CHAPTER II

MATERIALS AND METHODS

2.1. Equipments

Autoclave: Model HA-30, Hirayama Manufacturing Corporation, Japan.

Autopipette: Pipetman, Gilson, France.

Balance: Sartorius LC 620, Germany.

Centrifuge: Refrigerated centrifuge: Model J-21C, Beckman Instrument Inc., USA.

Centrifuge: Microcentrifuge High Speed: Model 1110 Mikro 22R, Hettich Zentrifugen, Germany.

Centrifuge: Minimicro DW 41.

Gel document: Syngene, A division of Synoptics Ltd., UK.

Incubator: Heraeus, Germany.

Incubator shaker: Innova 4000, New Brunwick Scientific Co., Inc., USA.

Laminar flow: Issco, USA.

pH meter: Precisa pH900, Precisa instrument AG company, Switzerland.

Refrigerator: Upright Ultra Freezer; Model MDF-U4086S, Sanyo Electric Trading Co.Ltd., Japan.

Spectrophotometer: Jenway 6400, England.

Vortex: Model 232, Fisher Scientific Inc., USA.

Water bath: MEMMERT GmbH + Co. KG, Germany.

U.V. transilluminator: 2011 MA Crovue, San Gabrial, USA.

Transformation apparatus: Gene Pulser™, Biorad, USA.

High performance liquid chromatography: Shimadzu, Japan.

Power supply: Model EC 135-90, E-C Apparatus Corperation, USA.

2.2. Chemicals

α-, β- and γ-cyclodextrins, Sigma, USA.

Absolute alcohol, Merck, Germany.

Acetonitrile (HPLC grade), Lab Scan, Ireland.

Agar, Merck, Germany.

Agarose, SEAKEM LE Agarose, FMC Bioproducts, USA.

Ampicillin, Biobasic Inc., Thailand.

Boric acid, Merck, Germany.

Bovine serum albumin (BSA), Sigma, USA.

Bromophenol blue, Merck, Germany.

Calcium chloride, Merck, Germany.

Chloroform, Sigma, USA.

Di-Sodium hydrogenphosphate, Fluka, Switzerland.

DNA marker, Lamda (λ) DNA digested with *Hin*dIII: Biobasic Inc., Thailand.

Ethidium bromide, Sigma, USA.

Ethylenediamine tetraacetic acid (EDTA), Fluka, Switzerland.

Glacial acetic acid, BDH, England.

Glucose, Sigma, USA.

Glycerol, Univar, Australia.

Hydrochloric acid, Lab Scan, Ireland.

Iodine, Baker chemical, USA.

Kanamycin, Bio101 Inc., USA.

Methanol, Scharlau, Spain.

NucleoTrap Gel Extraction Kit, BD Biosciences, USA.

Orthophosphoric acid 85%, Carlo Erba, Italy.

Phenol, Pierce, USA.

Polyethylene glycol 6000, Fluka, Switzerland.

Potassium iodide, Mallinckrodt, USA.

QIAprep Spin Miniprep Kit, Qiagen, Germany.

Sodium acetate, Carlo Erba, Italy.

Sodium carbonate, BDH, England.

Sodium chloride, Univar, Australia.

Sodium dihydrogen orthophosphate, Carlo Erba, Italy.

Sodium dodecyl sulfate, Sigma, USA.

Sodium hydroxide, Carlo Erba, Italy.

Soluble starch, Scharlau, Spain.

Tetracyclin, Sigma, USA.

Tris-base, USB, USA.

Tryptone, Merck, Germany.

Yeast extract, Scharlau, Spain.

2.3. Bacterial strains

Escherichia coli JM109 [*F', traD36, proA*$^+$, *proB*$^+$, *lacI*^{q}, *lacZΔM15/recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,Δ(lac-proAB), mcrA*] was used for DNA manipulation.

E. coli BMH 71-18 *mutS* [*F', proA*$^+$, *proB*$^+$, *lacI*^{q}, *lacZΔM15/mutS:Tn10, thi, supE, Δ(lac-proAB)*] was used for mutagenesis.

M13K07 bacteriophage was used for preparing single-stranded DNA.

2.4. Plasmid vector

A plasmid pVR328, a derivative of pUC119 containing the β-CGTase gene from *Bacillus circulans* A11 (Pongsawasdi and Yagisawa, 1987), was used for mutagenesis and constructing the recombinant plasmids containing various combination of the mutation regions.

2.5. Enzymes

Restriction endonucleases: *Nde*I, *Nsi*I, *Sph*I, *Hin*dIII, *Sac*II, *Sal*I, and *Bam*HI, were purchased from New England Biolabs Inc.; USA, and *Sca*I was from Fermentas Inc., USA.

T4 DNA ligase and T7 DNA polymerase was purchased from Fermentas Inc., USA.

T4 polynucleotide kinase was purchased from New England Biolabs Inc., USA.

T7 DNA polymerase was purchased from Fermentas Inc., USA.

Amyloglucosidase [(14) - α-D-glucohydrolase, EC 3.2.1.3, from *Aspergillus niger*] was purchased from Fluka, Switzerland.

RNase A was purchased from Sigma, USA.

2.6. Media preparation

1. Luria-Bertani broth (LB medium)

LB broth consists of 1% (w/v) Bactotryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl, supplemented with 100 μg/mL ampicillin when needed. LB broth was used to cultivate *E. coli* cells.

2. LB-starch agar plate

LB-starch agar consists of 1% (w/v) Bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.6% (w/v) agar and 1% (w/v) soluble starch, supplemented with 100 μg/mL ampicillin when needed. LB-starch agar plate was used for plate-enzyme assays to detect the starch-hydrolyzing activity of CGTase.

2.7. General techniques in genetic engineering

2.7.1. Preparation of competent cells

A single colony of the *E. coli* JM109 or BMH71-18*mut*S was cultured as a starter in 5 mL of LB medium and incubated at 37 °C with 250 rpm shaking for 24 hours. The starter was 1:100 diluted in 200 mL of LB-broth in 500 mL Erlenmeyer flask, and the culture was incubated at 37 °C with 250 rpm shaking until the optical density at 600 nm of the culture reached 0.5-0.6 (~3-4 hours).

The culture was chilled on ice for 15 minutes and the cells were harvested by centrifugation at 6,000 rpm for 15 minutes at 4 °C. The supernatant was removed. The cell pellet was washed twice with 1 volume and 0.5 volume of cold sterile water, respectively. The cells were resuspended and centrifuged at 6,000 rpm for 15 minutes at 4 °C. The supernatant was discarded. The pellet was washed with 10

mL of ice cold sterile 10% (v/v) glycerol, and finally resuspended in a final volume of 0.6 mL of ice-cold sterile 10% glycerol. The cell suspension was divided into 40 μL aliquots and stored at –80 °C until used.

2.7.2. Electroporation

The competent cells were thawed on ice. The cell suspension of 40 μL was mixed with 1-2 μL of the mutagenesis or ligation mixture, mixed well and placed on ice for 1 minute. The mixture was electroporated in a cold 0.2 cm cuvette with the apparatus setting as 2.5 μF, 200 Ω of the pulse controller unit and 2.50 kV.

After one pulse was applied, the cells were resuspended in 1 mL of LB broth and incubated at 37 °C for 1 hour. The cell suspension was spread on the LB-starch agar plate.

2.7.3. Plasmid preparation (alkaline lysis method)

Cells harboring plasmid were cultured in 1.5 mL LB medium and harvested by centrifugation at 8,000g, 4 °C for 2 minute. The packed cells were resuspended in 100 μL of solution I (25 mM Tris-HCl, pH 8.0, 10 mM EDTA and 50 mM glucose) by vigorous vortexing. Then 200 μL of solution II (1% SDS, 0.2 N NaOH) was added, mixed by inversion until the mixture was clear. The mixture was neutralized by adding 150 μL of solution III (3 M sodium acetate, pH 4.7), mixed by inversion and kept on ice for 10 minutes. After centrifugation at 10,000g for 10 minutes, 10 mg/mL of RNaseA was added to the supernatant to give a final concentration of 10 μg/mL and incubated at 37 °C for 20 minutes. The supernatant was extracted with one volume of phenol:chloroform (1:1). Two volumes of absolute alcohol were added, mixed by inversion several times and stored at –20 °C for 20 minutes. The plasmid was pelleted by centrifugation at 10,000g, washed with 70% ethanol, and vacuum-dried for 10 minutes. The pellet was dissolved in 50 μL TE buffer and stored at –20 °C.

For DNA sequencing, the QIAprep Spin Miniprep kit was used to prepare the plasmid DNA, and performed according to the kit protocol. Briefly, the plasmid-harboring cells were cultured in 1.5 mL LB medium and harvested by centrifugation at 8,000g, 4 °C for 1 minute. The bacterial cell pellet was resuspended in 250 μL Buffer P1 by vortexing until the cell clumps were not visible. Buffer P2 of

250 µL was added, and gently inverted the tube 4-6 times. The mixture became viscous and slightly clear. Buffer N3 of 350 µL was added. The solution became cloudy after gently inverting the tube 4-6 times. The preparation was centrifuged for 10 minutes at 10,000g. The supernatant was then applied to the QIAprep spin column and centrifuged for 1 minute. The flow-through was discarded. Buffer PB and PE were added and centrifuged for 1 minute, respectively. Finally, the column was placed in a 1.5 mL microcentrifuge tube and added Buffer EB to the center of the QIAprep spin column to elute the DNA, the column was left stand for 1 minute, and then centrifuged for 1 minute.

2.7.4. Agarose gel electrophoresis

DNA was analyzed by using 1% agarose gel in TBE buffer (98 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA, pH 8.2). DNA samples with 1× tracking dye were loaded into the wells. The gels were run at 100 volts for 1 hour or until bromophenol blue reached the bottom of the gel. After electrophoresis, the gels were stained with 2.5 µg/mL ethidium bromide solution for 2-5 minutes, and the DNA bands were visualized under the UV light. The sizes of DNA fragments were determined by comparing the relative mobilities with those of standard DNA fragments (λ/*Hin*dIII marker).

2.7.5. Extraction of the DNA fragment from the agarose gel

The Nucleotrap gel extraction kit was used for extracting DNA fragment from the agarose gel, and performed according to the kit protocol. Briefly, a gel piece containing the DNA fragment was excised from an agarose gel. The weigh of the gel slice was measured and transferred to a 1.5 mL microcentrifuge tube. Three volumes of Buffer NT1, and 4 µL of Nucleotrap suspension for each 1 µg of DNA to be purified were added. The mixture was incubated at 50 °C for 5-15 minutes and vortexed briefly during the incubation time. The sample was centrifuged at 10,000 rpm for 30 seconds at room temperature. The pellet was washed with 500 µL of Buffer NT2 and 500 µL of Buffer NT3, respectively. Finally, the pellet was added Buffer NE to elute DNA by incubating at room temperature for 10-15 minutes and occasional vortexing during the incubation time. The DNA fragment was collected by centrifugation.

2.7.6. Preparation of single-stranded plasmid

Single-stranded plasmid was prepared from pVR328, a pUC119 plasmid carrying the β-CGTase gene from *B. circulans* A11, by using helper phage M13KO7. A single colony of *E. coli* JM109, containing pVR328, was inoculated into LB broth containing ampicillin 100 μg/mL and incubated with shaking at 37 °C for 2-3 hours or until the cell density was very slightly turbid. Then, the culture was added the helper phage to 10^7-10^8 pfu/mL and grown at 37 °C for 1-2 hours with vigorous shaking. The culture was added kanamycin to 70 μg/mL to select for the phage-infected cells and further grown at 37 C for 12-18 hours with vigorous shaking. The 1.5 mL of the cell culture was centrifuged for 5 minutes at 10,000g in a microcentrifuge. The supernatant of 1.2 mL was removed to a new tube. The supernatant was added 200 μL of a PEG solution containing 20% PEG8000 and 2.5 M NaCl, mixed, and incubated to allow the phage particles to precipitate on ice for 30 minutes. The mixture was centrifuged for 15 minutes at 10,000 rpm to collect the phage precipitate. The pellet was resuspended in 90 μL of TE buffer and 10 μL of 3 M sodium acetate, pH 5.5. The suspension was extracted with one volume phenol:chloroform (1:1) and one volume chloroform, respectively. Two volumes of absolute alcohol were added, mixed and stored at –20 °C for 30 minutes. The single-stranded plasmid was pelleted by centrifugation at 10,000 rpm, washed with 70% ethanol and dried. The pellet was dissolved in 30 μL TE buffer.

2.7.7. Phosphorylation of oligonucleotide primers

Two mutagenic oligonucleotides, named A and B, were purchased from Biobasic Inc., Thailand, and the other two mutagenic oligonucleotides, named C and D, were purchased from Proligo Singapore Pty Ltd, Singapore. They were phosphorylated before use in the mutagenesis procedure. Approximately 100-200 pmol of an oligonucleotide was kinased in 10 μL reaction containing 1× kinase buffer, 1 mM ATP, and 10 U T4 kinase, and incubated at 37 °C for 1 hour. The reaction was stopped by heating at 70 °C for 15 minutes. Then, the reaction was spun for 1 minute and stored at –20 °C.

2.8. Mutagenesis of β-CGTase gene using the USE (Unique Site Elimination) procedure

The four mutagenic primers, A, B, C and D, were designed according to the amino acid regions of β-CGTase that were different from those of other thermostable CGTases. The nucleotide sequences of the four primers are shown in Fig. 2.1 For the screening of the mutants, primers A, B, C, and D were also designed to contain the *Bam*HI, *Sal*I, *Sal*I, and *Hin*dIII, respectively.

PRIMER A (47mer)

*Bam*HI
5′ C GTG ATA AGC CGT GGA TCC GCC GAA GGT GGA ATC GTT GAT CAC GCT G 3′

PRIMER B (41mer)

*Sal*I
5′ C GTG ATA AGC CGT CGA CCC GCT GGC ATC GTT GAT CAC GCT G 3′

PRIMER C (51mer)

*Sal*I
5′ GGA CTC GTT AGC GAA GTA ATG ATT GTT CGG GTC GAC CTC ATT GAC GCC AAG 3′

PRIMER D (56mer)

*Hin*dIII
5′ C CAG CGC CTG CTC AAG CTT CCG $TG^{T}/_{G}$ GTC GCC CTC $G^{C}/_{T}C$ $G^{T}/_{A}T$ GTA GAA ACG CTC CAT G 3′

Fig. 2.1. The mutageneic oligonucleotides used to produce the mutations in the USE mutagenesis procedure. The restriction recognition sites, which allow rapid screening of the potential mutants, are shaded.

The USE mutagenesis procedure is summarized in Fig. 2.2. The procedure works by simultaneously annealing two oligonucleotides primers to a single-stranded plasmid. One primer introduces the desired mutation, and the other primer mutates a restriction site unique to the plasmid for the purpose of selection. The selection primer, called *Sca*I primer, eliminates the *Sca*I site at the ampicillin resistant gene in the plasmid. Each of the four kinased mutagenic primers, the kinased *Sca*I primer and 40 ng of the single-stranded plasmid were mixed in 10 μL reaction containing

annealing buffer (200 mM of Tris-HCl, 100 mM of $MgCl_2$ and 500 mM NaCl). The reaction mixture was heated at 95 °C for 5 minutes, and then placed at room temperature for 15 minutes. Next, it was added 6 µL of an enzyme mixture containing 1× polymerase buffer, 1 U T7 polymerase, 5 U T4 ligase, 2.5 mM dNTPs, and 10 mM ATP. It was incubated at 37 °C for 1-2 hours. The reaction mixture was then transformed into an *E. coli* strain BMH71-18*mut*S. A mixture of plasmids were generated by culturing the transformation mixture in the presence of 100 µg/mL ampicillin overnight, pelleting the cells and preparing the plasmids. The plasmid mixture was digested with *Sca*I to linearize most of the wild type plasmid, and transformed into an *E. coli* strain JM109 to separate individual clones. Colonies were cultured for plasmid miniprep and screened for the plasmids with the added restriction sites by using restriction enzyme digestion.

The mutation regions were confirmed using DNA sequencing service by Bioservice unit, National Center for Genetic Engineering and Biotechnology (BIOTEC). The correctness of DNA sequences around the mutation regions was also determined. The DNA sequences were subcloned into the same location in the original plasmid (pVR328) to avoid possible other mutated sequenced in the mutated plasmids.

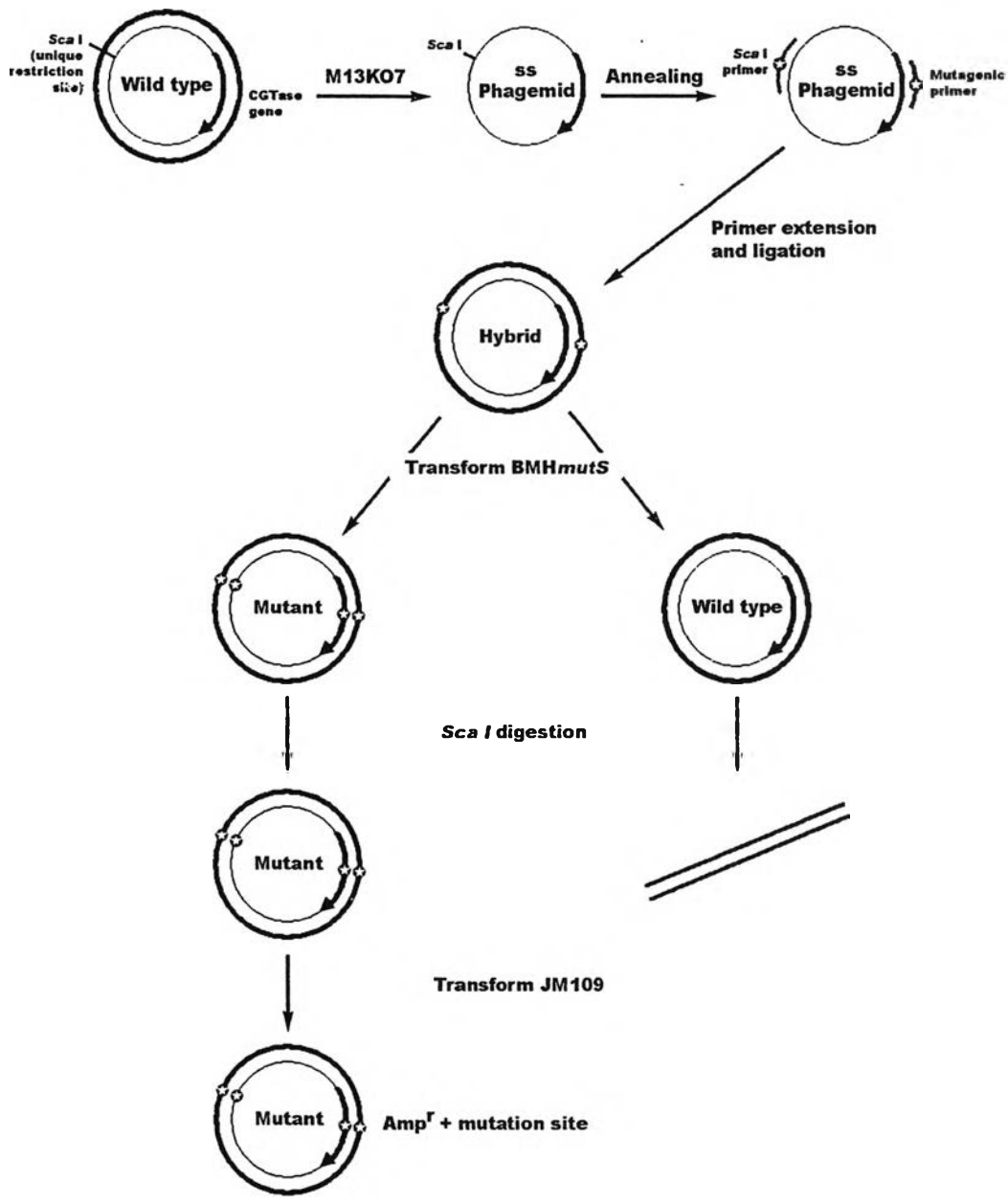

Fig. 2.2. Schematic diagram of USE mutagenesis protocol.

2.9. Construction of the recombinant plasmids containing various combination of the mutant regions

The mutated regions in the mutated plasmids were subcloned into the same regions in pVR328. The plasmids were digested with the appropriate restriction enzymes to yield DNA fragments containing the mutant regions. The DNA fragments

were gel purified using agarose gel electrophoresis and eluted with Qiaquick gel extraction kit. The eluted fragments from mutant plasmids and the restriction enzyme-digested pVR328 were mixed and ligated with T4 DNA ligase. The ligation reactions were incubated for 16-24 hours at 22 °C. The ligation mixture was used to transform the *E. coli* JM109 by electroporation. The transformants were plated on the LB agar containing 100 μg/mL of ampicillin at 37 °C for an overnight. Each transformant was grown in the LB broth in the presence of 100 μg/mL ampicillin. In order to verify the presence of the mutation, the recombinant plasmids were screened for the mutant regions by restriction enzyme digestion. They were checked for the cloning sites as well. The recombinant plasmids were constructed such that they contained all possible combinations of the mutant regions.

2.10. Detection of the mutant CGTase activity

2.10.1. Dextrinizing activity

1. Halo zone on LB-starch agar

E. coli JM109 cells, containing a CGTase plasmid, were plated on a LB-starch agar plate and incubated at 37 °C for 48 hours. The halo formation was observed after the iodine solution (0.02% (w/v) I_2 in 0.27% (w/v) KI) was poured onto the agar.

2. Dextrinizing activity assay

The *E. coli* JM109 cells, containing the CGTase plasmid, were grown at 37 °C for 16 hours in LB broth containing ampicillin. The culture was centrifuged to remove the cells, and the supernatant liquid was used as crude enzyme preparation.

CGTase activity was measured as the dextrinizing power according to the method of Fuwa (1954) with slight modification. The reaction mixture was performed by pre-heating the 500 μL of 0.2% soluble starch in 0.2 M phosphate buffer, pH 6.0, at each temperature (40, 50, 60, 65, 70, and 80 °C) for 1 minute. Then 100 μL of appropriately diluted (1:10-1:50) enzyme solution was added and incubated at that temperature for 10 minutes. To stop the enzymatic reaction, 1 mL of 0.2 N HCl

was added to the reaction mixture. The iodine-starch complex (blue color) was developed by mixing 400 μL of the reaction mixture with 4 mL of 0.01% (w/v) I_2 in 0.1% (w/v) KI. The solution was placed at room temperature for 20 minutes, and the absorbance at 600 nm was measured. The experiments were done in duplicate at each temperature. The results were expressed as the mean values of the duplicate experiments.

One unit of enzyme is defined as the amount of enzyme, which produces 10% reduction in the intensity of the blue color of the starch-iodine complex per minute under the described condition. The relative activity was calculated by using a no enzyme control with the highest absorbance as 0%, and zero absorbance as 100%.

2.10.2. CGTase thermostability testing

The thermostability was determined according to the method of Lemmhuis *et al.* (2003) with slight modification. Appropriately diluted crude enzyme of 100 μL was incubated for 10 minutes at various temperatures (40, 50, 60, 65, 70, and 80 °C), and placed at 40 °C for 1 minute. Then 500 μL of 0.2% soluble starch in 0.2 M phosphate buffer, pH 6.0, pre-incubated at 40 °C were added and further incubated for 10 minutes. The enzymatic reaction was stopped by adding 1 mL of 0.2 N HCl.. The iodine-starch complex (blue color) was developed by mixing 400 μL of the reaction mixture with 4 mL of 0.01% (w/v) I_2 in 0.1% (w/v) KI. The solution was placed at room temperature for 20 minutes, and the absorbance at 600 nm was measured. Duplicate experiments were done at each temperature. The results were expressed as the mean values of the duplicate experiments.

2.10.3. CD forming activity

The *E. coli* JM109 cells, containing the CGTase plasmid, were grown at 37 °C for 18 hours in LB broth containing ampicillin. The culture was centrifuged to remove the cells, and the supernatant liquid was used as crude enzyme preparation. The protein content of each crude enzyme preparations was determined by Coomassie

blue dye binding method. The enzyme preparations were diluted with 0.2 M phosphate buffer, pH 6, such that the concentrations of the protein were 80 μg/mL.

Then, 200 μl of the crude enzyme from each mutant plasmid was incubated with 0.5 mL of 1% soluble starch at 37 °C for 12 hours. The reaction was stopped by boiling for 10 minutes, and clarified by centrifugation. The mixture was treated further for four hours with 6U of glucoamylase to digest the remaining starch and oligosaccharides formed from CGTase reaction. The glucoamylase reaction was stopped by boiling for 10 minutes, and clarified by centrifugation. The cyclodextrin samples were filtered by syringe through the 0.45 μm Sartolon polyamide membrane (Sartorius, Germany) prior to HPLC analysis. The samples were analyzed using the HPLC with a Hypersil-APS2 (NH_2) column (0.46×250 mm), acetonitrile/water (63:37 (v/v)) at flow rate 1.0 mL/min and a refractometric detector. The cyclodextrin standard was a mixture of α-, β- and γ-cyclodextrins (10 mg/mL each). For quantitative analysis, peak area corresponding to each cyclodextrin was used to calculate the cyclodextrin product ratios.

2.11. Protein determination

The protein sample (100 μL) was mixed with 1 mL of Coomassie blue reagent (Appendix A), and left stand at room temperature for 5-10 minutes. The absorbance at 595 nm was measured. The protein concentration was calculated from a standard curve of bovine serum albumin.