หอสมุดกลาง สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER 2

MATERIALS AND METHODS

2.1 Instruments

Autoclave, HA-32, Manufacturing corporation, Japan

Automatic micropipette, Pipetman; P2, P20, P100, P200, P1000, Gilson Medical Electrics S. A., France

BioChiller™ 2000, Fotodyne Inc., USA

Camera, Pentax Super A

Centrifuge, refrigerated centrifuge: Model J2-21C, Beckman, USA

Geiger counter, USA

Gel dryer, model 583, Bio-Rad, USA

Gene Pulser™, Bio-Rad, USA

GS GENE LINKER™ UV CHAMBER, Bio-Rad, USA

High speed microcentrifuge, Kubota 1300, Kubota Corporation, Japan

Horizontal gel electrophoresis apparatus for 8.5 x 12.5 cm gel former

Hybridization oven, Bio-Rad, USA

Incubator shaker, Controlled environment: Psyco-Therm, New Brunswick Scientific Co., USA

Incubator, BM600, Memert GmbH, W., Germany

pH/ ION meter, PHM 95, Radiometer, Copenhagen

Power supply, Power PAG 300, 3000, Bio-Rad, USA

Sequencer, SQ3, Hoefer, England

Sonicator: Model W375, Heat systems-ultrasonics

Spectrophotometer UV-240, Shimadzu, Japan, and DU Series 650, Beckman, USA

Standard cassette, 35 x 43 cm, Okamoto, Japan

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Univap concentrator centrifuge, Univapo 100H, Uniequip

UV transilluminater: 2011 MA crovue, San Gabrial, USA

Vacuum Blottor: Model 785, Bio-Rad, USA

Vortex: Model K-550-GE, Scientific Industries, USA

2.2 Supplies

Black and white print film, Tri-Xpan 400, Eastman Kodak Company, Rochester, USA

Filter paper, HA 0.45 μM pore size, Millipore Corporation, USA

MagnaCharge, Nylon Transfer Membrane, Micron Separations Inc., MA

NENSORB™ 20 Nucleic Acid Purification Cartridges, Dupont, USA

Whatman paper, 3MM, Whatman International Ltd., Maid Stone, England

X-ray film, X-Omat XK-1, Eastman Kodak Company, Rochester, USA

2.3 Chemicals

Acetic acid, E. Merck, Germany

Acrylamide, E. Merck, Germany

Agarose, Seakem LE, FMC Bioproducts, USA

Ammonium persulphate, Bio-Rad, USA

Boric acid, BDH, England

Chloroform, BDH, England

Developer and Fixer, Eastman Kodak Company, Rochester, USA

Ethanol, Carlo Erba, Italy

Ethidium bromide, Sigma, USA

Disodium ethylenediamine tetraacetic acid (EDTA), Fluka, Switzerland

Isoamyl alcohol, E. Merck, Germany

Lamda phage DNA, New England Biolabs, USA

Methanol, E. Merck, Germany

N, N′ methylene bis acrylamide, Sigma, USA

Phenol, Carlo Erba, Italy

Sodium chloride, E. Merck, Germany

Sodium dodecyl sulphate (SDS), Sigma, USA

TEMED, BDH, England

Trizma base, Sigma, USA

Urea, Carlo Erba, Italy

Reagents for DNA manipulation and bacterial cell culture were autoclaved or passed through a 0.45 μm filter into sterile container before used.

2.4 Enzymes

Restriction endonucleases *Eco*RI and *Hin*dIII were purchased from Boehringer Mannheim. *Bam*HI, *Bst*EII, calf intestine phosphatase (CIP), *Kpn*I, *Pst*I, *Sal*I, *Sma*I, T4 DNA ligase, T4 polynucleotide kinase and *Xba*I were purchased from New England Biolabs.

2.5 Bacterial strains and plasmids

Bacillus subtilis TISTR25, an isolated bacteria from soil of Thailand, was kindly provided by Thailand Institute of Scientific and Technological Research (TISTR).

Escherichia coli JM109, genotype: F′ *traD36 lacI*^{q} *lacZΔM15 proA*$^+$*B*$^+$ e14$^-$ (McrA$^-$)*Δ(lac-proAB) thi gryA96* (Nal^r) *endA1 hsdR17* ($r^-_k m^+_k$) *relA1 supE44 recA1*, was used as a host for transformation in a general cloning procedure.

Escherichia coli DH5α, genotype: F′/*endA1 hsdR17* ($r^-_k m^+_k$) *supE44 thi-1 recA1 gryA* (Nal^r) *relA1 Δ(lacIZYA-argF) U169 deoR* (ϕ80 *d lacΔ (lacZ) M15)*, was used as a host for harboring pUC18 and pCSBC14.

M13mp18 was used as a vector for cloning and transformation into *E. coli* JM109 (Appendix 1).

Recombinant plasmids, pCSBC14, was a clone of chromosomal DNA fragment from *B. subtilis* TISTR25 in pUC18 (Tanunat, 1995).

The pNC3 (5.354 kb), a clone of neutral protease (*nprE*) gene, was used as a neutral protease probe for hybridization (Appendix 2).

2.6 Alkaline extraction of plasmid DNA

Recombinant plasmid, pCSBC14, was prepared by using the minipreparation method modified from Sambrook *et al.* (1989). A single colony of *E. coli* JM109 containing plasmid pCSBC14 was grown in 1.5 ml LB broth (1% Bacto-tryptone, 0.5% Bacto-yeast extract and 1% NaCl) with vigorous shaking at 37°C overnight. The cells were harvested by centrifugation at 8,000 rpm for 10 min. The pellet was resuspended in 100 µl of solution I (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose and 5 mg/ml of lysozyme). The mixture was incubated for 10-15 min on ice. 200 µl of solution II (0.2 M NaOH and 1% SDS) was added. The mixture was mixed gently by inversion until the solution was transparent and placed on ice for 10 minutes. Subsequently, 150 µl of solution III (3 M NaOAc, pH 4.8) was added, mixed gently and placed on ice for 30-60 minutes. The mixture was centrifuged at 8,000 rpm for 10 minutes. The supernatant was transferred to a new tube and extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was precipitated with two volumes of absolute ethanol at -20°C for at least 1 hour, centrifuged at 12,000 rpm for 10 min and washed with 70% ethanol. The DNA pellet was air-dried and dissolved in 20-50 µl of TE (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA).

2.7 Measurement of DNA concentration

Electrophoresis was the standard method used for separation DNA fragments on the basis of their molecular weight. The rate of movement in the gel was inversely proportional to the log of the molecular weight. The size of DNA fragments can be estimated by comparison with a standard curve of the distance migrated through the gel versus the log of the molecular weight of the known DNA fragments.

The DNA was run on 0.7% agarose gel in 1xTBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM Na_2EDTA, pH 8.3). The gel was prepared by adding

0.35 g of agarose to 50 ml of 1xTBE buffer. Agarose was solubilized by heating in a microwave oven and then allowed to cool to 50 - 60°C before pouring gel into a plastic gel former (8.5 x 12.5 cm) with a preset well-forming comb. Electrophoresis was run at about 10 volts/cm. After electrophoresis, the gel was stained with ethidium bromide. DNA concentration was estimated from the fluorescent band by comparing to the intensity of the red fluorescent bands of the standard DNA (500 ng lambda phage DNA digested with *Hin*dIII). The red fluorescent intensity of the standard DNA bands of 23.1, 9.4, 6.6, 4.4, 2.3, 2.1 and 0.6 kb correspond to 240, 98, 67, 45, 24, 21 and 6 ng DNA, respectively.

2.8 Subcloning of DNA fragments from pCSBC14 into M13mp18

2.8.1 Preparation of DNA fragments

2.8.1.1 Digestion of pCSBC14 with *Hin*dIII and *Eco*RI

The recombinant plasmid pCSBC14 was digested with 2 restriction enzymes, *Hin*dIII and *Eco*RI. The digestion was carried out in 50 μl reaction which consisted of 5 μg of pCSBC14, 1x recommended buffer for both *Hin*dIII and *Eco*RI, 10 units of each *Hin*dIII and *Eco*RI, and sterile distilled water. The reaction was incubated at 37°C for 3-5 hours. Then, a portion of the sample (0.3 μg) was analyzed for the completion of digestion by agarose gel electrophoresis.

From the restriction map of pCSBC14 in Figure 2.1, five DNA fragments, approximately 0.1, 0.2, 0.7, 0.9 and 1.6 kb, were generated by *Hin*dIII and *Eco*RI digestion.

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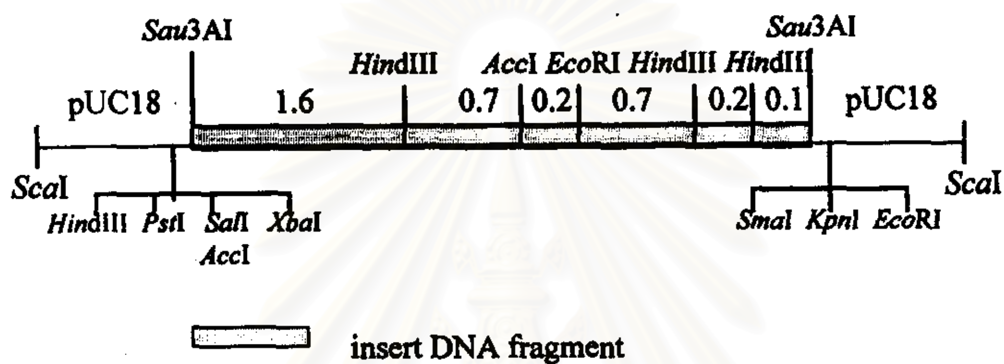

Figure 2.1 Restriction map modification of pCSBC14

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2.8.1.2 DNA fragment elution

Three DNA fragments of 0.7, 0.9 and 1.6 kb were recovered from the agarose gel by using QIAEXII Agarose Gel Extraction Kit. After electrophoresis, the desired DNA fragment was excised as gel slice from the 0.7% agarose gel with a clean, sharp scalpel and put in a microcentrifuge tube. 3 volumes of buffer QX1 was added. Suspended QIAEXII was added to the sample. The mixture was incubated at 50°C for 10 minutes with occasional mixing by vortexing. After the gel slice was dissolved completely, the color of the mixture should be yellow. The sample was centrifuged at 12,000 rpm for 30 seconds. The supernatant was carefully removed with a pipette. The pellet was washed once with 500 µl of buffer QX1 and then twice with 500 µl of buffer PE. The pellet was air-dried for 10-15 minutes or until the pellet became white. DNA was eluted by addition of 20 µl of 10 mM Tris-HCl, pH 8.5, resuspended by vortexing and incubated at room temperature for 5 minutes. After that, the sample was centrifuged at 12,000 rpm for 30 seconds. The supernatant, contained the purified DNA fragment, was carefully pipetted into a clean tube.

2.8.2 Preparation of vector DNA (M13mp18)

2.8.2.1 Digestion with *Hin*dIII and *Eco*RI

3 µg of M13mp18 was digested with *Hin*dIII and *Eco*RI. The digestion was carried out in 50 µl of the reaction mixture as described in section 2.8.1.1. Subsequently, 1/10 volume of 3 M NaOAc was added and extracted once with phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was precipitated with 2 volume of absolute ethanol and dissolved in 20 µl of TE buffer.

2.8.2.2 Digestion with *Hin*dIII

3 µg of M13mp18 was digested with *Hin*dIII as described in section 2.8.1.1. The linearized M13mp18 was dephosphorelated by adding 1 unit of calf intestine phosphatase, 1x the recommended buffer and sterile distilled water to

the total volume of 100 μl. The mixture was incubated at 37°C for 1 hour. Subsequently, 1/10 volume of 3 M NaOAc was added and then extracted once with phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was precipitated with 2 volume of absolute ethanol and dissolved in 20 μl of TE buffer.

2.8.3 Ligation of DNA fragments to M13mp18

Each of three DNA fragment (0.7, 0.9 and 1.6 kb) was ligated to M13mp18 in 20 μl reaction containing 750 ng of DNA fragment, 250 ng of digested M13mp18, 1x T4 DNA ligase buffer, 1 mM ATP, 10 unit of T4 DNA ligase and sterile distilled water. The reaction mixture was incubated at 14°C for 12 to 18 hours. The ligation reaction was terminated by incubating at 65°C for 5 minutes.

2.8.4 Transformation by electroporation (Dower *et al.*, 1988)

2.8.4.1 Cell preparation

Starter *E. coli* JM109 was grown in 1 ml of L-broth (1% Bacto-tryptone, 0.5% Bacto-yeast extract and 0.5% NaCl) with vigorous shaking at 37°C overnight. The culture was diluted in 100 ml of L-broth and continued to grow to an OD_{600} of 0.5. Cells were harvested by chilling briefly on ice and centrifuging at 4,000 rpm for 15 min at 4°C. The cells were resuspended in 100 ml of cold water, centrifuged as above, resuspended in 50 ml of cold water, centrifuged and resuspended in 2 ml of 10% glycerol. After that, the cells were centrifuged, and finally resuspended in 0.2-2 ml of 10% glycerol. This concentrated suspension was aliquots into 45 μl portions. The cells could be used immediately or stored at -70°C for later use.

2.8.4.2 Transformation

The concentrated cells were thawed on ice for 5 minutes. 1 to 2 μl of ligation mixture was added to give a final concentration of DNA from 10 pg/ml to 7.5 μg/ml. The suspension was mixed gently and placed on ice. The electrical pulses were generated by a Gene Pulser™ set at 25 μF capacitor, 2.5 kV and 200 Ω.

The cell/DNA mixture was transferred to a chilled 0.2 cm electroporation cuvette. The suspension was shaken to the bottom of the cuvette. The cuvette was placed in the chamber. One pulse was applied. This should result in a pulse of 12.5 kV/cm with a time constant of 4.5 to 5 msec. The cells were immediately removed from the cuvette and mixed with 3 ml of top agar (LB + 0.8% Bacto-agar), 200 µl of overnight *E. coli* JM109 cell culture, 20 µl of 20 mg/ml X-gal and 20 µl of 25 mg/ml IPTG. The suspension was mixed and poured on LB bottom agar plate (LB + 1.5% Bacto-agar).

2.8.5 Selection of transformants

The transformants of ligated M13mp18 could be selected from the white plaques. The DNA was extracted from the white plaques. The insert DNA size was determined.

2.8.5.1 Extraction of M13mp18 replicative form DNA

E. coli JM109 was grown in LB-broth with shaking at 37°C overnight and diluted 1:50 in 1.5 ml of LB-broth. The white plaque was picked with a toothpick into the diluted culture. The culture was shaken at 37°C for 5-8 hours and transferred to a microcentrifuge tube. The cells were harvested by centrifugation at 4,000 rpm for 10 min. The RF form was extracted from cell pellet by alkaline extraction as described in section 2.6.

2.8.5.2 Determination of the insert DNA size

The insert DNA size of transformants were determined by digestion with *Hin*dIII for 1.6 kb DNA fragment or *Hin*dIII and *Eco*RI for 0.7 and 0.9 kb DNA fragments.

2.9 DNA sequencing

The sequence of a deoxyribonucleic acid molecule were elucidated using enzymatic method based on the ability of a DNA polymerase to extend the DNA

primers on template-primer DNAs and occasionally incorporated terminating nucleotides. Sequence determination was carried out as a set of four separate reactions. Each of which contained all four deoxyribonucleotide triphosphates (dNTPs) supplemented with a limiting amount of a different dideoxyribonucleotide triphosphate (ddNTP) per reaction. Because ddNTPs lack the 3'OH group necessary for chain elongation, the growing oligonucleotide was terminated selectively at G, A, T or C, depending on the respective dideoxy analog in the reaction. The resulting fragments, each with a common origin but ending in a different nucleotide, were separated according to size by high resolution denaturing polyacrylamide gel electrophoresis.

2.9.1 Preparation of single-stranded M13 DNA

E. coli JM109 was grown in LB-broth with shaking at 37°C overnight and diluted 1:50 in 1.5 ml of LB-broth. The white plaque was picked with a toothpick into the diluted culture. The culture was shaken at 37°C for 5-8 hours and transferred to a microcentrifuge tube. The cell suspension was centrifuged at 4,000 rpm for 10 min. The phage-containing supernatant was removed to a new tube containing 200 µl of 20% polyethylene glycol 6000 (PEG6000)/2.5 M NaCl solution and 150 µl of 3 M NaOAc. The phage was precipitated 15 minutes at 4°C and centrifuged at 10,000 rpm for 10 minutes. The supernatant fluid was carefully removed with a pipette. As much PEG as possible should be removed at this point. The phage was resuspended in 100 µl of TE buffer and extracted with 100 µl of phenol. After centrifugation, the top aqueous layer was removed to a new tube, extracted once with the phenol/chloroform (3:1) and once with 100 µl of chloroform. The final aqueous layer was added 0.1 volume of 3 M NaOAc, 2.5 volumes of absolute ethanol and chilled at -70°C for 30 minutes. The single-stranded DNA was precipitated by centrifugation at 12,000 rpm for 15 minutes, then it was washed with 70% ethanol, dried and dissolved in 20 µl of TE buffer.

2.9.2 Sequencing reaction

The dideoxynucleotide chain termination method of Sanger *et al.* (1977) as in Sequenase Version 2.0 DNA Sequencing Kit (Amersham) was used.

2.9.2.1 Sequencing of single-stranded DNA templates

Annealing reaction: The annealing reaction mixture (10 µl) contained 1 µg of template DNA, 1 µl of primer (0.5 pmol), and 2 µl of 5x sequence buffer. The mixture was heated at 65°C for 2 min, then cooled to room temperature over 15-30 min.

Labelling reaction: A single 'labelling' reaction was used for all four termination reactions. To the annealing mixture was added 1 µl of 0.1M DTT, 2 µl of diluted labelling mix (1:5), and 0.5 µl of 10 µCi/µl [α-^{35}S]dATP. Labelling was begun by the addition of 2 µl of diluted polymerase (1:8, 1 µl of Sequenase Version 2.0 polymerase, 0.5 µl of pyrophosphatase and 6.5 µl of enzyme dilution buffer). The mixture was incubated at room temperature for 5 min. Four aliquots of this 'labelling' reaction mixture were used for the 'termination' reactions.

Extension-termination reaction: Four separate dideoxy 'termination' mixtures were prepared in 0.5 ml microcentrifuge tubes. 2.5 µl of the ddGTP termination mix was placed in the tube labelled G. Similarly the A, T and C tubes were filled with 2.5 µl of the ddATP, ddTTP and ddCTP termination mixs, respectively. The tubes were closed to prevent evaporation and warmed at 37°C a minute before the reactions were started. 3.5 µl of the above labelling reaction mixture was added to each termination mixture and incubated at 37°C for 5 min. Four microlitres of stop solution is added. The mixtures were heated at 75°C for 2 min immediately prior to loading 2-3 µl onto the sequencing gel.

2.9.2.2 Sequencing double-stranded DNA templates

The plasmid DNA (3-5 µg) was denatured by adding 0.1 volumes of 2 M NaOH, 2 mM EDTA and incubating 30 minutes at 37°C. The

mixture was neutralized by adding 0.1 volumes of 3 M NaOAc, pH 4.8 and the DNA precipitated with 2 volumes of ethanol (-70°C, 15 minutes). After washing the pelleted DNA with 70% ethanol, it was redissolved in 7 µl of distilled water. 2 µl of sequenase reaction buffer and 1 µl of primer were added. Annealing could be done in exactly the same manner as for single-stranded DNA or by warming at 37°C for 15-30 minutes.

2.9.3 Sequencing gel preparation and electrophoresis

The DNA products of sequencing reactions were separated in denaturing polyacrylamide gels as a function of the log of their molecular weights. As a result, the distances between the smaller fragments were greater than those of larger fragments. The rate of migration of the DNA fragments in the gel was a function primarily of the voltage gradient (volts/cm of gel length). Thus, the longer gels require a greater voltage to achieve a given speed of separation.

2.9.3.1 Preparation of the gel

The glass plates were cleaned thoroughly with cleaning solution. After the glass plates were dried, a few millilitres of silanizing solution (Rain-X) was applied with wiping paper over the inner surface of the shorter plate which would contact the gel and left dry in the air. Inner surfaces of both plates were then cleaned with 70% ethanol, and polished thoroughly. The gel plates were assembled with two spacers and sealed on both sides and the bottom with sealing tape. For a 43 cm x 0.35 mm x 33 cm gel, 60 ml 6% denaturing acrylamide gel mix (7.6 M urea, 5.7% acrylamide, 0.3% *N, N* methylene bis acrylamide and 1x TBE), 300 µl of 10% ammonium persulphate and 42 µl TEMED were added and mixed. The solution was then poured into the gel cast using a 50 ml disposable syringe. During this time, the mould was kept at about 45° to the horizontal. The flow of gel mix between the glass plates was kept continuously, as this would minimize the risk of trapping air bubbles. The gel cast was laid almost horizontally. The gel comb was inserted to a

depth of about 0.5 cm. The gel cast was clamped with clips directly over the spacers. The gel should polymerize within 30 minutes.

2.9.3.2 Gel electrophoresis

The plastic seal along the bottom of the casted gel was removed. The gel plate was placed in the gel running tanks, and clamped securely into place. 1x TBE buffer was poured into the upper and lower buffer chambers. The gel comb was removed. The wells were flushed with running buffer using a pasture pipette. The samples were denatured by heating at 75°C for 2 min. Immediately prior to loading, the sample wells were flushed again to remove any residual urea. The samples were loaded with gel-loading tips. When gel loading was completed, the cover was placed over the gel and the power supply was turned on. The gel was run at constant power of 35-40 watts for a 40 cm-long gel for 2-3 hours (100-200 bases) and 5-8 hours (300-400 bases).

2.9.3.3 Autoradiography

After gel electrophoresis, the shorted plate was removed. The gel must be soaked in 5% acetic acid, 15% methanol for 15-30 min to remove urea. Then a piece of Whatman 3MM paper was laid over the gel and slowly peeled back to lift the gel onto the paper. The paper was cut to a suitable size, covered with plastic wrap and dried the gel under vacuum at 80°C for 2 hours. Then the gel was exposed to the X-ray film for 72 hours in a cassette, at room temperature. The X-ray film was developed using the method recommended by the supplier.

2.10 DNA sequence analysis

The nucleotide sequences were analysed by computer programming. Data handling and sequence analysis were conducted using the DNA strider 1.2 software (Marck, 1988). To search for similar sequence the BLAST (Altschul *et al.*, 1990, Altschul *et al.*, 1997) program was used. Searching using the BLAST program was performed at the National Center of Biotechnology Information (NCBI) in the non-

redundant GenBank from the NCBI updated Feb 24, 1998 using the BLAST network service (http://www.ncbi.nlm.nih.gov). ClastalX (1.64b) program (Higgins and Sharp, 1988) was used for sequence alignment. Phylip version 3.5c program was used for constructed phylogenetic tree. The deduced amino acid sequence was predicted three-dimensional structure by using Swiss-Model at the wepsite http://www.expasy.ch/Swissmod/SWISS-MODEL.html, Swiss-PdbViewer and Rasmol programs.

2.11 Assay of L-glutamine D-fructose-6-phosphate amidotransferase

2.11.1 Protein preparation

E. coli DH5α harboring pUC18 or pCSBC14 was grown in 100 ml of LB medium with or without 100 μl of 25 mg/ml IPTG at 37°C for 18 hours with vigorous aeration. The cultures were harvested by centrifuging at 4,000 rpm for 15 min, washed with 0.05 M Tris-HCl, pH 8.0, and resuspended in 0.05 M Tris-HCl, pH 8.0 (4 ml/g of packed cell). These suspensions were sonicated for 1 min on ice by using a sonicator. The extracts were centrifuged at 4,000 rpm for 15 min. The supernatant fluids were used for the enzyme assay.

2.11.2 Enzyme assay

Enzyme activity was measured by the method of Ghosh *et al.* (1960). The assay mixture normally contained, in a final volume of 1 ml, 20 mM fructose-6-phosphate, 15 mM L-glutamine, 37.5 mM potassium phosphate buffer, pH 7.0, 2.5 mM EDTA and cell lysate. After incubation at 37°C for 30 min, the reaction was stopped by heating in a boiling water bath for 2 min, cooled and centrifuged. 0.80 ml of the supernatant fluid was used for the determination of glucosamine-6-phosphate.

Glucosamine-6-phosphate was determined by using a modification of the Elson-Morgan procedure described by Kenig (Kenig *et al.*, 1975). Samples were treated, respectively, with 0.10 ml of saturated $NaHCO_3$ solution and 1 ml of ice-cold, acetic anhydride in acetone (1%, v/v). After vigorous shaking, the tubes were

maintained at room temperature for 3 min, heated in a boiling water bath for 3 min to destroy excess acetic anhydride, and cooled to room temperature. The samples were treated with 0.20 ml of 0.80 M dipotassium tetraborate, pH 9.0, mixed, and heated in a boiling water bath for 3 min. After cooling, the samples were treated with 6.0 ml of Ehrlich's reagent (1.0 g of ρ-dimethylaminobenzaldehyde added to 1.25 ml of 10 N HCl and then diluted to 100 ml with glacial acetic acid). Finally, the samples were incubated at 37°C for 20 min and cooled. The optical densities were measured in a spectrophotometer at 585 nm. In each experiment, two control samples, one without cell lysate and one without substrate, were assayed in the same way. The absorption reading for the control without substrate, or the sum of the readings of the two controls, was substracted from the readings obtained from the samples of the complete reaction mixtures. Solutions of glucosamine-HCl (0.1 to 1.0 mM) were assayed simultaneously for the standard curve of glucosamine. One unit of enzyme is defined as the quantity that produces 1 μmole of glucosamine-6-phosphate in 30 minutes under the condition of the assay described above.

2.12 Protein determination

The protein was estimated by the method of Bradford (1967). Protein solutions containing 1 to 10 μg protein in a volume up to 0.1 ml were pipetted into eppendorf tubes. The volume was adjusted to 0.1 ml with appropriate buffer. One millilitre of protein reagent (100 mg of Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, 100 ml of 85% phosphoric acid and water to a final volume of 1 liter) was added to each tube and mixed by vortexing. After 2 min, the absorbance at 595 nm was measured.

2.13 Southern-blot hybridization analysis of *B. subtilis* TISTR25 chromosomal DNA with neutral and alkaline protease probes

2.13.1 Isolation of *B. subtilis* TISTR25 chromosomal DNA

One colony of *B. subtilis* TISTR25 was grown in 100 ml LB at 37°C overnight. The cells were harvested by centrifugation at 4,000 rpm for 15 min. Then, the cell pellet was washed, resuspended in 10 ml of SET buffer (20% sucrose, 50 mM Tris-HCl, pH 7.6 and 50 mM EDTA) and centrifuged at 4,000 rpm for 10 minutes. The cells were placed at -70°C for 10 minutes and shaken in warm water at 65°C. The cell pellet was resuspended in 2 ml of SET buffer. 0.2 ml of 5 mg/ml lysozyme and 0.1 ml of 10 mg/ml RNaseA were added. The reaction mixture was incubated at 37°C for 30 minutes. Then, 0.05 ml of 25% SDS was added and incubated at 37°C for 1 hour. Subsequently, 0.3 ml of 2 mg/ml Pronase was added and incubated at 37°C for 15 minutes. An equal volume of chloroform/isoamyl alcohol (24:1) was added and shaken gently at 37°C for 10-16 hours. Then, 1 ml of sterile distilled water and 10 ml of chloroform/isoamyl alcohol were added, and the tube was inverted gently for about 5 minutes. Phases were separated by centrifugation at 4,000 rpm for 10 minutes. The upper phase was transferred to a sterile 15 ml tube by using a wide-bored pipette and extracted with an equal volume of chloroform/isoamyl alcohol. The upper phase was separated as above. 5 M NaCl was added to the final concentration of 0.1 M. The DNA was precipitated by adding 2 volumes of cold absolute ethanol. The solution was mixed thoroughly and stored at -20°C overnight. The DNA was spooled out of solution with a glass rod, dipped into a tube of absolute ethanol and dissolved in 2 ml of TEN buffer (10 mM Tris-HCl; pH 7.6, 1 mM EDTA; pH 8.0, 10 mM NaCl). 0.1 ml of chloroform was added and stored at 4°C.

2.13.2 Southern blotting

B. subtilis TISTR25 chromosomal DNA was digested separately with 8 restriction endonucleases, *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sma*I and *Xba*I. Each reaction consisted of 1 μg chromosomal DNA, 1x restriction buffer, 1x BSA, 5

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unit restriction enzyme and sterile distilled water. The time for digestion was overnight. After digestion, size fractionation of the DNA was carried out by an agarose gel electrophoresis. The DNA fragment in the agarose gel was treated to ensure efficient transfer and to generate single-stranded DNA suitable for hybridization. The gel was placed in a depurination solution (0.25 M HCl) for 15 minutes at room temperature with gentle shaking. Then, the gel was rinsed twice with sterile distilled water and placed in denaturing solution (0.5 N NaOH) for at least 30 minutes at room temperature with gentle shaking. The DNA fragments were then transferred to a nylon membrane. The transfer was carried out by vacuum blotting.

2.13.2.1 Vacuum blotting

A sheet of Whatman 3MM paper 0.5-1.0 cm larger than the gel was pre-wet in 10x SSC (1.5 M NaCl and 150 mM sodium citrate) before placing onto the porous screen. The nylon membrane was cut to the same size or slightly larger than the gel, pre-wet in 10x SSC and positioned on the filter paper. Then, the rubber mask was pre-wet in distilled water and positioned over the membrane. The rubber mask should have a template cut such that the window was 2-5 mm smaller than the gel or enough to provide for a good seal. The gel was transferred carefully into position over the opening in the rubber mask in contact with the membrane. The cover lid was placed on the base of the unit. The locking levers was turned into position, and vacuum of 5 mm of Hg was applied . Subsequently, the transfer buffer 10x SSC was poured gently on to the surface of the gel. The meniscus formed by the gel should contain sufficient buffer for complete transfer. The transfer was allowed to proceed for approximately one and half hours. When the transfer was completed, the pump was turned off and the remaining transfer buffer was removed. The apparatus was dismantled, the membrane was removed and placed in 2x SSC for 5 minutes at room temperature with gentle shaking. The membrane was dried at room temperature on a piece of filter paper. Finally, the DNA was fixed to the nylon membrane by UV crosslinking.

2.13.3 Southern blot hybridization

B. subtilis TISTR25 DNA fragments were hybridized with both neutral and alkaline protease probes. Neutral protease probe was approximately 600 bp which generated by digesting pNC3 with *Eco*RI and *Bgl*I. Alkaline protease probe, an oligonucleotide with 20 bases (5'-TGTCGCAAGCACCGCACTAC-3'), was designed from the nucleotide sequence of *Bacillus* alkaline protease gene (Van Der Laan *et al.*, 1991; Takami *et al.*, 1992) based on melting temperature and synthesized by automatic synthesizer.

2.13.3.1 Probe labelling

2.13.3.1.1 Nick translation

The 600 bp neutral protease probe was labelled by using nick translation kit (Amersham). The DNA was diluted to a concentration of 2-50 ng/µl in TE buffer. 50 ng of the DNA was placed into a clean microcentrifuge tube, and 10 µl of the nucleotide buffer solution, 33 pmol of [α-^{32}P]dATP, 5 µl of enzyme solution (0.5 units/µl DNA polymerase I and 10 pg/µl DNase) and sterile distilled water were added for a reaction volume of 50 µl. The reaction mixture was mixed gently by pipetting up and down, and the tube was capped before spinning for a few seconds to bring the content to the bottom of the tube. The reaction was incubated at 15°C for 60 minutes. Finally, the reaction was stopped by the addition of 5 µl of 0.2 M EDTA, pH 8.0.

2.13.3.1.2 5' end labelling

The oligonucleotide of alkaline protease probe was 5' end labelled with [γ-^{32}P]dATP. The 10 µl reaction consisted of 10 pmol of DNA probe, 1 µl of 10x T4 kinase buffer, 5 units of T4 polynucleotide kinase, 50 µCi of [γ-^{32}P]dATP and sterile distilled water. The reaction mixture was incubated at 37°C for 30 minutes and then stopped by incubating at 65°C for 10 minutes.

2.13.3.2 Purification of labelled probes

Both of labelled neutral and alkaline protease probes were purified with the NENSORB™ 20 cartridges to separate unincorporated radioactive nucleotides. Firstly, the NENSORB™ 20 cartridges was clamped to a secure support (Figure 2.2) and rinsed with 2 ml of absolute methanol. The disposable plastic syringe was filled with air and attached to the adapter. Constant gentle pressure was applied to push methanol slowly through the cartridge. Secondly, 2 ml of reagent A [0.1 M Tris-HCl, 10 mM Triethylamine (TEA), 1 mM disodium EDTA, pH 7.7] was loaded into the column and pushed through the column with gentle pressure. 20 μl of the labelled probe and 380 μl of reagent A were mixed and carefully loaded directly to the top of the column bed by using micropipette. Thirdly, 3 ml of reagent A and 3 ml of a water were used, respectively, to wash the column. Finally, the labelled probe was eluted with 1 ml of reagent B (20% n-propanol for neutral protease probe or 50% methanol for alkaline protease probe) from the column bed by the gentle force. The effluent in 200 μl (9-11 drops) fractions were collected in eppendoef tubes for using in hybridization.

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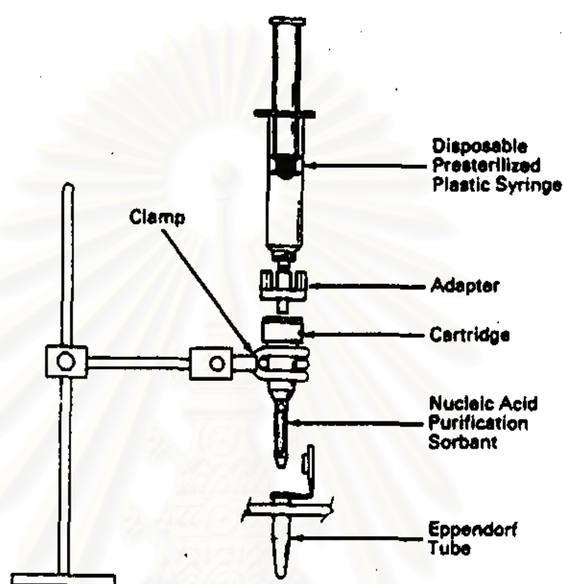

Figure 2.2 Manual use of NENSORB™ 20 Nucleic Acid Purification Cartridge (DuPont)

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2.13.3.3 Hybridization

Hybridization was performed in bottles to maximize user safety and to minimize probe volumes. The membrane was inserted into a Hybaid bottle, and 30 ml of prehybridization buffer [5x SSC (0.75 M NaCl, 75 mM sodium citrate), 2x Denhardts solution (0.1% Ficoll, 0.1% Polyvinylpyrrolidone, 0.1% BSA) and 0.5% SDS] was added. Salmon sperm DNA was denatured by boiling for 5 minutes and immediately chilling on ice. The denatured salmon sperm DNA was added to a final concentration of 100 µg/ml to the buffer. The bottle as well as a balance bottle were clipped into places in the rotisseric. The membrane was prehybridized for at least one hour at 55°C. After prehybridization, the purified labelled probe was denatured by heating to 100°C for 5 minutes, immediately chilled on ice and added directly to the prehybridization buffer in the bottle. Hybridization was carried out for approximately 16 hours at 42°C (for alkaline protease probe) or 55°C (for neutral protease probe). Then, the washing steps were carried out by using at least 50 ml of the following solutions which were pre-warmed to the required temperature: 30 minutes twice with 2x SSC, 0.2% SDS at room temperature, 30 minutes with 0.2x SSC, 0.2% SDS at room temperature and 30 minutes with 0.1x SSC, 0.2% SDS at 45°C. After washing, the membrane was wrapped in plastic wrap and autoradiographed at -70°C in a cassette with an intensifying screen for approximately 12 hours (or overnight).

2.13.4 Reprobing of the membrane

The membrane were kept wet at all time. The probe on the membrane was removed by boiling the membrane in 0.1x SSC, 0.1% SDS for 5 minutes. The residual probe radioactivity was checked by Geiger counter and autoradiographed before initiating the next hybridization experiment as described above.