CHAPTER III

MATERIALS AND METHODS

1. Source of microorganisms

Twenty-four strains of *Pseudomonas pseudomallei* used in this study were clinical isolates from Chulalongkorn Hospital and Songkhla Hospital during 1985 to 1989. Three additional strains (NCTC 7431,NCTC 4845 and NCTC 1688) were kindly supplied by Dr. D.A.B. Dance ,Wellcome Company, while working in the Department of Tropical Medicine,Mahidol University,Bangkok,Thailand. The total of twenty-seven strains were listed in Table 1.

2. Selection for protease-producing strains

2.1 Media and culture conditions

All strains of *P. pseudomallei* were cultured on blood agar plates for isolated colony. One colony was picked as representative of each strain and transferred into a 13 x 100 mm screw-capped test tube containing 1 ml of glycerol beef extract broth (see appendix II.,1.1) and shaken at 180 rpm, at 37° C for 48 hours. Supernatant was collected by centrifugation at 8,000 g for 10 minutes (Microcentrifuge,HSC-10 K, Savant instrument ,U.S.A.).

Table 1. Sources of *Pseudomonas pseudomallei* , 24 clinical isolates and 3 NCTC strains.

number	code number	source	place, year isolated
1	C1/85	human, blood	Chulalongkorn Hospital,1985
2	C2/85	human, blood	Chulalongkorn Hospital,1985
3	C1/86	human, blood	Chulalongkorn Hospital,1986
4	C2/86	human, blood	Chulalongkorn Hospital,1986
5	C3/86	human, pus	Chulalongkorn Hospital,1986
6	C4/86	human, pus	Chulalongkorn Hospital,1986
7	C5/86	human, sputum	Chulalongkorn Hospital,1986
8	C7/86	human, blood	Chulalongkorn Hospital,1986
9	C1/87	human, pus	Chulalongkorn Hospital,1987
10	C2/87	human, blood	Chulalongkorn Hospital,1987
11	C5/87	human, blood	Chulalongkorn Hospital,1987
12	S1/87	human, pus	Songkhla Hospital, 1987
13	S3/87	human, blood	Songkhla Hospital, 1987
14	S6/87	human, sputum	Songkhla Hospital, 1987
15	C1/88	human, blood	Chulalongkorn Hospital,1988
16	C2/88	human, blood	Chulalongkorn Hospital,1988
17	C3/88	human, blood	Chulalongkorn Hospital,1988
18	C4/88	synovial fluid	Chulalongkorn Hospital,1988
19	C6/88	human, blood	Chulalongkorn Hospital,1988
20	C7/88	human, pus	Chulalongkorn Hospital,1988
21	K1/88	human, pus	Chulalongkorn Hospital,1988
22	E1/88	human, pus	Chulalongkorn Hospital,1988
23	C2/89	human, blood	Chulalongkorn Hospital,1989
24	C4/89	human, blood	Chulalongkorn Hospital,1989
25	NCTC 7431	human	NCTC
26	NCTC 1688	human	NCTC
27	NCTC 4845	monkey	NCTC

2.2 Detection of protease activity

Protease activity was detected by its ability to hydrolyze casein in skim milk. Six microlitres of culture supernatant (from 2.1) of each strain was applied into a 3 mm diameter well on skim milk plate (see appendix II,2.1) and incubated at 37° C for 1 hour. Clear zone surrounding the well, as indication of proteolytic activity, was measured. The strain which gave highest clear zone diameter was selected for further studies.

3. Enzyme assays

3.1 Protease assay

3.1.1 Skim milk plate test : Protease activity was determined by the ability of enzyme to hydrolyze skim milk. Skim milk plate (see appendixII,2.1) was punched to obtain 3 mm diameter wells, each plate consists of 45 wells. This method was from Schumacher's (69) protease assay using a single-diffusion technic . For the test, 6 µl of sample solution was applied into the well on skim milk plate then incubated at 37° C for 1 hour. A clear zone diameter around the well was measured. This method was used for screening test to select the protease producing-strains and for determining the protease in fractions from chromatography column.

3.1.2 Azocasein assay : Protease activity was measured using azocasein as a substrate, by the method of Honda et.al. (70). The reaction mixture contained 100 µl of 0.5 mg azocasein in

phosphate buffer pH 7.0 and 100 µl of appropriate dilution of enzyme solution. The reaction mixture was incubated for 30 min at 37° C in a water bath. The reaction mixture was terminated by the addition of 5% trichloreacetic acid. After standing for 10 min at room temperature , the supernatant was collected by centrifugation at 2500 rpm for 10 min. Then 0.4 ml of 0.5 N NaOH was added to 0.4 ml of the supernatant and the absorbance at 420 nm was measured. One unit of protease activity (PU) is defined as the amount of enzyme that give an increase in an absorbance of 0.1 at 420 nm in 30 min at 37° C.

3.2 Collagenase assay

Purified protease was assayed for collagenase activity by the method from Smyth and Arbuthnott (58) except for using phosphate buffer pH 7.0 instead of Tris-maleic acid buffer pH 7.0. Ten milligram of azocoll powder (Sigma) were weighed into 13 X 100 mm test tube and was added with 1.9 ml of 0.01 M phosphate buffer, pH 7.0, then incubated at 37° C for 5 min. A 0.1 ml of test solution (purified protease ,ca. 10 and 20 PU) was added and incubated for an additional 1 hour. The remaining insoluble azocoll was sedimented by centrifugation at 2000 rpm for 10 min. The supernatant was assessed for collagenase activity by liberating a dye in solution. A commercial collagenase type 1 A, 1mg/ml (Sigma) was used as a positive collagenase activity and reagent blank was a negative control.

3.3 Elastase assay

Elastase activity was determined by the method modified Sachar's (71) using elastin congo red (Sigma) as a substrate. The reaction mixture contained 5 mg of elastin congo red in 0.25 ml of 0.2 M Tris-HCl pH 8.8, 0.25 ml of distilled water and 0.25 ml of enzyme solution (purified protease)in 13 x 100 mm test tube. Purified protease (ca.10, 20 and 50 PU) was tested for elastase activity. After the mixture was incubated at 37° C for 20 min ,an aliquot (1 ml) of 0.7 M phosphate buffer pH 6.0 was added. The insoluble substrate was removed by centrifugation and filtrate was assessed for elastase activity through the color released. Elastase type 1 (Sigma) was used as a positive elastase activity and reagent blank was used as negative control.

4. Growth and protease production

P. pseudomallei was grown in glycerol beef extract broth (GB) (see appendix II,1.3). A 0.2 ml of broth-culture (optical density at 600 nm, 0.5 ; ca. 2 x 10^8 CFU/ml) was inoculated into 200 ml of broth and culture was incubated at 37° C in a shaking incubator at 180 rpm. Growth was monitored turbiditrically with spectrophotometer (Hitachi U-200) and the culture filtrate was assayed for protease activity. The culture filtrates were collected for protease activity at every 2 and 4 hour interval for 24 hours, later at every 24 hour interval until 168 hours(day 7). Every time the sample was taken the purity of organism was tested.

5. Effects of media on protease production

Four different broths were used to determine the production of protease at 37° C, shaking 180 rpm up to 7 days. The media were glycerol beef extract broth(GB), glycerol beef extract containing 0.5% glucose(GBG), glycerol beef extract containing calcium ion (GBC) and beef extract broth(BB) (see appendix II,1.1-1.4), and adjusted the initial pH at 7.0 . Each medium was prepared in 100 ml broth in a 250 ml Erlenmeyer flask, and was inoculated with 0.1 ml of *P. pseudomallei* strain C2/85 (ca.2 x 10^7 cells). Supernatant was collected at day 1,2,3,5 and 7 and kept at -20° C for testing protease activity. All of the supernatants were tested in the same time, using azocasein as the substrate.

6. Optimal growth condition

P. pseudomallei was grown in a selected medium at different temperature, aeration and shaking rates. The culture supernatant was collected at day 1,2,3,5 and 7 and kept at -20° C for protease activity determination.

7. Isolation of *P. pseudomallei* protease

7.1 Preparation of culture filtrate

P. pseudomallei (ca. 2 x 10^8CFU) was grown in five 500 ml-Erlenmeyer flasks containing 200 ml of glycerol beef extract(GB)

and were incubated statically at 37°C for 72 hours. The culture filtrate was collected by centrifugation at 6000 rpm for 30 min (Beckman J2-21 ,U.S.A., rotor JA-10). The supernatant obtained was added with 0.005 M sodium azide and filtered through a 0.45 um millipore membrane. This cell free filtrate was kept for further experiment.

7.2 Ammonium sulfate fraction

Solid ammonium sulfate (Merch W. Germany) was slowly added with gentle agitation by a magnetic stirrer into the filtrate to give an initial concentration of 40 % saturation (243 gm/liter) and stand for at least 1 hour. All steps were done at ca. 4°C. The precipitate was collected by centrifugation at 8000 g for 30 min and dissolved in a small amount of 0.01 M phosphate buffer pH 7.0, dialyzed overnight with several changes against the same buffer. The remaining supernatant, after the 40 % ammonium sulfate precipitation, was added with ammonium sulfate to raise the concentration to 70 % and 90 % respectively. The precipitates were dissolved and dialyzed as described above. The preparation from ammonium sulfate precipitation was clarified by centrifugation if necessary. Hence three samples of ammonium sulfate fraction were kept for estimation of protease activity using azocasein as substrate. The fraction with highest protease activity would be used as the material for further purification.

7.3 Diethylaminoethyl (DEAE) cellulose column chromatography

The crude protease (protein content ca. 80 mg) was applied to DEAE-cellulose (DE52) column (2.5 x 25 cm) which equilibrated with 0.02 M Tris-HCl pH 8.0 . The column was washed with 300 ml of the same buffer at a flow rate of 50 ml/hr (Peristaltic pump,LKB,Sweden) and then eluted with a linear gradient of 0.02 M Tris-HCl pH 8.0 versus 1 M NaCl in the same buffer (500 ml each). Ten millilitres per fraction was collected by a fraction collector (2211 SuperRac, LKB, Sweden) and assayed for protein content by measuring the absorbance at 280 nm.Protease activity was determined by skim milk plate test. Fractions with a protease activity were pooled and concentrated to 3 ml with Amicon membrane PM 10 and kept for further purification.

7.4 Gel filtration column chromatography

A concentrated material from DE-52 (3ml) was applied to a Sephadex G-200 column (1.5 x 95 cm) previously equilibrated with 0.02 M phosphate buffer pH 7.0 . The column was eluted with 500 ml of the same buffer at a flow rate of 15ml/hr and 5 ml fractions were collected. The collected fractions were then assayed for protein content and protease activity as above. A peak of protease activity was pooled and kept at -20° C,as a purified *P. pseudomallei* protease.

8. Determination of protein

8.1 **Lowry method:** Protein content was determined by Lowry method (72) with bovine serum albumin (BSA, Sigma) as a standard. To 0.5 ml of sample was added 3 ml of solution C (see appendix II,3.3). The mixture was mixed and stand for 10 min at room temperature, 0.3 ml of solution D (see appendix II,3.4) was then added and immediately mixed ,incubated for 30 min at room temperature. The solution was measured for absorbance at 650 nm.

8.2 **The absorbance 280 nm:** The protein content of fractions from column chromatography was monitored by UV-spectrophotometer (Beckman ACTA III UV-visible) at 280 nm.

9. Analysis of protease

9.1 SDS-PAGE analysis

The *P. pseudomallei* protease was analyzed on PAGE containing 0.1 % SDS. The separation gel was 10 % polyacrylamide (see appendix II, 6) prepared in a glass 16 x18 cm slab gel system (Hoeffer, U.S.A.). The stacking gel was 3 % (see appendix II,7). Thirty microlitres of purified protease was mixed with 15 µl of sample buffer (see appendix II,5.7), and denatured by heating in boiling water for 5 min before applying 25 µl into the gel (1.5 µg protein). The electrophoresis was performed in electrode buffer pH 8.3

(see appendix II,5.6), with constant current at 15 mA until the marker dye had moved to the end of the gel. The gel was stained with Coomassies Brillient blue R 250 solution (see appendix II,5.8) at least 1 hour, then destained with a destaining solution (see appendix II,5.9) until the background was clear.

9.2 Polyacrylamide gel electrophoresis(PAGE)

9.2.1 Anionic system : The anionic system of Davis (74) was performed. The reagents and the gel were prepared (see appendix II,11) on a vertical slab. Thirty microlitres of the purified protease (98µg/ml) was added with 15 µl of sample buffer (see appendix II,11) and 30 µl of this mixture was applied into the gel. The electrophoresis was carried out in the electrophoretic buffer pH 8.3 at constant current of 10 mA for 2 1/2 hours. The gel was stained with Amido Black 10B solution (see appendix II,10) for overnight and destained with 7% acetic acid solution until background was clear.

9.2.2 Cationic system : The cationic system of Reisfield and Lewis and Williams (73) was used to analyze the protease. The reagents and the gel were prepared (see appendix II,8,9). Thirty microlitres of the purified protease (98 µg/ml) was added with 15 µl of 30% glycerol, then 30 µl of this mixture was applied in duplicates. An anode was mounted at the top, a cathode to the bottom of the electrophoretic system. The electrophoresis was run in an

electrode buffer pH 4.5 (see appendix II, 8) with constant current at 10 mA for 2 1/2 hr. After the electrophoresis , one of them was stained with Amido Black 10B (see appendix II,10) and the other was kept for assessing protease activity. In determining the protease from PAGE , the gel was soaked into a 0.3 M phosphate buffer pH 6.0 for 5 min and overlaid on a skim milk plate (see appendix II,2.1) then incubated at 37°C for 2 hours.

9.3 Immunoelectrophoresis (IEP)

IEP was performed in 1.5 % noble agar (see appendix II, 12.3) in sodium barbital buffer pH 8.2 (see appendix II,12.1) at 150 volt for 90 min. After electrophoresis ,a trough was punched and filled with crude protease antiserum (see method 11), then kept overnight in a moist chamber. To stain the precipitin line, the agar was dialyzed against dialysing solution (see appendix II,12.4) overnight then stained with Coomassie blue and destained with destaining solution (see appendix II,12.5) until the background was clear.

10. Estimation of molecular weight

10.1 Molecular weight estimation by Sephadex G-200

Sephadex G-200 column (1.5 x 95 cm) was equilibrated with 0.05 M phosphate buffer pH 7.0 containing 100 mM KCl operating at 4°C. The column was calibrated with blue dextran 2000 ,carbonic

anhydrase, albumin, alcohol dehydrogenase and β-amylase whose molecular weight were 2,000,000, 29,000, 66,000, 150,000 and 200,000 daltons respectively. Each protein marker was applied in the same volume (2 ml). To determine a void volume (V_o) , blue dextran was applied and monitored by a spectrophotometer at 280 nm and measured a volume of effluent collected from the point of sample application to the center of effluent peak. Through the same process, elution volume (V_e) of each marker protein was also determined. A calibration curve was prepared by plotting the logarithms of MW of protein standards versus their respective V_e /V_o values, according to the method of Andrew (75). The V_e of the purified protease was determined in the same buffer and the same volume. The V_e /V_o of the protease was calculated and the molecular weight was determined from the standard curve.

10.2 Molecular weight estimation by SDS-PAGE

Molecular weight of a protein was estimated by the relative mobility according to the method described by Laemmli (76). SDS-PAGE marker proteins (Pharmacia ,Sweden) was applied at the same time as unknown protein (purified protease). The relative molibity (R_f) of the marker proteins: phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inbihitor and alpha-lactalbumin whose MWs were 94,000, 67,000, 43,000, 30,000, 20,000 and 14,000 daltons respectively, was determined from the following formular

$$R_f = \frac{\text{distance of protein migrated from origin}}{\text{distance from origin to marker dye}}$$

The R_f values were plotted against MWs on a semi-logarithmic paper as a standard MW curve. The R_f value of the protease was calculated and estimated for the MW from this standard curve .

11. Preparation of antiserum against protease

A New Zealand white rabbit was immunized with a crude protease by footpad injection. The crude protease containing about 100 ug protein was emulsified with an equal volume of Freund's complete adjuvant (Gibco,U.S.A.) and used for the injection. The second dose was injected at four week later by the same route. The rabbit was bled one week after the second dose and the presence of antibody against protease was checked by single immunodiffusion (Ouchterlony).

12. Determination of optimal pH

Protease preparation was adjusted to the same protease unit, ca. 10 PU with the following buffers: 0.1 M HCl-glycine, pH 3.0 and pH 4.0; 0.1 M sodium acetate buffer, pH 5; 0.1 M phosphate buffer, pH 6.0 to 8.0; 0.1 M Tris-HCl, pH 8.0,9.0 and 0.1 M sodium bicarbonate buffer pH 10.0 (see appendix II,4). Azocasein (0.5%) was prepared and 100 μl of this substrate was mixed with 100 μl of prepared protease sample and the protease activity was determined.

13. **Heat stability**

A purified protease was treated at various temperatures as follows: 50°C,15 min., 60°C, 15 min., 75°C ,15 min. and 100°C for 10 min. After the heat treatment, the protease activity was determined.

14. **Effects of protease inhibitors, metal ions and chelating agents**

Protease inhibitors [PMSF (phenylmethylsulfonyl fluoride) and HMB (para-hydroxymercuribenzoate)] were prepared at the concentration of 2 and 10 mM. In the case of soy bean trypsin inhibitor, the concentration of 1 and 2 mg/ml were prepared. Also, various metal ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, Hg^{2+}, Ba^{2+}, and Cu^{2+},), chelating agents such as EDTA (ethylene diamine tetraacetic acid) and PHE (1-10 phenanthroline) at the concentration of 2 and 10 mM were prepared .

The *P. pseudomallei* protease, ca. 10 PU , was mixed with equal volume (50 µl/ 50 µl) of each inhibitor , metal ion and chelating agent. Then the solutions were incubated in water bath at 37°C for 15 min before adding 100 µl of 0.5 % azocasein to determine the enzyme activity as described above.