CHAPTER V

DISCUSSIONS

In the study for the screening of protease production using skim milk plate test , all 24 clinical isolates and 3 reference NCTC strains of *Pseudomonas pseudomallei* possessed extracellular protease (Fig. 1). The proteolytic activity could be detected by clear zone which was roughly proportional to the amount of existing protease. However, the degree of protease production by each strain could not be distinguished because of the difference in cell number of each colony. This protease seemed to be an extracellular enzyme since the protease activity in the culture supernatant increased as a function of time before bacterial lysis (Fig. 2 and Fig. 3).

The finding of the effect of glycerol on protease production (Fig.3) is consistent with the report (51) that glycerol was important for the production of toxin which was found later to possess proteolytic activity (22). This might be because either the alkaline pH which developed in the medium without glycerol was unfavorable or the glycerol was a requisite metabolite for the protease/toxin production.

P. pseudomallei protease was produced at the highest amount in GBC (glycerol beef extract broth containing calcium ion) medium

(Fig.4). The similar result has been reported in *P. aeruginosa* (77) that calcium ion was required for the production of proteinase and the enzyme contained 1-2 gram atom of calcium per mole of the enzyme. Our result showed that calcium ion did not stimulate the protease activity (Table 6). Hence, *P. pseudomallei* protease might require calcium ion for enhancing protease production.

P. pseudomallei protease production was less when the organism was grown in medium containing 0.5% glucose. In other reports, the synthesis of protease by several bacterial species including *Aeromonas sp.* (78), *Bacillus sp.* (79) and *Vibrio sp.* (80), was repressed by glucose.

The protease production of *P. pseudomallei* was highest in shaking condition at 30° C (Fig.5). This is consistent with the report from Morihara (26) that proteinase production of *P. aeruginosa* was achieved at 20°-30° C after shaking for 3-5 days.

The protease from *P. pseudomallei* was purified by sequential procedures. The specific activity of the final preparation was 17 times that of the crude extract (Table 3). The fraction achieved by 40-70 percent of ammonium sulfate precipitation containing about 80 mg of protein was applied to DEAE-cellulose column. The protease activity was detected in fractions 11-38 and was not detected after elution in a linear gradient of 1 M NaCl (Fig.7).

Eventhough the protease was not adsorbed by DEAE-cellulose, at least other nondesirable proteins were removed. It is one of the method described in Laboratory Manual of Ion Exchange Chromatography, Pharmacia, and has been used in the purification of *P. aeruginosa* protease by Wretlind (61). Various ionic strength and buffer systems have been used ,e.g. 0.01, 0.05 M Tris-HCl buffer pH 8.0 and 0.005, 0.01 M phosphatebuffer pH 7.0. However, it was found that these buffers failed to make the protease adsorb the ion exchanger column (data not shown). In the experiment of Heckly (22), neither toxin nor proteolytic enzyme of *P. pseudomallei* was adsorbed by DEAE and carboxymethyl (CM) cellulose column.

Comparing to the protein contents in the fractions, the protease activity was relatively higher in fractions 20-35 than that in fractions 1-19 (Fig.7). Therefore, pooled fraction numbers 20-35 were used for further purification using Sephadex G-200 and it revealed a single peak (Fig.8). Fraction numbers 31 and 33 had the same protease activity (Fig.8) although they had different absorbance. This may be due to the limited rate of protease diffusion in skim milk plate.

The purification steps in table 3 showed that the relative activity of protease in DEAE-cellulose was higher than that in Sephadex G-200. This result may be because the longer process in purification was a cause of enzyme denatured. Hence, the proteolytic activity was low when compared to the proteolytic activity in DEAE-cellulose column.

Analysis of protease on SDS-PAGE showed only one major band of MW 39,000 daltons and two faint bands of MW 35,000 and 33,000 daltons. The faint protein bands might be contaminated protein since the protease seemed to have no subunit as evaluated by gel filtration. On the other hand, it might be splitted products of protease itself. The molecular weight of protease was about 39KD by SDS-PAGE and 50KD by gel filtration. The molecular weight estimated by gel filtration was higher than by SDS-PAGE. This might be due to the difference in methodology and the configuration of the native protease. However, the molecular weight results implied that the protease was a single-unit protein. Other proteases such as those from *P. aeruginosa*, *V. vulnificus*, and *S. marcescens* possessed the MWs of 33KD, 45KD and 56KD respectively (64,28,27).

P. aeruginosa protease has been reported in different types and numbers (55,61,64). Jensen et.al.(55) suggested that this difference might be due to strains and culture conditions. In our study, only one strain of *P. pseudomallei* was selected to purify its protease. It is interesting to study whether different strains of *P. pseudomallei* produce the same protease or not.

The purified protease failed to migrate to polyacrylamide gel (PAG) (anionic system). It is surprising since most proteins are negatively charged in this alkaline system but *P. pseudomallei* protease is an exception. Analysis of purified protease on cationic PAGE system revealed a single band which possessed protease

activity. This result demonstrated that the native protease possessed a positive net charge, hence the molecule most likely contained many basic amino acid residues. In addition, the isoelectric point of protease should be more than 7.5, since it could be separated only on cationic PAGE. It is thus not surprising that why the protease was unable to adsorb in DEAE or CM column. This result indicated that a considerable purification might be obtained by a cationic exchanger column with a suitable buffer system or isoelectrofocusing. However,the purified protease in this experiment was quite satisfied since it was almost homogeneous in SDS-PAGE. There was only a single band on PAGE and a single precipitin arc against crude protease antisera.

The protease was heat-labile and the optimal pH was 5 therefore it seemed to be an acid protease. In general, an acid protease is described by its acidic optimal pH and is usually produced by fungi, e.g. *Aspergillus sp.*, *Penicillium sp.* and *Mucor sp.* (52). Some fungi such as *Trichophyton rubrum* (81) and *Neurospora crussa* (82) produced alkaline protease. However, a similar result was demonstrated by Heckly (22) that the proteolytic enzyme of *P. pseudomallei* was destroyed by an alkaline reagent, ammoniumhydroxide, but stable in an acidic solution.

P. pseudomallei protease was sensitive to chelating agents such as EDTA and 1-10 phenanthroline, but not to other protease inhibitors. Since chelating agents had effects on the enzyme

activity, this protease was a metalloprotease. Generally, some or all chelating agents inhibit metallo-enzyme, either by removing the metal or by forming a complex with it in situ (83). However, the inhibition of enzyme by chelating agent does not constitute conclusive evident that it is a metallo-enzyme. This can be demonstrated definitely only by metal analyzer (83).

The protease activity was not inhibited by soy bean trypsin inhibitor which was well known that it was an inhibitor of trypsin. Hence, the structure of the active site of enzyme was different from that of trypsin. Furthermore, the proteolytic activity was not inactived by specific -SH-inactivating agent like HMB. Thus, the -SH group was not essential for the enzyme activity of the protease.

The heavy metal ions such as Cu^{2+}, Hg^{2+} inactivated the proteolytic activity. Morihara (52) reported that heavy metal ions such as Ag^{2+}, Cu^{2+}, Pb^{2+} and Fe^{2+} inhibited the proteinase of *P. aeruginosa*. Miyoshi et. al. (28) reported that Cu^{2+} and Hg^{2+} inactivated the protease of *V. vulnificus* and proposed that these metal ions affected enzyme activity by being substituted for elements which took part in enzyme activity.

The purified protease was unable to digest elastin as a substrate but able to digest azocoll indicating a collagenase activity. Heckly and Nigg (22) reported that a crude culture filtrate of *P. pseudomallei* possessed an elastolytic activity and also

collagenase activity. Mandl (84) could not comfirm the collagenase activity against native collagen from the crude filtrate of *P. pseudomallei* sent from Heckly. In his review of bacterial collagenase, only *Clostridium histolyticum* and *Cl. perfringens* type A strains attacked native collagen. In general, if collagenase attacks undenatured native collagens, it also attacks denatured collagen (hide powder, azocoll, gelatin)(84). Therefore, the purified protease showed only collagenase activity against azocoll. However, the collagenase activity of the purified protease should be confirmed by testing with native collagen.

According to Heckly (24,25) the necrotoxicity of *P. pseudomallei* appeared to be a function of the proteolytic activity. However, all attempts to separate enzymatic and dermal necrotic activity had failed. He suggested that the necrotoxin might be associated with the proteolytic activity. Our results showed the isolation of almost homogeneous protease. This protease might be the same as the one associated with necrotoxin of Heckly (24). Thus, the biological activity of the protease from *P. pseudomallei* should be further investigated for necrotic activity by intradermal injection for necrotic lesions (85). This would prove whether the protease is associated with necrotoxin or not.

Conclusion

This thesis described the production, purification and some of the physicochemical properties of an extracellular protease produced by *P. pseudomallei* (strain C2/85). Each of the twenty-seven strains of *P. pseudomallei* produced protease. *P. pseudomallei* was able to produce protease in a medium containing beef extract but better production was achieved in glycerol broth containing calcium ion at 30°C with shaking .

The protease isolation and purification was achieved by precipitation with 40-70% ammonium sulfate, DEAE-cellulose and Sephadex G-200 column chromatography. Analysis of the protease by SDS-PAGE showed almost homogeneity and PAGE(cationic system) showed only one band. One precipitin band appeared on IEP against crude protease antiserum. The isoelectric pH of the protease might be over 7.5 since the mobility could be achieved only in cationic PAGE system. Molecular weight determination of protease by SDS-PAGE and gel filtration showed that the MW was 39KD and 50KD,respectively.

The optimal pH activity was pH 5; however, this protease had activity at wide pH range from pH4-9. No activity was detected in a strong alkaline solution. The protease was a heat-labile protein enzyme, but it remained most activity after incubation at 60°C for 15 min. The protease activity was not affected by trypsin

inhibitor, serine protease inhibitor and thiol-protease inhibitor but was sensitive to chelating agents. The sensitivity of enzyme to EDTA and phenanthroline and its acid optimal pH suggested that this protease was an acid metalloprotease. Metal ions such as Hg^{2+} and Cu^{2+} inhibited the protease activity but Ca^{2+}, Mg^{2+}, Ba^{2+} and Zn^{2+} did not.

According to the procedure of purification and cationic PAGE, it could be assumed that the protease possessed many basic amino acid groups in its molecule. Also, a metal ion was essential for its activity since chelating agents could inhibit its activity. *P. pseudomallei* protease had collagenase but not elastase activity. This biological activity should be an important factor of *P. pseudomallei* infection.