CHAPTER IV

RESULTS

1. Protease produced by *Pseudomonas pseudomallei*

All twenty-seven strains of *Pseudomonas pseudomallei* produced protease on skim milk plate (Fig. 1). Clear zone size was used to determine the amount of protease activity. The protease activities of *P. pseudomallei* in wells 4,5,9,10,13,15 and 22 were relatively higher than those of *P. pseudomallei* in well 2, 11,and 26. *P. pseudomallei* strain C2/85 which was a clinical isolate from a septicemic patient corresponded to well 9, was one of the relatively high protease-producing strain. This strain was selected for the study of protease production and isolation in further experiments.

2. Growth and protease production

Growth was followed turbidimetrically and protease activity was assayed using azocasein as substrate . In GB medium, a lag phase was longer when compared to a lag phase in BB medium (Fig.2 and 3), although protease was detected almost at the same time (at 16 hour). The protease activity was observed from the media in the exponential phase and early stationary phase of growth. Protease activity in BB medium (Fig 2) was high at 48 hours and could not be detected

after 50 hours. On the other hand, protease activity was still detectable until day 7 when the organism was grown GB medium.

3. Effects of media on protease production

Four media (BB, GB, GBG and GBC) were used to determine the protease production at 37°C, with shaking at 180 rpm up to 7 days. The highest protease activity was detected when organism was grown in GBC medium (Fig.4) when compared to those in GB, GBG and BB . The protease activity could be detected up to day 7 in all media except in BB medium. From this result, it could be seen that protease activity in GB medium was higher in medium containing Ca^{2+} but less in medium containing glucose.

4. Optimal condition for protease production

GBC medium was used to observe the optimal condition for protease production. At various conditions as shown in Fig. 5, it was shown that protease activity at 30° C with shaking condition was high from day 2 through day 7. Growths at other conditions (at 37° C, both static and shaking) were less than that of the above condition. Protease activity was also less when the organism was grown at 30° C at static condition.

5. Isolation of protease

5.1 Ammonium sulfate fractionation : Crude protease was precipitated with ammonium sulfate at 0-40%, 40-70% and 70-90% saturation. All precipitates were dissolved and dialyzed against 0.01 M phosphate buffer, pH 7.0 and determined for the protease activity against azocasein. The protease activity in each fraction was shown in table 2. The recovery of protease activity was about 3.5 %, 48.3 % and 2.5 % in 0-40 % , 40-70 % and 70-90 % ammonium sulfate saturation respectively. Therefore, most of *P. pseudomallei* proteases were precipitated by 40-70 percent ammonium sulfate (Table 2 and Fig.6).

5.2 DEAE-cellulose chromatography : Crude protease material from 40-70% ammonium sulfate fraction was separated on DEAE-cellulose column. Fig. 7 showed the result of a typical elution profile and protease activity. The majority of protease was eluted from the column in fraction tube number 9-38 and no protease was adsorbed on this column. However, most of other contaminated materials were separated by adsorption to the column. The protease activity was shown to be high in the second peak. Thus , fractions of number 20-35 were pooled for further purification.

5.3 Sephadex G-200 chromatography : Material from DEAE-cellulose was applied on Sephadex G-200 and typical results was shown in Fig. 8. Only one major peak of protein with protease

activity was eluted. This peak was pooled and used as a purified *P.pseudomallei* protease.

The purification steps were summerized in Table 3. By these procedures,the final recovery was about 1.6% and the relative activity was increased about 17 times.

6. Molecular weight of *P.pseudomallei* protease

6.1 SDS-PAGE determination: The molecular weight of pseudomallei protease was estimated in 10 % gel containing 0.1% SDS. It was found that its MW was about 39 KD (Fig.9).

6.2 Sephadex G-200 determination The protease was estimated for its molecular weight by gel filtration on Sephadex G-200 column. Fig.10 showed that the MW of protease was about 50 KD from its Ve/Vo by using various standard markers.

7. Analysis of *P.pseudomallei* protease

7.1. SDS-PAGE analysis : SDS-PAGE analysis of purified protease revealed one major band of protein at MW 39 KD and two faint bands at MW 35 KD and 33 KD respectively (Fig.11). This result showed that the purified protease retained almost homogeneous.

7.2 PAGE analysis : Anionic PAGE system showed that the purified protease did not migrate in this gel condition (Fig.12). In contrast to the cationic PAGE system, a single band which possessed a protease activity was detected (Fig. 13). This result implied that undenatured *P. pseudomallei* protease had a positive net charge and isoelectric point might be over 7.5. However,no other band of protein could be seen in this cationic PAGE system. This made confidence that purified protease was almost homogeneous.

7.3 Immunoelectrophoresis (IEP) : The purity of *P.pseudomallei* protease preparation was demonstrated by immunoelectrophoretic analysis of the crude and purified protease . After the electrophoresis, rabbit antiserum prepared against the crude protease produced only one precipitin line with the purified protease. In contrast, there were many precipitin lines when the crude protease were used (Fig.14). The protease exhibited a relative high electrophoretic mobility and migrated to the cathode . It is thus implied that the native protease was a positive charge protein.

8. Optimal pH of *P.pseudomallei* protease

P. pseudomallei protease was active over the pH range from 4-9 but exhibited maximal activity at pH 5 (Fig. 15).The wide range of pH for the protease activity was between acid pH to neutral pH. However, the strong protease activity was achieved in an acid pH. This suggested that this protease seemed to be an acid-protease.

9. **Heat stability**

As shown in Table 4, 98-100 % of the protease activity was lost after heating for 10 minutes at 100° C.The activity remained almost all when the protease was heated at 50° C for 15 minutes. Heating for 15 min. at temperature above 60° C slightly affected the protease activity .

10. **Effects of protease inhibitors and chelating agents**

The effects of various protease inhibitors were shown in Table 5. *P. pseudomallei* protease (about 10 PU) was preincubated for 15 min. with each protease inhibitor before assaying the protease activity. It was found that all inhibitors did not affect the protease activity. The protease activity was inhibited by EDTA and 1-10 phenanthroline in a varying degree, depending on the concentration of chelating agents.

11. **Effects of metal ions**

As shown in Table 6, both Hg^{2+} and Cu^{2+} at 5 mM inhibited *P.pseudomallei* protease almost completely . However, 1 mM of Cu^{2+} only slightly affected the enzyme activity. Zn^{2+} at the indicated concentration, was little affected on the protease activity. Other divalent cations such as Ca^{2+} ,Mg^{2+} and Ba^{2+}, at the indicated concentration, did not inhibit the protease activity.

12. **Elastase activity**

P. pseudomallei protease (ca. 10 ,20 and 50 PU) was determined for its ability to degrade elastin congo red . As shown in Fig 16, protease at the highest concentration used (50 PU) was unable to dissolve elastin congo red while a commercial enzyme (elastase type 1: Sigma, ca. 80 ug;7 units/ml) showed elastase activity. It was indicated then, that *P.pseudomallei* protease did not possess elastase activity in the condition tested.

13. **Collagenase activity**

Purified *P. pseudomallei* protease was also tested for collagenase activity using azocoll as substrate. In this procedure, protease could digest azocoll to release an azo dye into the solution, indicating a positive collagenase activity. In comparison, a commercial collagenase type 1A (Sigma), ca. 20 units, was used and showed a positive collagenase activity (Fig. 17).

Table 2 Precipitation of protease from the culture supernatant of *P.pseudomallei* strain C2/85 with ammonium sulfate.

Percentage of saturated $(NH_4)_2SO_4$	Total activity per fraction (PU)	Total protein per fraction (mg)	Specific activity (PU/mg)	Percentage recovery
0	58410	2218	26	-
0-40	2075	26	80	3.5
40-70	28224	77	367	48.3
70-90	1560	12	130	2.7

* Protease unit (PU) was defined as the amount of enzyme that digest azocasein to give an increase 0.1 O.D. at 420 nm.

Table 3 **Purification of *Pseudomonas pseudomallei* protease**

Step	Volume (ml)	Total protein (mg)	Total activity (PU)	Specific activity (PU/mg)	Relative activity	yield (%)
supernatant	990	2217	58410	26	1	100
40-70%$(NH_4)_2SO_4$	24	76.8	28224	367	14	48
DEAE-cellulose	4.8	3.17	1584	499	19	3
Sephadex G-200	1.8	2.16	936	433	17	1.6

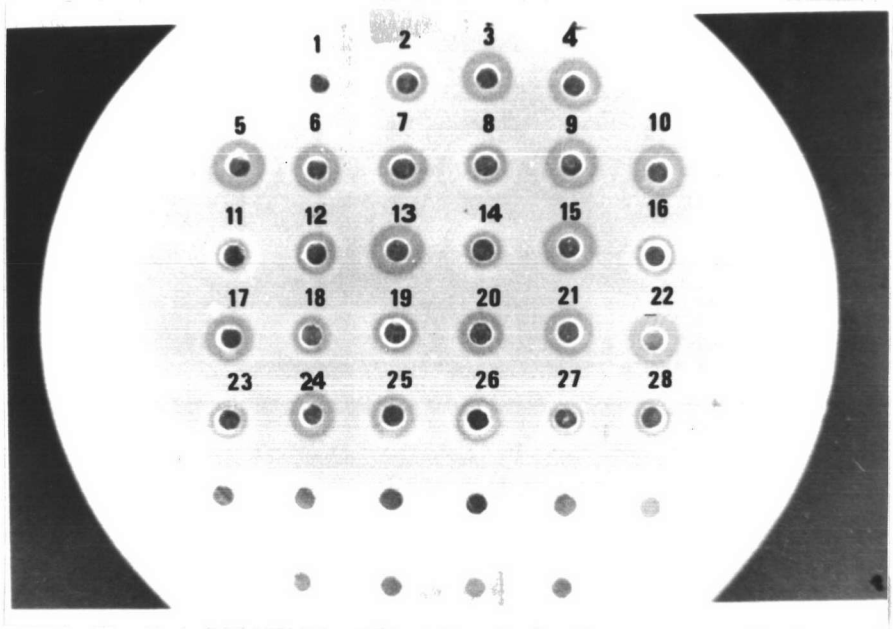


Fig. 1 Protease activity in skim milk agar; well 1, glycerol broth, well 2 to well 28, various culture filtrates of *P. pseudomallei*.

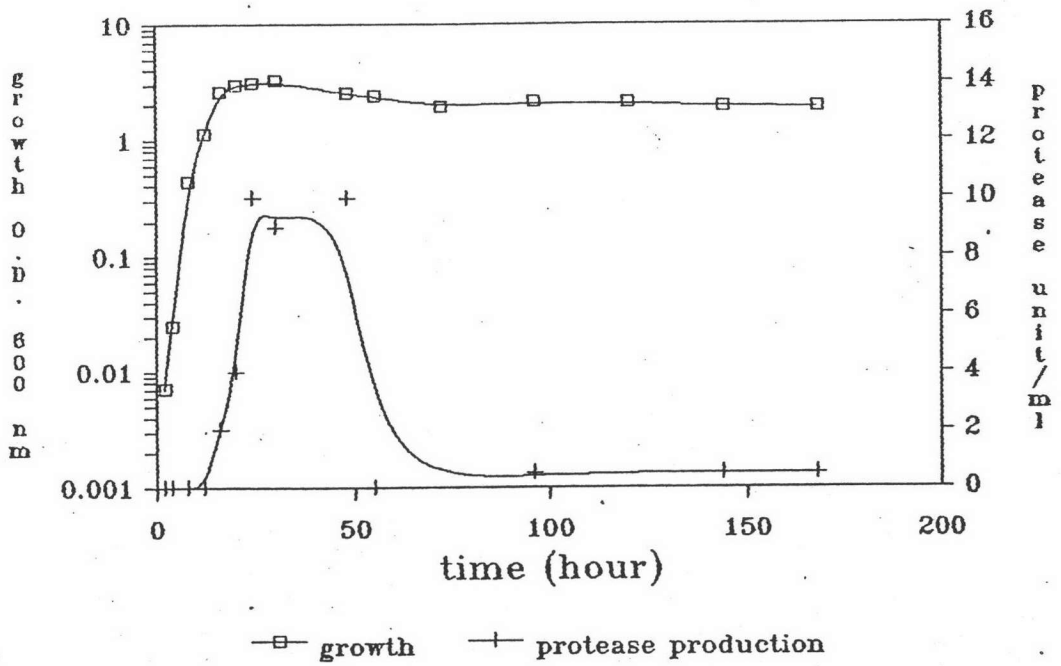


Fig. 2 Growth and protease production by *P. pseudomallei*. Five 500 ml-Erlenmeyer flask containing 200 ml of broth (1% beef extract) were inoculated with seed culture suspension (ca.2 x10^8 cells) and incubated at 37° C on a incubater shaker operating at 180 rpm. Growth was measured turbidimetrically at 600 nm with a spectrophotometer and the culture supernatant fluid obtained after centrifugation was assessed for protease activity.

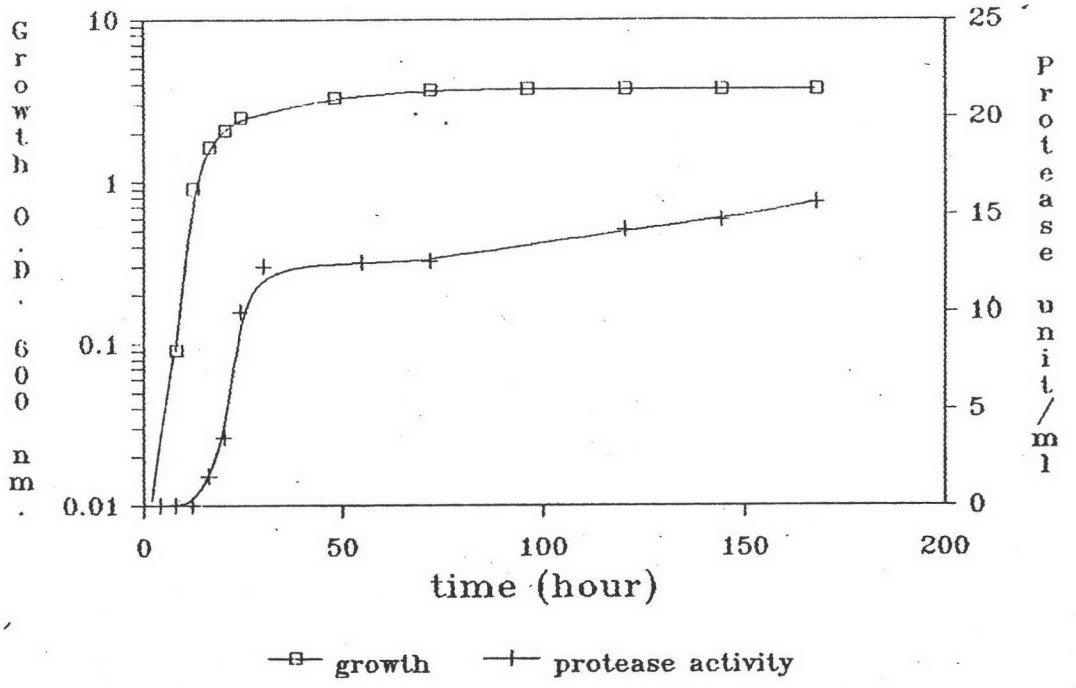


Fig. 3 Growth and protease production by *P. pseudomallei* in glycerol broth. The condition was the same as in Fig. 2 except the medium used.

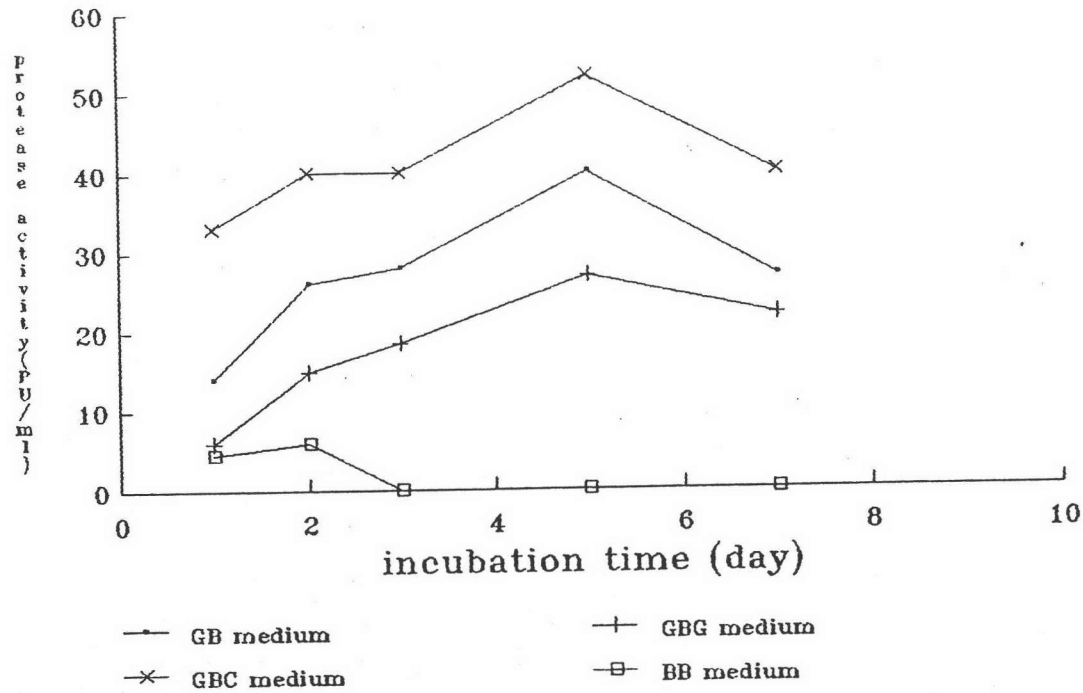


Fig. 4 Protease production by *P. pseudomallei* strain C25/85 in various media: GB, glycerol broth; GBG, glycerol broth containing 0.5% glucose; GBC, glycerol broth containing 0.005 M calcium ions and BB, beef extract broth at 37° C with shaking 180 rpm.

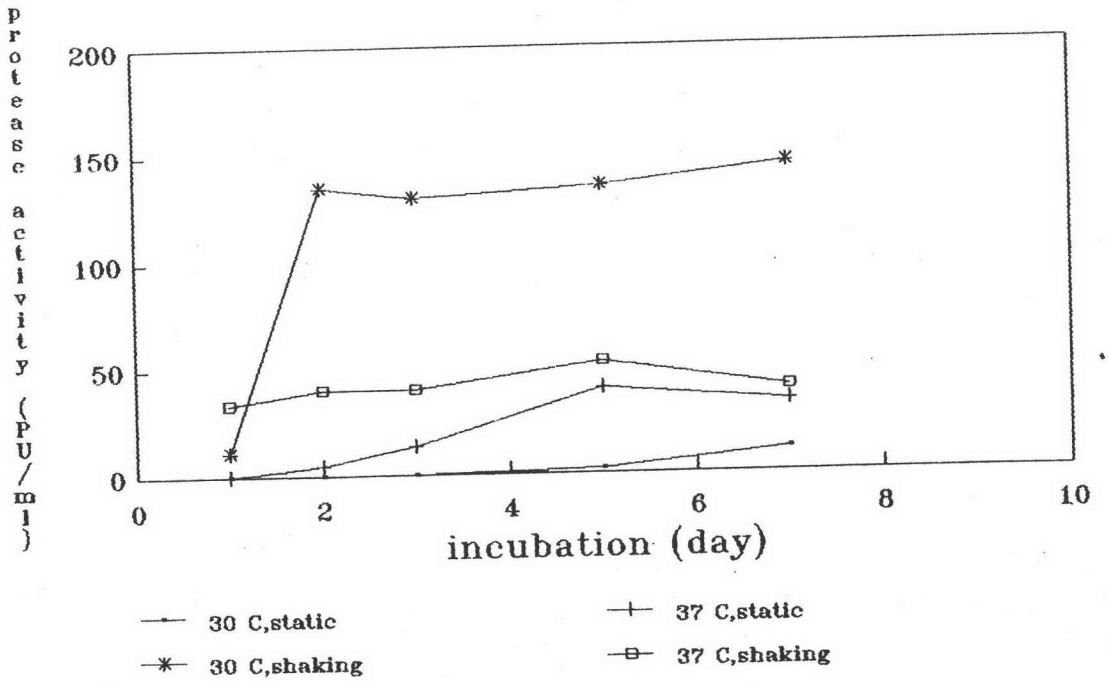


Fig. 5 Protease activity of *P. pseudomallei* strain C2/85 was determined in glycerol broth containing calcium ions (GBC), under various conditions.

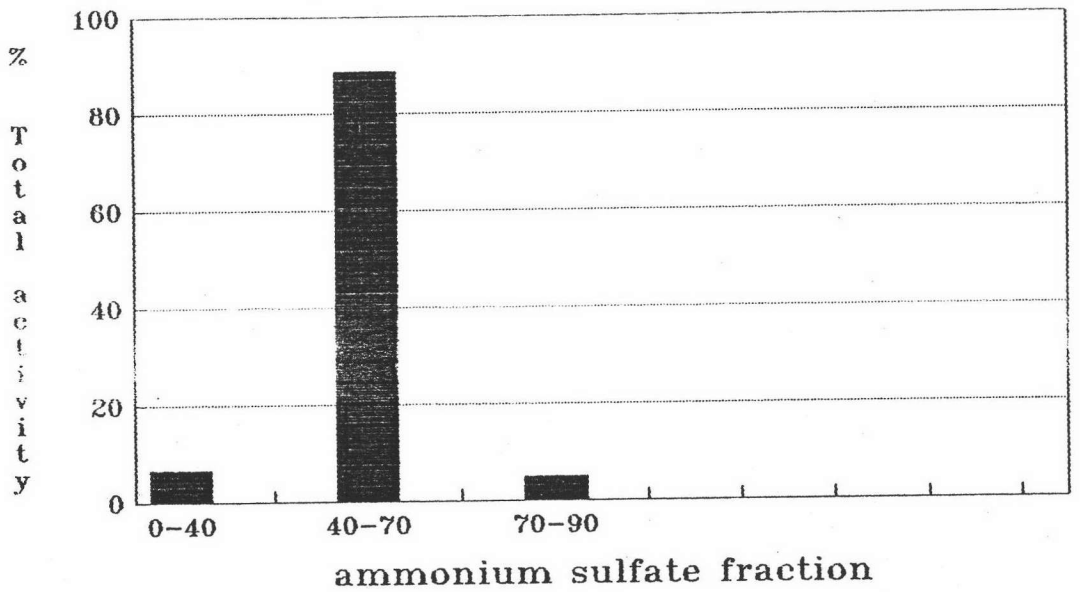


Fig. 6 Percentage of total protease activity in 0-40, 40-70, and 70-90 % ammonium sulfate saturation respectively.

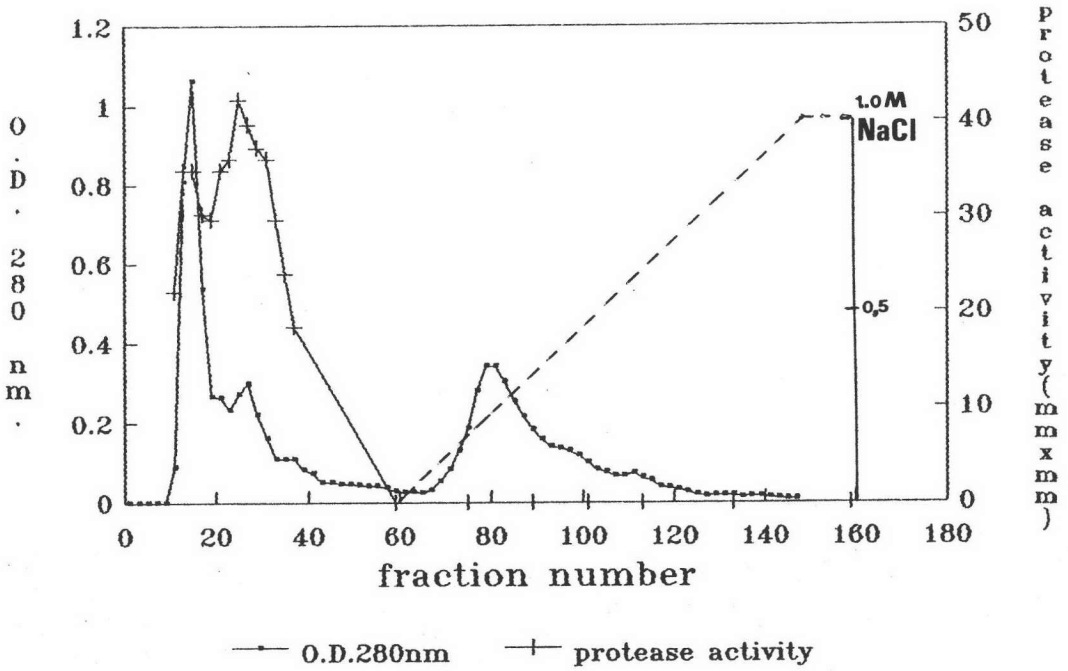
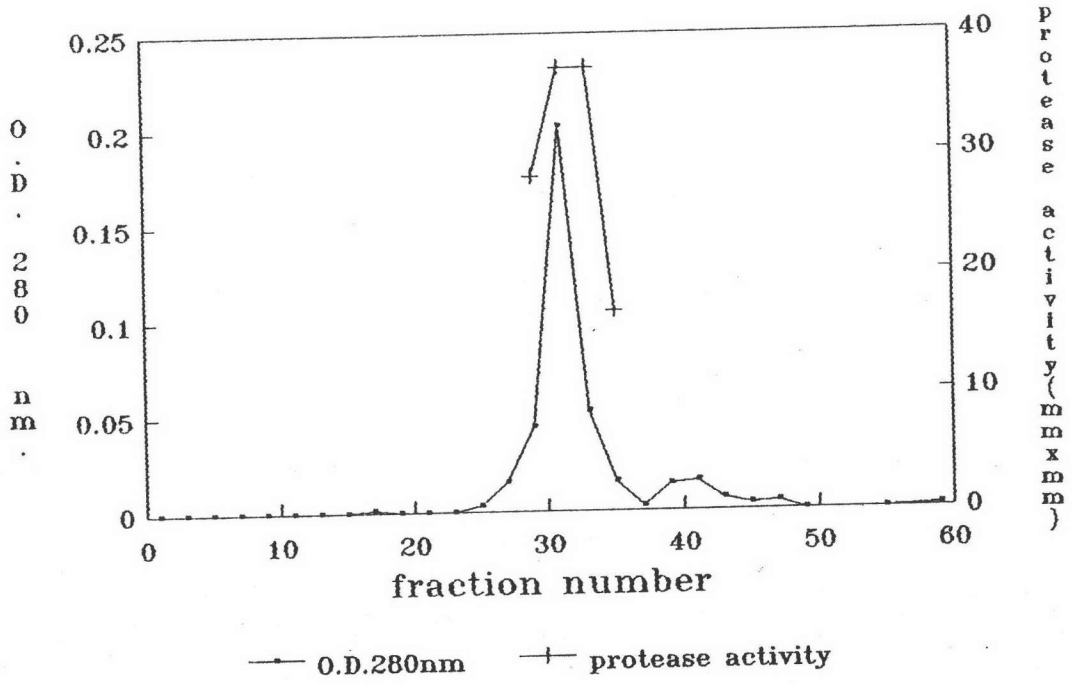


Fig. 7 Typical elution profile of *P. pseudomallei* protease from DEAE-cellulose (DE52) column. The fraction tube no. 20-35 were collected for next step. Protease activity was illustrated as the aquare of clear-zone diameters measured by skim milk plate test.



from DE 52, fraction No.20-35

Fig. 8 Typical elution profile of *P. pseudomallei* protease from Sephadex G-200 column . Protease activity was illustrated as the square of clear-zone diameters measured by skim milk plate test.

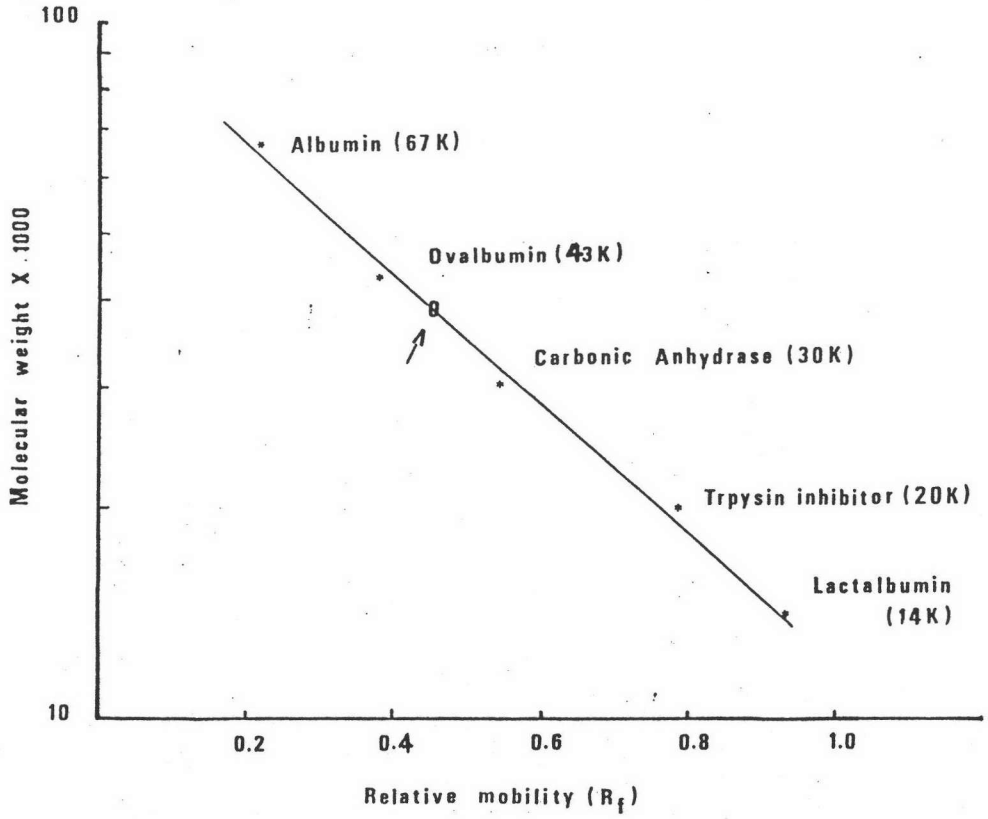


Fig. 9 Estimation of the molecular weight of protease by SDS-PAGE (10% gel). The arrow denoted the position of the protease of *P.pseudomallei*.

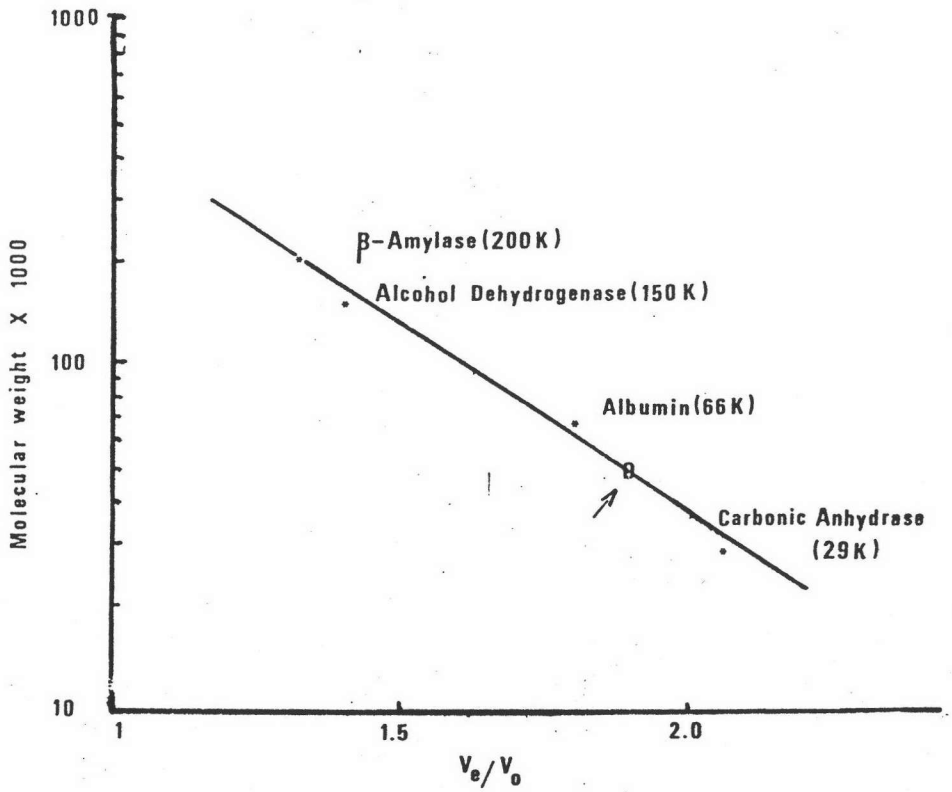


Fig. 10 Determination of the molecular weight of *P. pseudomallei* protease strain C2/85 by Sephadex G-200 column. Standard molecular weights were plotted against their Ve/Vo ratios. Protease was eluted between bovine albumin and carbonic-anhydrase and its calculated molecular weight was about 50 KD.

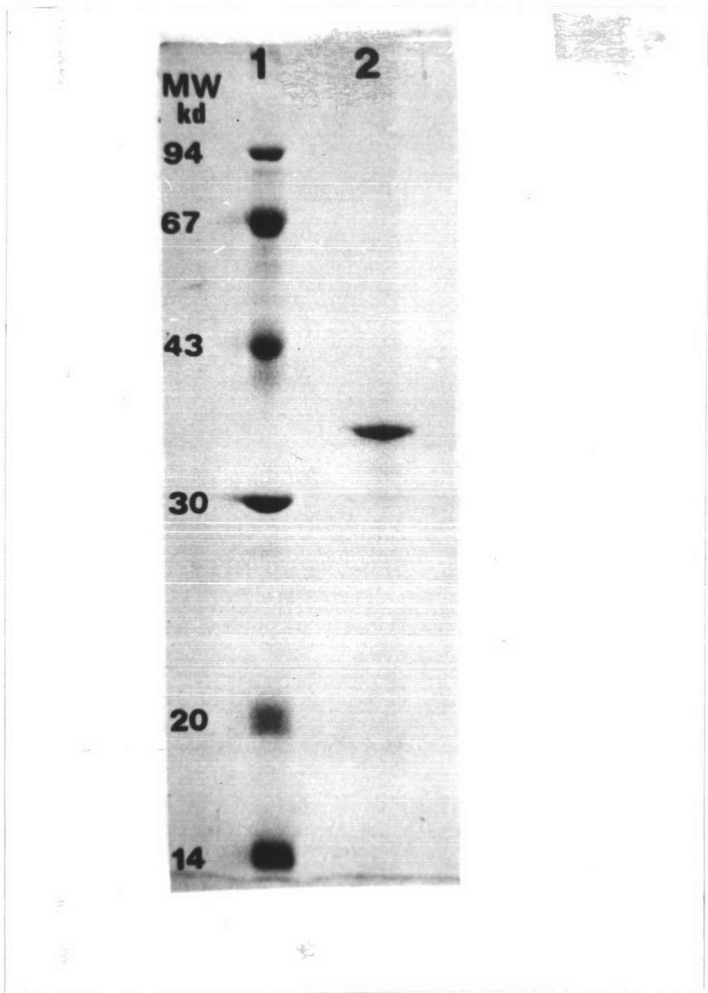


Fig. 11 SDS-PAGE analysis of *P.pseudomallei* protease. Lane:1; molecular weight markers : phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin respectively, Lane 2 ; purified protease from *P.pseudomallei*.

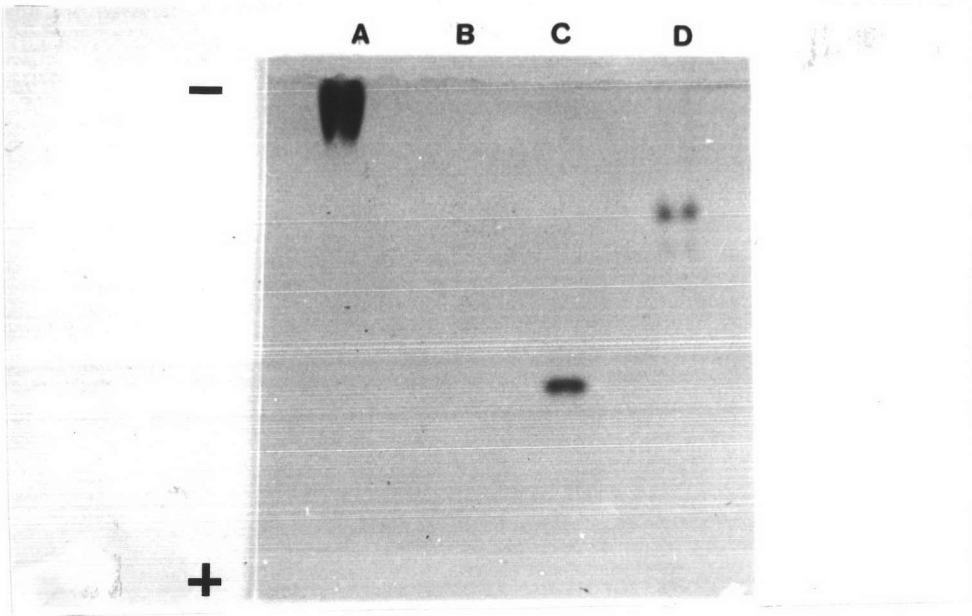


Fig. 12 Anionic PAGE system in 7.5 % gel . Lane A, human IgG; lane B, purified protease (2 μg); lane C, albumin; and lane D, carbonic anhydrase.

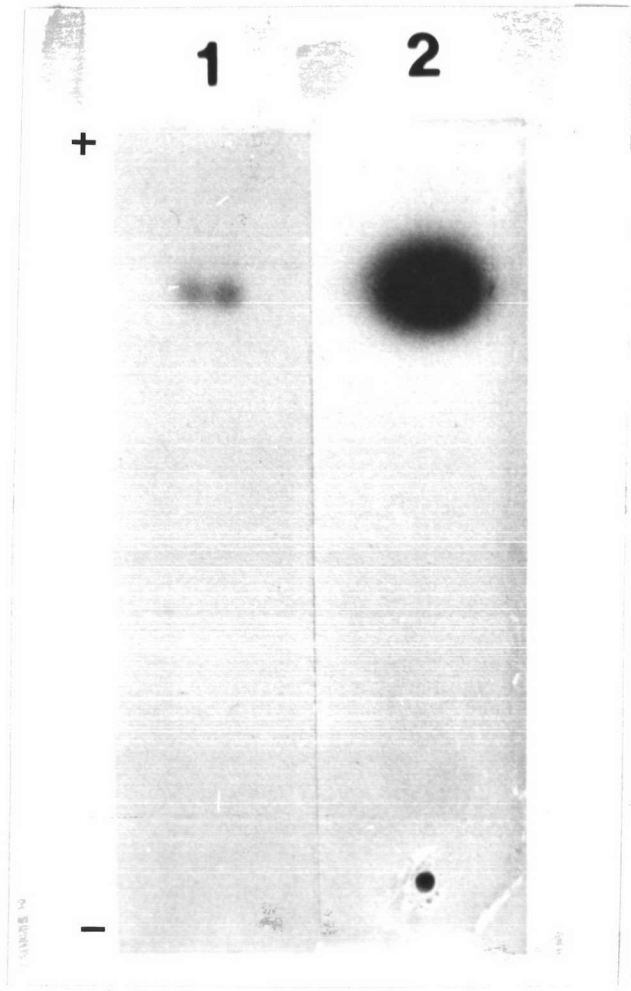


Fig. 13 Slab polyacrylamide gel (cationic system) electrophoresis of purified protease . Lane 1, purified protease (2 µg) Coomassie stained ; lane 2, zone of hydrolysis by gel overlaid on skim milk plate (1.5 % skim milk in 1 % noble agar).

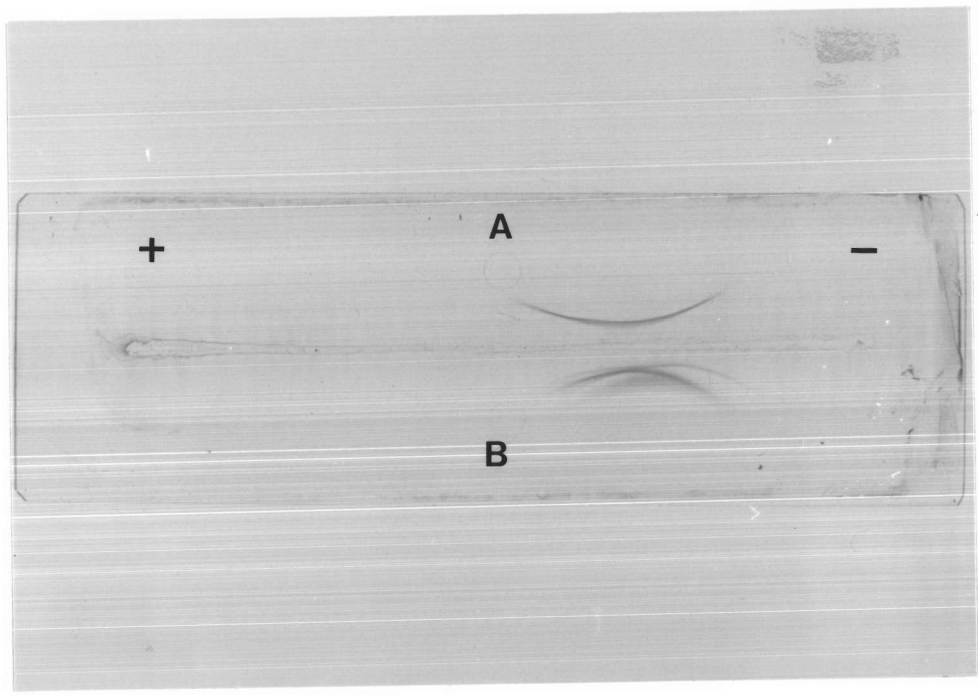


Fig. 14 Immunoelectrophoresis of purified *P.pseudomallei* protease (well A) and a crude protease (well B). The trough contained rabbit antiserum prepared against the crude protease (from 40-70 % ammonium sulfate).

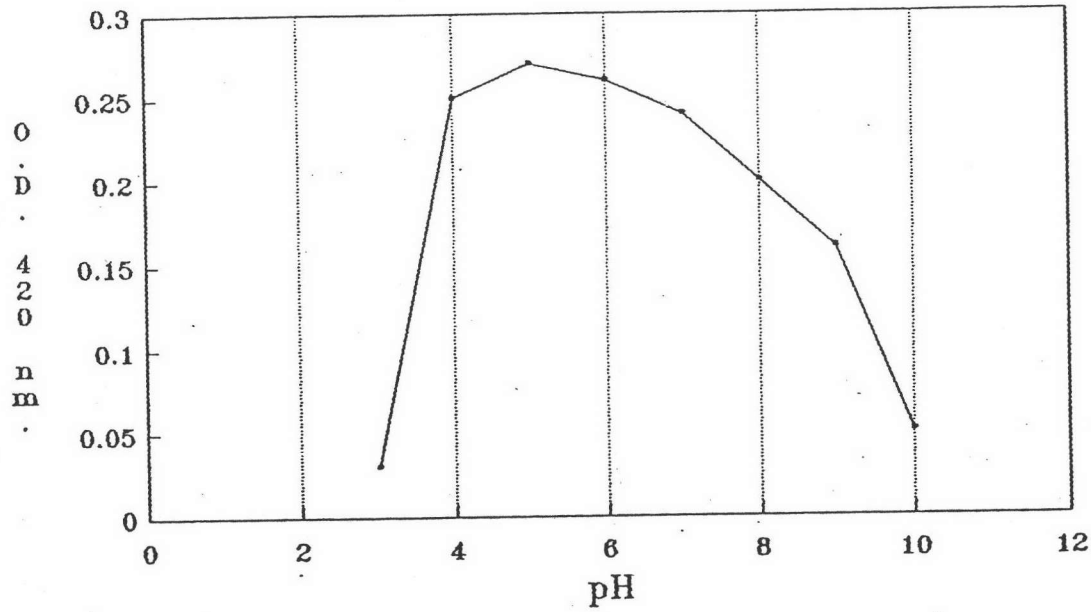


Fig. 15 Effect of pH on protease activity of *P.pseudomallei*. Buffers of HCl-Glycine (0.1M), Acetic acid-sodium acetate (0.1M), Potassium phosphate (0.1M), Tris-HCl (0.1M) and Sodium bicarbonate (0.1M) were used for pHs of 3.0-4.0, 5.0 , 6.0-7.0, 8.0-9.0 and 10.0 respectively.

Table 4. Effect of heat treatment on protease activity of *P. pseudomallei*

Temperature(° C) duration time(min.)	Protease * activity	Activity remaining (%)
unheating	0.301	100
50° C, 15 min.	0.305	100
60° C, 15 min.	0.178	59
75° C, 15 min.	0.043	14
100° C,10 min.	0.008	0

* Absorbance at 420 nm. in azocasein assay.

Table 5 . Effects of chelating agents and protease inhibitors on the activity of purified *P. pseudomallei* protease

Compound [a]	concentration (mM)	activity remaining (%)[b]
EDTA	1	62
	5	8
PHE	1	6
	5	0
PMSF	1	101
	5	106
HMB	1	92
	5	89
Trypsin inhibitor	1 *	104
	0.5	106

* Trypsin soy bean inhibitor 0.5 and 1.0 mg/ml.

a, abbreviation: EDTA = ethylenediamine tetraacetic acid,
PHE = 1-10 phenanthroline,
PMSF = phenylmethylsulfonyl fluoride,
HMB = p-hydroxymercuribenzoate.

b, Ten protease units in the final concentration for 15 min preincubation period.

Table 6 Effects of divalent cations on the activity of a purified protease from *P.pseudomallei* strain C2/85.

Compound	Concentration [a] (mM)	Activity remaining (%) [b]
$CaCl_2$	1	106
	5	103
$MgCl_2$	1	99
	5	104
$ZnCl_2$	1	83
	5	80
$HgCl_2$	1	10
	5	0
$BaCl_2$	1	106
	5	101
$CuCl_2$	1	78
	5	11

a, Concentration given was for the 15 min.preincubation period.

b, Ten protease units in phosphate buffer instead of inhibitor was used as 100 % remaining activity.

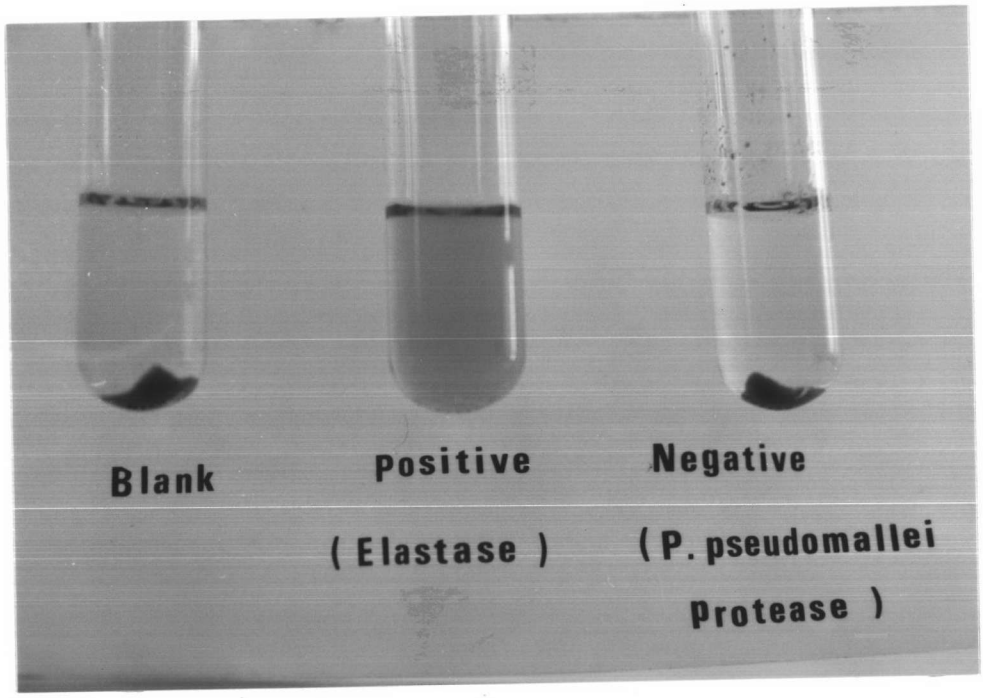


Fig. 16 Elastase activity using elastin congo red as a substrate. *P. pseudomallei* protease was used in concentration of 50 PU in the reaction mixture .

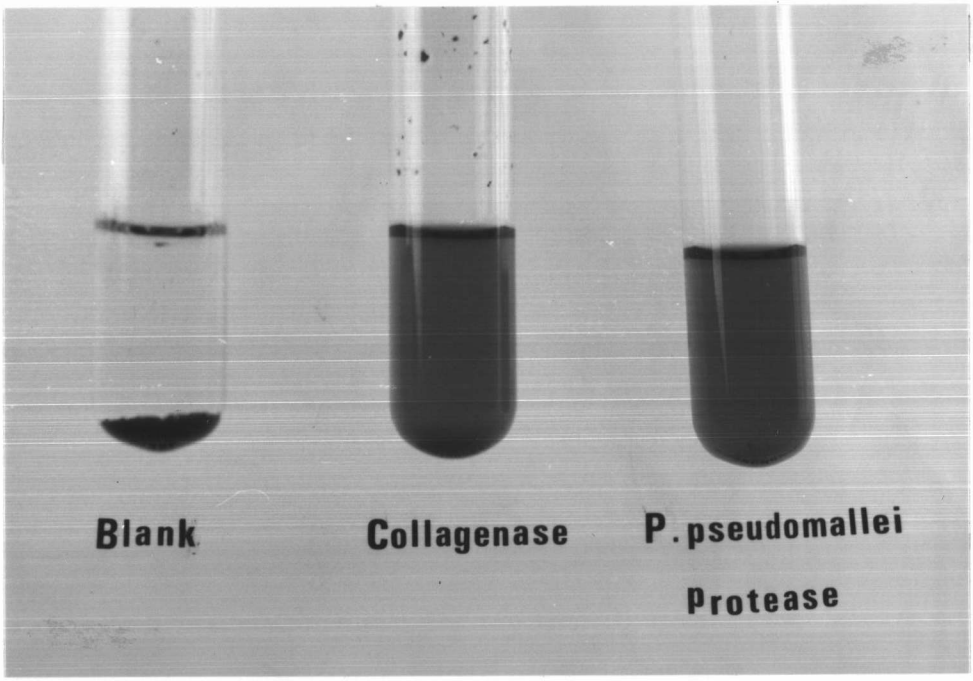


Fig. 17 Collagenase activity using azocoll as a substrate. *P. pseudomallei* protease (10 PU) could digest azocoll, comparing with collagenase type I A (Sigma) as a positive collagenase activity.