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.