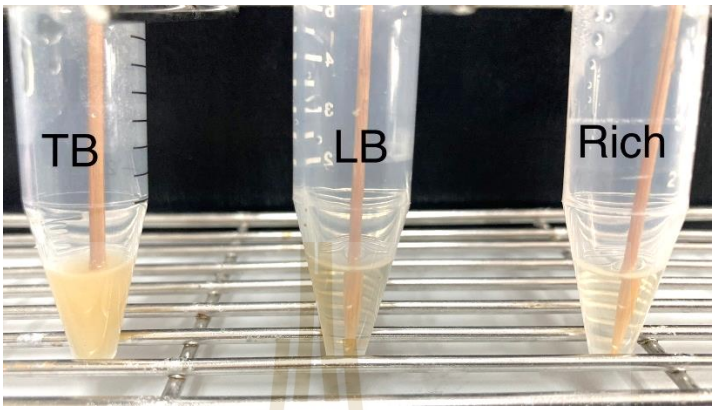

Figure 4.3 Comparison of the recombinant MBP-*Bs*CsnA in different bacteria growth media. The analysis is based on the turbidity of growth media.

Table 4.3 Comparison of chemical ingredients and the percentage of bacteria growth media

Components	TB (%)	LB (%)	Rich (%)
Tryptone	1.2	10	10
Yeast	2.4	0.5	0.5
NaCl	-	0.5	0.5
Glucose	-	-	0.2
Glycerol	0.5	-	-
KH_2PO_4	0.23	-	-
K_2HPO_4	1.25	-	-

4.4 Optimization, expression and purification of MBP-*Bs*CsnA

The recombinant MBP-*Bs*CsnA was successfully expressed in *E. coli* TOP10 and purified by one step purification using amylose beads. We optimized the conditions for enzyme expression and found that TB-Amp media is the optimal media to inoculate the recombinant MBP-*Bs*CsnA and the enzyme induction with 0.3mM IPTG at 27°C for 20hr can produce the maximum level of active enzyme. Cells were cultured in 100ml shaken flask and the recombinant enzyme was purified from culture supernatant. The enzyme was purified by amylose beads. The result indicated that the enzyme could be expressed in *E. coli* TOP10 as demonstrated by SDS-PAGE and zymogram analysis, respectively (Figure 4.4 and Figure 4.4.1).

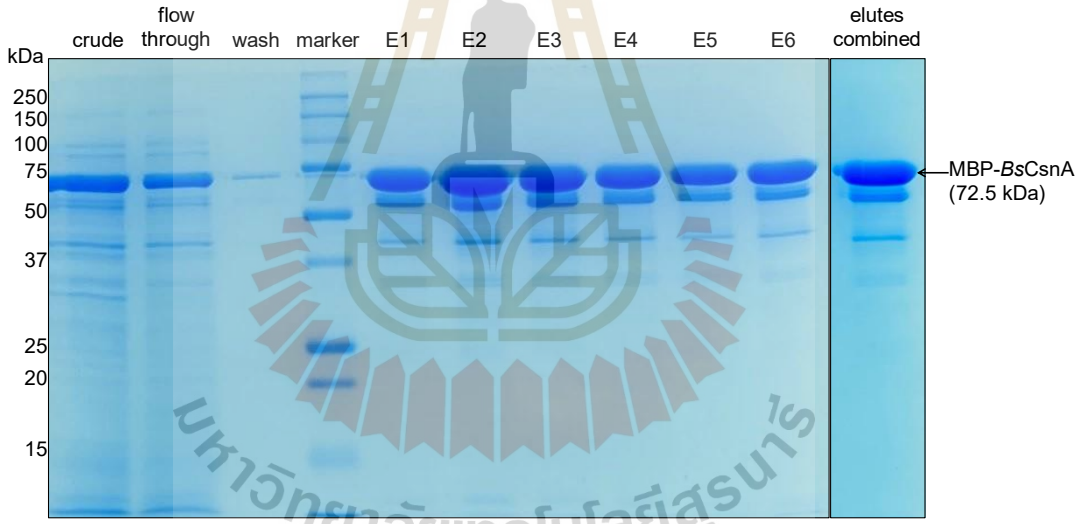

Figure 4.4 Expression and purification of recombinant MBP-*Bs*CsnA. SDS-PAGE analysis of MBP-*Bs*CsnA purified from crude supernatant. About 20 μL of crude, flow through, wash, elutes (E1-E6) and elutes-combined were loaded as indicated. MBP-*Bs*CsnA was seen at the expected size (72.5 kDa). The gel was stained with Coomassie brilliant blue and All blue Prestained Protein Standards (BioRad #1610373, U.S.A.) was used as a marker.

The SDS-PAGE analysis confirmed the extracellular expression of the enzymes MBP-*Bs*CsnA (Fig. 4.4). The molecular weight of MBP-*Bs*CsnA was predicted to be 72.5 kDa, which consists of 42.5 kDa of MBP and 30 kDa of *Bs*CsnA. Chitosanase activity of the purified recombinant enzyme was shown by in-gel activity staining (Figure 4.4.1) as well as by standard chitosanase assay. The specific activity of purified enzyme was 400 U/mg. The purification of MBP-*Bs*CsnA and His-*Bs*CsnA was summarized in Table 4.4.

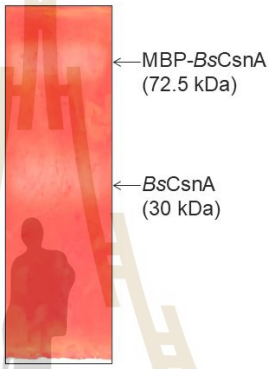

Figure 4.4.1 Zymogram analysis of purified recombinant MBP-*Bs*CsnA by transfer gel-zymography. Mixed protein MBP-*Bs*CsnA with loading buffer (Laemmli buffer) without reducing agent and heating. The protein gel was laid on top of a polyacrylamide gel, containing 0.1% chitosan (low molecular weight). The gel was stained with 2% Congo Red. White band indicated chitosanase activity.

This study presents an interesting approach to express and characterize a fusion protein, MBP-*Bs*CsnA, in *E. coli* TOP10. The gene encoding the *Bacillus* chitosanase can be fused successfully with Maltose Binding Protein using pMAL vector. Recombinant chitosanase with maltose binding protein fusion can be expressed and purified successfully using pMAL protein fusion and purification system. It means that pMAL vector, protein fusion, and purification system have ability to clone, express, and purify *Bs*CsnA. Therefore, *Bs*CsnA can be secreted efficiently by *E. coli* expression system.

Table 4.4. Comparison of purified MBP-*Bs*CsnA and His-*Bs*CsnA

Purified enzyme	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Moles of protein (nmol)	Specific activity (U/nmol)
MBP-*Bs*CsnA	3.15	1236	400	13.7	28.64
His-*Bs*CsnA	5.6	5063.2	900	33.33	27.13

When estimated from the specific activity of the purified enzyme (400U/mg), about 150000 U of crude enzyme can be obtained from a 1-L culture in a shake flask, indicating the enzymatic efficiency of the recombinant chitosanase fusion protein. However, it is noted that this specific activity of the purified enzyme is lower than previously reported His-tagged fusion with *Bs*CsnA, which had a specific activity of 900U/mg (Pechsrichuang et al., 2013). This discrepancy could arise from differences in fusion tags and purification methods.

4.5 Effect of temperature and pH on MBP-*Bs*CsnA

The optimal temperature for recombinant enzyme activity was 55°C under standard assay conditions (Fig. 3a, purple solid line). The enzyme was stable up to 50°C after incubation for 30 min at pH 6.0, without substrate. Less than 10% of residual activity could be detected after incubation at 60°C under these conditions (Fig. 3a, dashed line). The optimal pH of recombinant enzyme was 6.0 (Fig. 3b, solid line). Notably, the chitosanase activity was stable within pH 2-9 after incubation at 30 °C for 24 h (Fig. 3b, dashed line).

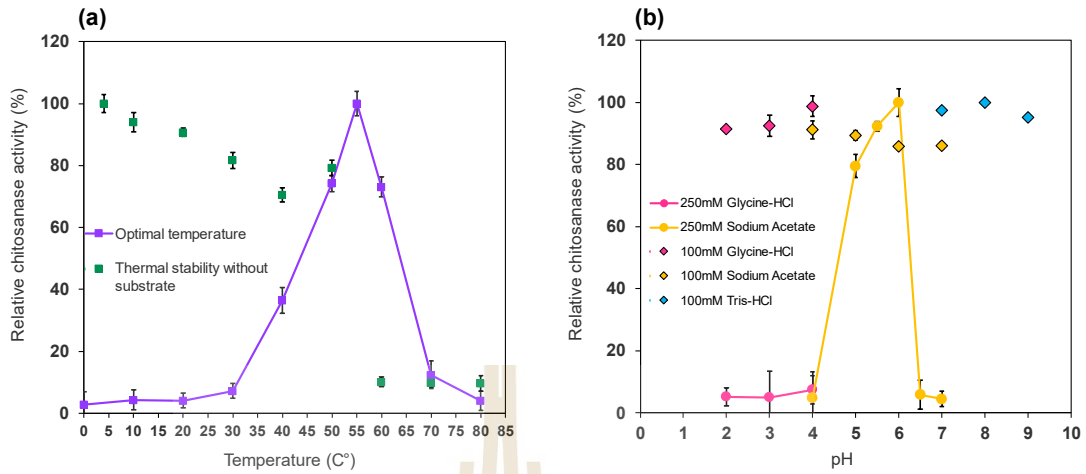

Figure 4.5 Effect of temperature and pH on MBP-chitosanase activity. (a) The optimal temperature for MBP-*Bs*CsnA (purple solid line) was determined using 0.5% chitosan (practical grade) in 200 mM sodium acetate buffer (pH 5.5) at indicated temperatures for 5 min incubation. The chitosanase activity (%) compared to the highest activity within the temperature range was plotted on the x-axis and the temperature values on the y-axis. The effect of temperature on stability of MBP-*Bs*CsnA in the absence of chitosan substrate was tested using 50 mM sodium acetate buffer (pH 6.0) for 30 min, using the standard assay. The chitosanase activity (%) compared to the activity at storage temperature (4°C) was plotted on the x-axis and the temperature values on the y-axis. Each dot represented the average of triplicates from two independent experiments and the error bars represented the ratio standard deviation of the mean. (b) The optimal pH for MBP-*Bs*CsnA was determined using 250mM glycine-HCl buffer (pink solid line) and 250mM sodium-acetate buffer (yellow solid line) at 50°C incubation. The effect of pH on stability of MBP-*Bs*CsnA was tested at pH 2-4 using 100mM glycine-HCl buffer (pink dotted line), pH 4-7 using 100mM sodium-acetate buffer (yellow dotted line) and pH 7-9 using 100mM Tris-HCl buffer (blue dotted line) at 50°C incubation. The relative chitosanase

activity (%) was plotted on the x-axis and the pH values on the y-axis. Each dot represented the average of triplicates from two independent experiments and the error bars represented the ratio standard deviation of the mean.

When the enzyme was incubated for 30 min at 50°C and pH 5.5, in the presence of chitosan, the residual activities of the enzymes were less than 100%. These results indicated that substrates could prevent thermal inactivation of the chitosanase activity. In addition, the thermal inactivation kinetics at 50°C showed that the enzyme was more stable in the presence and absence of 0.5% chitosan (low molecular weight) than *Bs*CsnA-10xHistidine tag, as shown in Fig. 4.5.1.

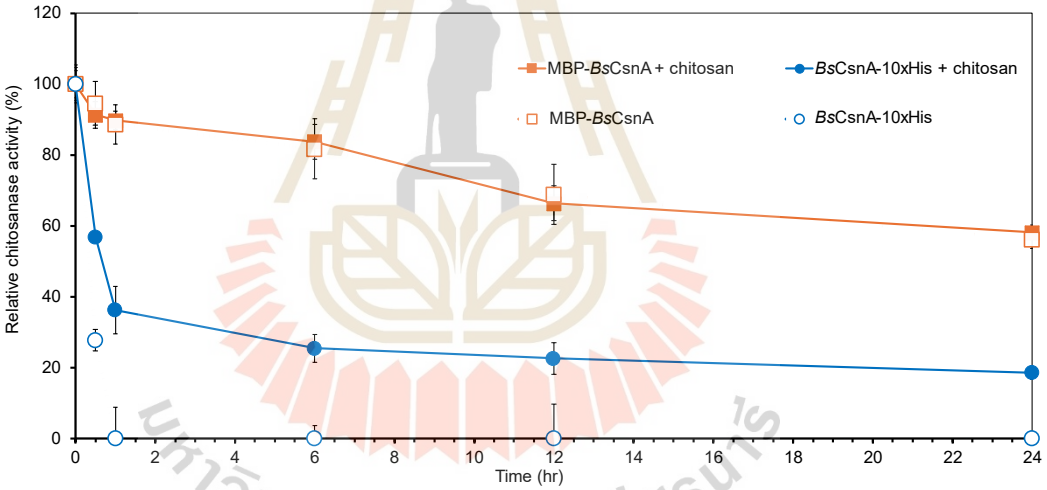

Figure 4.5.1 Thermostability of MBP-*Bs*CsnA in the presence and absence of chitosan substrate. Thermostability of MBP-*Bs*CsnA at 50°C in the presence (orange square, solid line) or the absence (orange square, dashed line) of 0.5% low molecular weight chitosan was measured at the indicated temperatures. Thermostability of *Bs*CsnA-10xHistidine tag in the presence (blue circle, solid line) or the absence (blue circle, dashed line) of chitosan was used as a control. Each dot represented the average of triplicates, and the error bars represented the ratio standard deviation of the mean.

In this study we show that the properties of MBP-*Bs*CsnA enzyme is similar to those of previously reported His-tagged fusion (Pechsrichuang et al., 2013), except that thermostability was significantly improved. The chitosanase activity was stable in the widest range of pH. It was not possible to determine the enzyme activity at pH > 7.0 because the chitosan substrates were insoluble at this condition. It is similar to those of previously reported His-tagged fusion (Pechsrichuang et al., 2013) while the thermostability was significantly improved. The enhancement of chitosanase thermostability by substrate was confirmed by thermal inactivation experiment. The thermal inactivation kinetics at 50 °C showed that the enzyme was more stable in the presence and absence of 0.5% chitosan (low molecular weight) than *Bs*CsnA-10xHis-tagged fusion. These results indicated that the recombinant MBP-*B. subtilis* chitosanase could be attractive for industrial applications because it is relatively thermo- and pH-stable.

4.6 Chito-oligosaccharide production using MBP-*Bs*CsnA

The hydrolysis reaction mixture was analyzed by thin layer chromatography (TLC). The TLC results (Fig. 5) showed that after 6 hours, Mor-CHOS and MB-CHOS, primarily DP3, DP4, and DP5, became detectable. After 48 hours of hydrolysis, Mor-CHOS and MB-CHOS were made up mainly of dimers and trimers. These results suggested that the MBP-*B. subtilis* chitosanase can cleave GlcN–GlcN links, which is common to all known chitosanases.

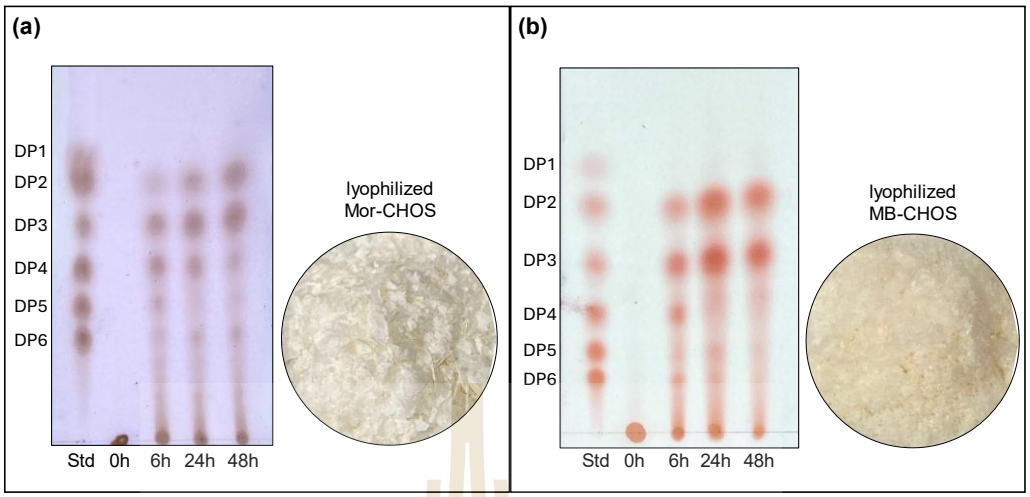

Figure 4.6 Thin layer chromatography analysis of chitosan hydrolyzed by MBP-*Bs*CsnA. The hydrolytic products of (a) 500-600 kDa Morena-chitosan and (b) Marine Bioresource-chitosan at indicated time points were analyzed by TLC. A mixture of 10 mM (DP1-DP2) and 5 mM (DP3-DP6) chitosan-oligosaccharides was used as a standard (Std). The lyophilized hydrolytic products of Morena-chitosan (Mor-CHOS) and that of the Marine Bioresource-chitosan (MB-CHOS) were also shown.

The findings from TLC analysis of chitosan hydrolysis by MBP-*Bs*CsnA provide valuable insights into the substrate specificity and catalytic mechanism of the enzyme. The conversion of chitosan hexamer into smaller oligomers suggests that the MBP-*Bs*CsnA has broad substrate specificity and exhibits endo-type hydrolytic activity by cleaving internal glycosidic linkages within chitosan chain. The MBP-*B. subtilis* chitosanase can cleave GlcN–GlcN links, which is common to all known chitosanases.

4.7 Anti-inflammatory activity of Chito-oligosaccharide in human macrophage cell

The results showed that Mor-CHOS at 25 and 50 ug/mL (Fig. 7a), and MB-CHOS at 100 µg/mL (Fig. 7b) significantly decreased the LPS-induced IL-1β release from THP-

1 monocytes ($p < 0.001$) in a dose-dependent manner. At the highest concentration (200 μg/mL) from both CHOS showed that it could stimulate IL-1β secretion. The maximum inhibitory effect obtained with these CHOS was comparable to the effect of 0.2 μg/ml (0.5 μM) dexamethasone.

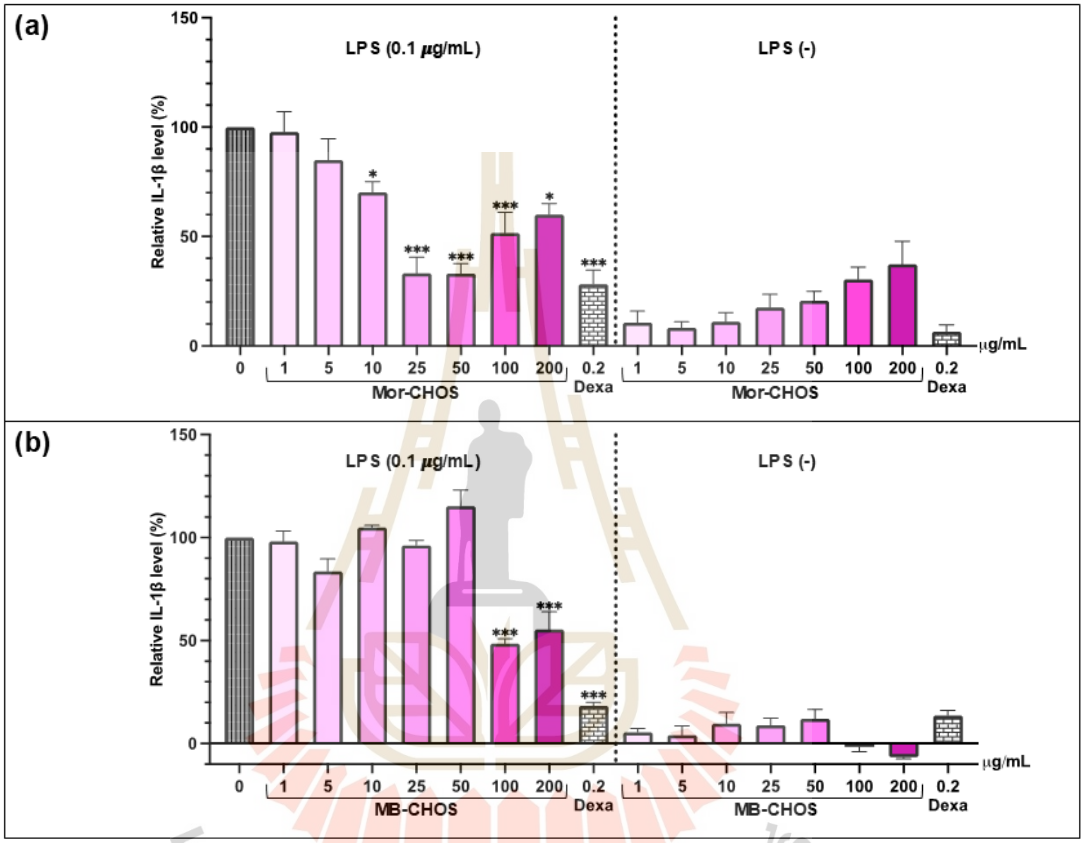

Figure 4.7 IL-1β response in vitD3-differentiated THP-1 monocytes pretreated with Mor-CHOS/MB-CHOS (1 - 200 μg/mL). The IL-1β response of THP-1 cells was compared between cells without pretreatment and (a) Mor-CHOS pretreated cells or (b) MB-CHOS pretreated cells. Dexamethasone (0.2 μg/mL) was used as a positive control. Each column represented the average value of triplicates from two independent experiments and the error bars represented the standard deviation of the mean. Statistical analysis was performed using Ordinary One-way ANOVA, Dunnett's multiple comparisons test with a single pooled variance and P value style: *P < 0.033, **P < 0.002, ***P < 0.001.

Chito-oligosaccharide, produced by bioconversion 1% chitosan dissolved in mild acid using MBP-*Bs*CsnA, showed anti-inflammatory activity. Mor-CHOS 25 and 50 μg/mL gave the best anti-inflammatory effect than MB-CHOS 100 μg/mL. At the highest tested concentration (200 μg/mL), the CHOS might stimulate inflammation in human THP-1 macrophages. The results indicated that the immunomodulatory property of CHOS depended on the chitosan source which could lead to biological effects on the human cells.

4.8 Production of cream

Since MB-CHOS has an anti-inflammation effect, it was used as an active ingredient in skincare products, especially cream. There were 2 variants of cream, cream with 100 ug/ml MB-CHOS and vanilla fragrance (Figure Xa) and cream with 100 ug/ml MB-CHOS, vanilla fragrance, and curcumin (Figure Xb). The cream was produced not only to help reduce inflammation of the skin but also to keep the skin healthy and moist. However, clinical trial is recommended to do for further test.

Figure 4.8 Cream products contain 100ug/ml of MB-CHOS with vanilla fragrance (a), and 100ug/ml of COS with vanilla fragrance and curcumin (b).

CHAPTER 5
CONCLUSION AND RECOMMENDATION

5.1 The recombinant chitosanase from *Bacillus subtilis* 168 was successfully cloned into pMAL-p5X expression vector and expressed in *E. coli* TOP10. The pure chitosanase can be obtained via Maltose Binding Protein (MBP) fusion and one-step purification using amylose beads.

5.2 The optimal temperature of recombinant Maltose Binding Protein- *Bacillus subtilis* Chitosanase fusion (MBP-*Bs*CsnA) was 55°C and stable up to 50°C after incubation for 30 min at pH 6.0, without substrate. The optimal pH was 6.0 and stable within pH 2-9 after incubation at 30°C for 24 hrs. The thermal inactivation kinetics at 50°C showed that the enzyme was more stable in the presence and absence of substrate than *Bs*CsnA-10xHistidine tag. MBP-*Bs*CsnA can cleave GlcN-GlcN links, which is common to all known chitosanases.

5.3 The chitosan-oligosaccharide (CHOS) generated by MBP-*Bs*CsnA and *Bs*CsnA-10-His constructs showed similar anti-inflammatory activity suggesting that MBP-*Bs*CsnA is an attractive format for industrial valorization of chitosan.

5.4 Recombinant MBP-*Bs*CsnA fusion is safe, efficient, and suitable for bioconversion of chitosan into value-added chitosan-oligosaccharide.

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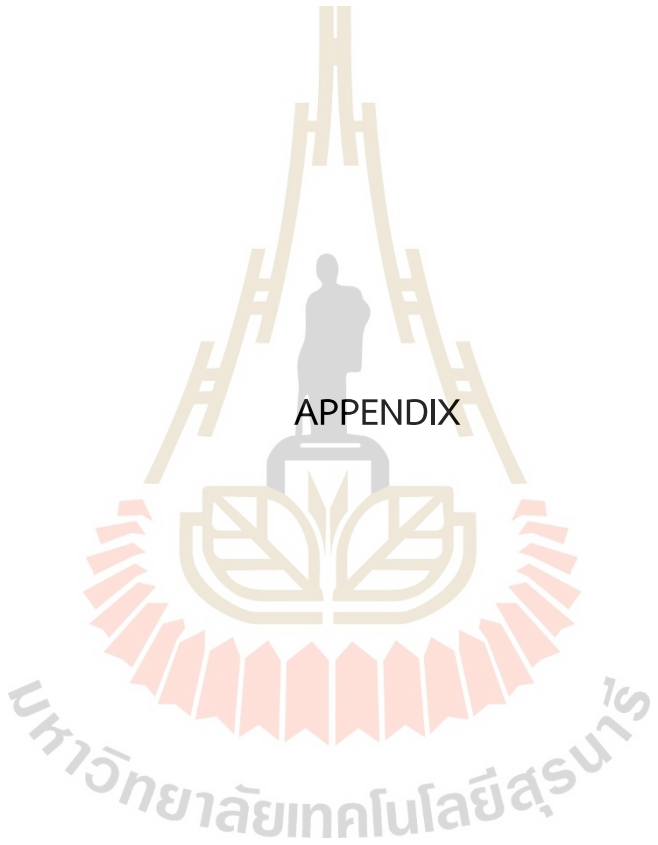
APPENDIX



APPENDIX

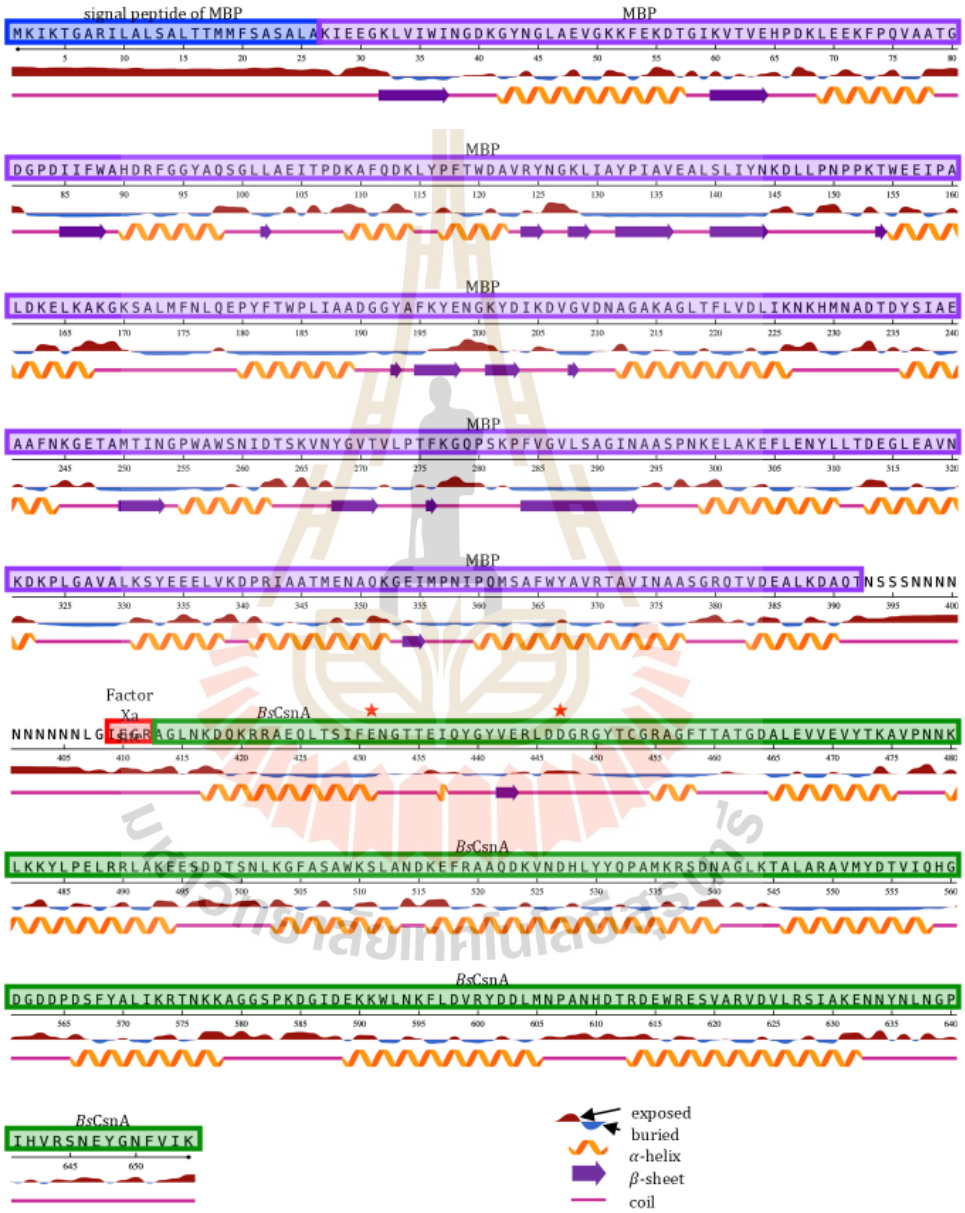


Figure X. A graphical representation of MBP-*Bs*CsnA showing relative surface availability (red is exposed and blue is buried) and the secondary structures (α-helix, β-sheet and coil). The signal peptide of MBP (blue), MBP (purple), Factor Xa site

(red) and *Bs*CsnA (green) were labelled accordingly. The two catalytic residues of *Bs*CsnA (Glu^{431} and Asp^{447}) were marked by red stars. of The recombinant MBP-*Bs*CsnA is made up of 654 residues and is about 72.5 kDa of size.

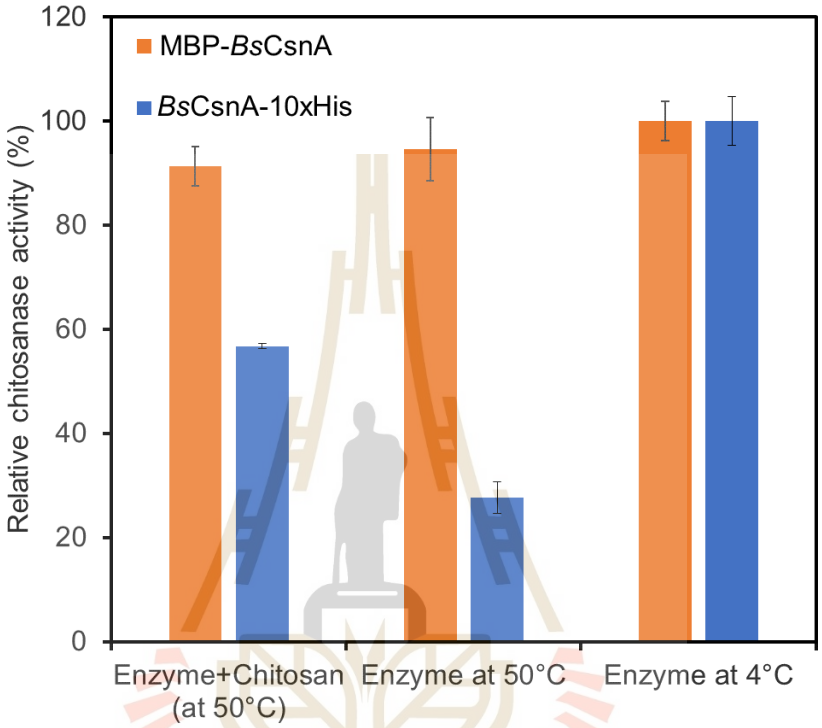


Figure X. The thermostability of MBP-*Bs*CsnA (orange bar) and *Bs*CsnA-10xHistidine (blue bar) at 50°C in the presence or absence of 0.5% low molecular weight chitosan for 30 min was compared to the activity at storage temperature (4°C). Each bar represented the average of triplicates from two independent experiments and the error bars represented the ratio standard deviation of the mean.

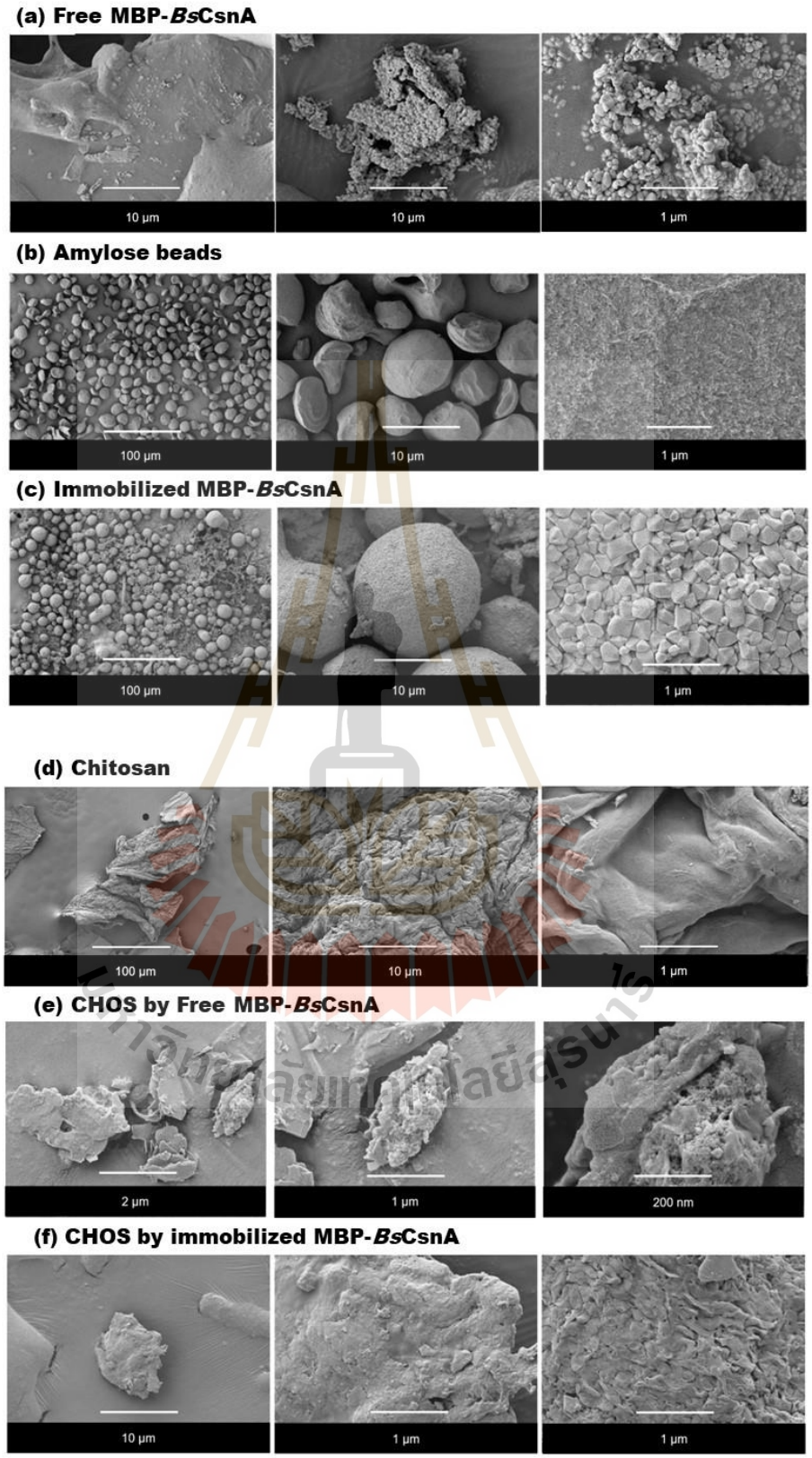


Figure X. FE-SEM analyses of amylose beads before and after immobilized enzyme.

VITAE

Miss Waheni Rizki Aprilia was born on April 2, 1993 in Semarang, Central Java, Indonesia. She graduated from bachelor's degree of Science, Department of Biology, Faculty of Science and Mathematics, Diponegoro University in 2016 with honors. She had work as research assistant at B2P2VRP, Indonesia. In 2018-2023, she received One Research One Grant (OROG) scholarship to study Doctoral Degree in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. Her research topic is production of chitosanase using food grade expression system. She had presented her research work in Thai Society for Biotechnology International Conference Online, April 29, 2022, and received first prize of poster presentation popular vote award (Poster presentation; Production of Recombinant *Bacillus subtilis* Chitosanase by Maltose Binding Protein Fusion). November 2022 and 2023, in Thai Society for Biotechnology International Conference, she had presented about Production and Characterization of Recombinant *Bacillus subtilis* Chitosanase Maltose Binding Protein Fusion (MBP-*Bs*CsnA), Suitable for Bioconversion of Chitosan into Value-added Chitosan-oligosaccharide (CHOS) in oral and poster, respectively. She received the second prize of World Technology Universities Network (WTUN) Student Competition 2022, the second runner-up of Falling Walls Lab Thailand 2023, and the gold award for postgraduate category in The International Biotechnology Competition and Exhibition 2024.

Publications in International Scientific Journals:

1. Yamabhai, M., Khamphio, M., Min, T. T., Soem, C. N., Cuong, N. C., Aprilia, W. R., . . . Eijsink, V. G. H. (2024). Valorization of shrimp processing waste-derived chitosan into anti-inflammatory chitosan-oligosaccharides (CHOS). *Carbohydrate Polymers, 324*, 121546.