SEPARATION OF ANTIGENIC GLYCOPROTEIN FRACTIONS FROM CELL-FREE HOMOGENATE OF *PSEUDOMONAS PSEUDOMALLEI* AND CHARACTERIZATION AS TYROSINEPHOSPHATASE

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Abstract. Cell-free extracts were prepared from *Pseudomonas pseudomallei* cells by freezing-thawing, sonication, and differential ultracentrifugation. The extracts were subjected to column chomatography with DEAE-sepharose to obtain glycoprotein fractions. The fractions showed acid phosphatase activity to p-nitrophenyl phosphate, tyrosine phosphate, serine phosphate, but not to threonine phosphate. They were highly antigenic when tested by immunofluorescence assay with the sera of melioidosis patients.

INTRODUCTION

Pseudomonas pseudomallei is a medically important microorganism in Southeast Asia as the causative agent of melioidosis (Leelarasamee and Punyagupta, 1989). Because of the rather endemic nature of the disease, the bacteriology of *P. pseudomallei* has been conducted mainly by local scientists on a limited scale. *P. pseudomallei* constitutes a particular group of *Pseudomonas* species together with *P. mallei* and *P. cepacia* (Gilardi, 1985; Moss, 1990). We have confirmed this fact from fatty acid analysis by gas liquid chromatography and the high acid phosphatase activity with characteristic pH activity pattern and heat-resistance (Dejsirilert *et al,* 1989; Kondo *et al,* 1991a).

In the course of our study on acid phosphatase activity of *Pseudomonas pseudomallei,* we came to an observation that the enzymes were excreted into the culture filtrate and the enzymatic fractions were obtained by ammonium sulfate precipitation, gel-filtration with Sephadex G-75, and column chromatography with DEAE-cellulose. The fractions reacted to the sera from melioidosis patients in gel-diffusion precipitation assay. A remarkable finding was that the phoshatase activity was highest with phosphotyrosine and phosphoserine as substrates (Kanai and Kondo, 1991).

As the extension of this study, an attempt was made to demonstrate such enzymatic fractions from the cells of *P. pseudomallei* and to characterize them.

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MATERIALS AND METHODS

Microorganisms

A local strain (UB 26) of *P. pseudomallei* was employed in this study. They had been isolated from melioidosis patients admitted in Sappasitthiprasong Ubon Hospital and identified on the basis of growth on the selective medium, peculiar order, characteristic colony morphology, and various biochemical tests including oxidase, fatty acid pattern of gas-liquid chromatography (GLC), and pH activity pattern of acid phosphatase (Kondo *et al,* 1991a). The further confirmation was made by immunofluorescence assay using antibodies against the protein fraction and endotoxin of *P. pseudomallei* (Petkanchanapong *et al,* 1992).

Culture medium and cultivation of microorganisms

Tryptone glucose extract (TGE) agar was employed to cultivate the strains. The medium was dispensed into plastic plates of 9 cm diameter. The overnight growth at 37°C on 100 plates was harvested to be suspended into 100 ml of 0.01 M EDTA solution in 0.1 M tris buffer pH 7.2.

Preparation of cell-free extract

The harvested cell suspension was subjected to 15 cycles of freezing and thawing in the temperature

range between –20°C and 35°C. Thus treated suspension was then sonicated for 3 minutes to promote cell disruption.

Mechanical fractionation of the extract

The extract obtained as above was centrifuged at 2,000 rpm for 10 minutes. The resulting supernatant was then subjected to recentrifugation at 5,000 rpm for 20 minutes. The supernatant obtained was preserved and the precipitate was washed with 30 ml of Tris buffer by centrifugation at 5,000 rpm for 20 minutes. The washings were combined with the preserved supernatant. The pooled supernatant was centrifuged at 12,000 rpm for 30 minutes. The resulting precipate was resuspended in 20 ml of water and washed by centrifugation at the same condition as before. The supernatant was subjected to ultracentrifugation at 100,000*g* for 60 minutes to yield precipitate and supernatant. The precipitate was preserved and the supernatant was ready for fractionation by column chromatography with DEAE-sepharose. All these procedures were conducted in a cooled environment (4–7°C). The precipitates obtained sequentially were designated as fractions A, B, C, D and the final supernatant as fraction E (Fig 1).

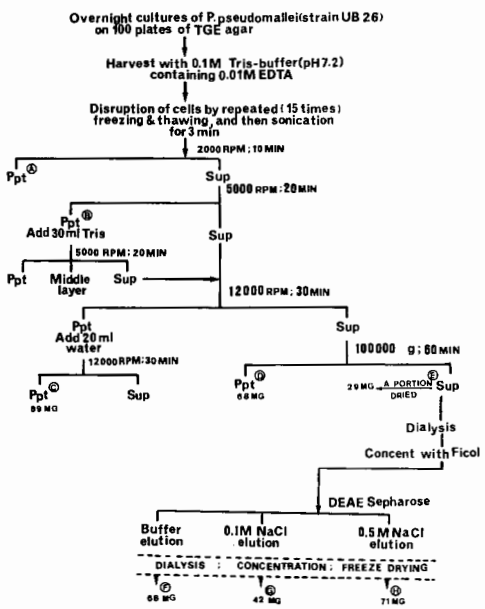

Fig 1–Flow chart of mechanical fractionation of *P. pseudomallei* cells.

Fractionation of the final supernatant

The final supernatant (fraction E) was dialysed against water at 4°C for 3 days, and then concentrated to a volume of 28 ml with Ficol (Sigma Chemical Co). A 3 ml portion of this material was set by and lyophilized. The remaining portion was divided into equal halves, and each portion was fractionated by column chromatography with DEAE-sepharose (1.5 × 10 cm). Before use, DEAE-sepharose (Pharmacia, Sweden) was equilibrated with 0.025 M Tris HCl buffer pH 7.4. Elution was made stepwise first with the above buffer, then 0.1 M NaCl, and finally with 0.5 M NaCl, each time using 90 ml. The eluate fraction size was 4 ml. Each eluate fraction was assayed for protein and sugar levels.

Lipid analysis

To get partial evidence that fraction D is a membrane fraction, lipid analysis was conducted aiming at detection of phospholipids. 8.4 mg of Fraction D was extracted with 2 ml of chloroform-methanol (2 : 1) and left to stand overnight at room temperature. The soluble fraction was separated and washed with 0.5 ml of a 1% solution of KCl by centrifugation at 3,000 rpm for 20 minutes. The lower layer was separated and again washed with 0.5 ml of 50% methanolic solution of 0.5% KCl according to Folch *et al* (1957).

The lower layer here obtained was separated and evaporated to dryness with a rotary evaporator. The residue of 2 mg was obtained, and dissolved in 100 µl of chloroform-methanol (2 : 1). An aliquot of this solution (25 µl) was subjected to thin-layer chromatography (TLC) with 5 × 20 cm plates of Silica Gel G (Merck, Darmstadt) which had been activated 110° C for 60 minutes before use. The plates were developed with the solvent system: chloroform-methanol-water-acetic acid (50 : 25 : 4 : 8). As reference materials, 200 µg of phosphatidyl ethanolamine dipalmitoyl and phosphatidyl glycerol dipalmitoyl (Sigma, St Louis, USA) were employed by dissolving in 10 µl of chloroform-methanol (2 : 1). The presence of spots was detected by iodine vaper, then spraying with 0.2% ninhydrin solution in ethanol and heating at 110°C .

Determination of protein and sugar contents

Protein levels were determined by the method of

Lowry *et al* (1951) or with Bio Rad protein assay reagent (Bio Rad Chemical Division, Richmond, CA, USA). Bovine serum albumin (fraction V, Sigma) was used as a protein standard. Sugar determination was made by the phenol-H_2SO_4 method as follows. A sample of 20 µl was placed in a test tube of 1.2 × 7 cm (Corning) with 780 µl of water, and added with 20 µl of 80% phenol. This mixture was then treated with 2 ml of concentrated sulfuric acid. After the temperature went down to 25-30°C, the developed color was read with a Coleman spectrophotometer set at 490 nm. Glucose was used as a sugar standard.

Phosphatase assay

Non specific phosphatase activity was assayed in a reaction mixture consisting of 0.8 ml of buffer, 0.1 ml of the enzyme sample, and 0.1 ml of p-nitrophenyl phosphatase solution (0.2%). For pH range 3.19 to 6.22, acetate buffer solutions were prepared at 0.1 M. For pH range higher than 6.5, 0.1 M Tris-HCl beffer solutions were employed.

The reaction mixture was incubated in a water bath at 40°C for 30 minutes. To stop the reaction and develop the yellow color of released p-nitrophenol, 1.0 ml of 0.5 M NaOH was used. After addition of one ml distilled water the reaction mixture was subjected to colorimetric determination with a Coleman spectrophotometer set at 420 nm. To the blank tube NaOH solution was added at zero time.

Phosphatase activity to aminophosphate substrates was determined with 10m M solutions of phospho-L-serine, phospho-L-threonine and phospho-L-tyrosine. These chemicals were obtained from Sigma Chemical Co (St Louis, MO, USA). The reaction mixture consisted of 0.1 ml of the substrate solution, 0.1 ml of enzyme sample and 0.4 ml of 0.1 M sodium acetate buffer of pH 5.8. After incubation at 37°C for 60 minutes, the amount of released phosphate was determined according to the colorimetric method of King (1932) with a Coleman spectrophotometer set at 685 nm.

Immunofluorescence assay (IFA) for antigenicity

Ninety microliters of each fraction dissolved or suspended in 2 mg/ml of 0.1 M Tris-saline buffer was mixed with 10 µl of latex beads (Sigma LB-8, average diameter 0.8 mm). After shaking for 15 minutes, the mixture was centrifuged at 4,000 rpm

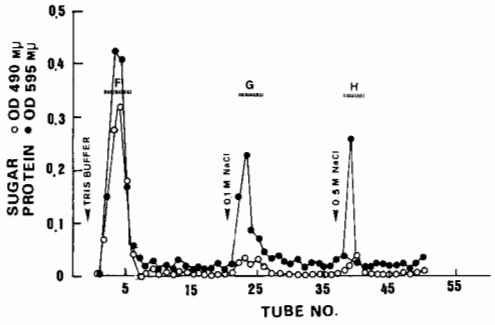

Fig 2–Column chromatography with Sephadex G-75 of 100,000 g/60 minutes supernatant (Fraction E in Fig 1).

for 20 minutes. The sediment was resuspended in 2 ml of the above buffer and employed for IFA.

As animal antisera, two samples were supplied by courtesy of Dr T Kurata, National Institute of Health, Tokyo, Japan. One was rabbit polyclonal antibodies against an endotoxin preparation of *P. pseudomallei* and another was mouse monoclonal antibodies against protein fractions purified from the culture filtrate.

As the human antisera, two samples (No. 134, No. 137) each from a melioidosis patient were provided from Sappasitthiprasong Ubon Hospital by courtesy of Dr Vipada Chaowagul.

RESULTS

Fig 1 shows the flow chart of fractionation of *P. pseudomallei* cells harvested from the overnight cultures on 100 plates of TGE agar. For disruption of the cells freezing and thawing was employed, since this is the better way to avoid the risk of aerosol than other methods. After 15 cycles of the operation, culture test detected no viable cells in the treated material. Then, it was sonicated for 3 minutes to improve the disruption and dispersion.

The sonicate was subjected to differential centrifigation to get cell components sequentially, and the final supernatant was ultracentrifugted at 100,000*g* for 60 minutes. The supernatant here obtained as a soluble component of *P. pseudomallei* cells was dialized, concentrated with Ficol and loaded on a column of DEAE-sepharose (1.5 × 10 cm).

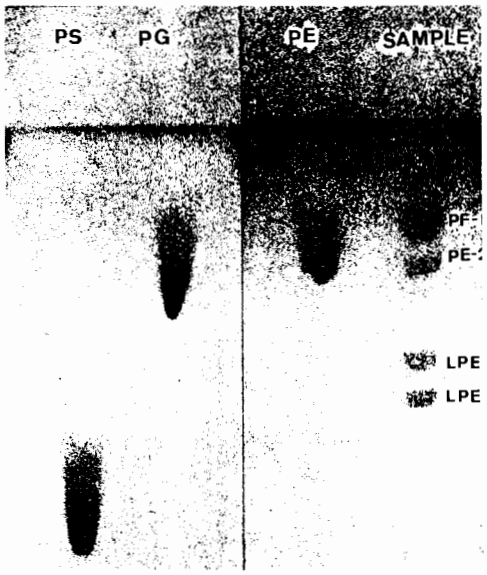

Fig 3–Detection of phospholipids in fraction D by thin-layer chromatography (TLD). The presence of lipid spots were visualized by iodine vapor as in this figure. Later, their identification as first-class amines was made by spraying 0.2% ninhydrin solution in ethanol and heating at 110 C : 10 minutes. PS : phosphatidyl serine; PG : phosphatidyl glycerol; PE : phosphatidyl ethanolamine; LPE : lysophosphatidyl ethanolamine.

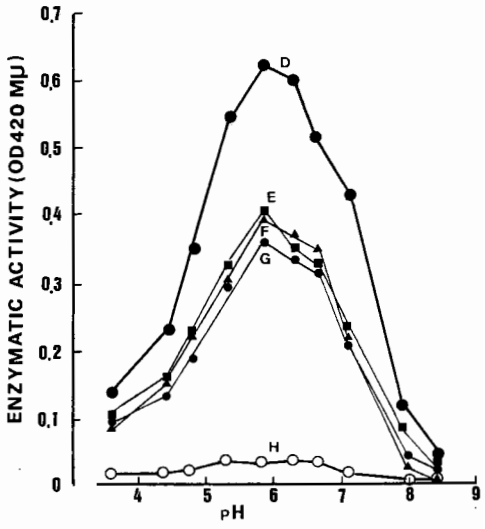

Fig 4–Comparative nonspecific acid phosphatase activity of the fractions (D, E, F, G, H in Fig 1) of *P. pseudomallei* by the assay with p-nitrophenyl phosphate as substrate.

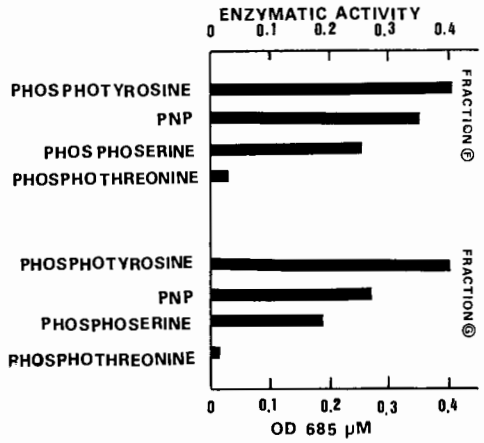

Fig 5–Specific acid phosphatase activity of fraction F and G in the presence of aminophosphates as substrate.

Elution was conducted sequentially with the Tris buffer, 0.1 M NaCl and 0.5 M NaCl. The protein and sugar determination of each eluate revealed three distinct peaks as shown in Fig 2.

They were protein fractions accompanied with the presence of sugar. The eluates of each peak were pooled, dialysed against water, concentrated with Ficol, and lyophilized. These three fractions were designated as F, G, and H in the eluated order, their yield being 68 mg, 42 mg, and 71 mg, respectively.

Fraction D was seemingly a membrane fraction in this fractionation scheme. This was confirmed by lipid analysis to demonstrate 2 ninhydrin-positive phospholipid spots with near Rf value as phosphatidyl ethanolamines and possibly their corresponding 2 spots of lysomolecules with the lower Rf value. They may be the molecular species different in constituent fatty acids (Fig 3).

Fractions F, G, and H, together with their starting material (fraction E) and fraction D (100,000*g* precipitate : a membrane fraction), were tested for their comparative acid phosphatase activity employing the same amount (50 mg). p-Nitrophenyl phosphate was used as substrate. The results are shown in Fig 4. As is clear in this figure, fraction D revealed the strongest reaction, and fractions E, F, and G were almost the same in the reaction intensity. Concerning their pH-activity patterns, however, there was no difference among them. Meanwhile, fraction H showed no acti-

Table 1

A summary of the properties of DEAE-sepharose fractions.

Fraction	Yield (mg)	Protein (μg) per 100 μg	Sugar (μg) per 100 μg	Intensity* in IFA test with antisera against LPS	GP	patient No.134	sera No. 137	** Acid phosphatase activity (OD_{420}mμ) at pH 5.8 in the presence of Antisera against LPS	GP	Control (no antiserum)
D	68	20.5	7.5	++++	++++	++++	++++	0.73	0.82	0.8
F	68	19.0	27.5	++++	++++	++++	++++	0.52	0.50	0.49
G	42	22.5	27.5	+++	++	++++	++++	0.43	0.45	0.45
H	71	18.5	30.0	±	-	++++	++++			No activity

Graded by arbitrary scale from (-) to (++++)
** 50 mg of each fraction (D, F,G) was exposed to 50 ml of antiserum.
LPS : lipopolsaccharide preparation, GP : glycoprotein fraction from culture filtrate (10)

vity in any pH environments employed here.

p-Nitrophenyl phosphate is for screening purpose to detect nonspecifically acid phosphatase activity. In view of our previous finding that *P. pseudomallei* has a specific acid phosphatase activity on some aminophosphate substrates (Kanai and Kondo, 1991), an attempt was made to examine the enzymatic activity of fraction F and G with phosphotyrosine, phosphoserine, and phosphothreonine as substrates. Each fractions was used in the amount of 70 mg and the assay was conducted at pH 5.8. The results are shown in Fig 5. Though phosphothreonine was not effective as the substrate at all, the other two aminophosphates were highly effective. Especially, the activity with phosphotyrosine was stronger than that with p-nitrophenylphosphate in both fraction F and G.

Finally, the antigenicity of fractions D, F, G, and H was examined by immunofluorescence assay (IFA) with the sera of immunized animals and of bacteriologically confirmed melioidosis patients. The patient sera, No. 134 and No. 137, had high titers against the whole cell of *P. pseudomallei,* the former being>1 : 4,096 and the latter 1 : 512. The animal sera were the rabbit antisera to lipopolysaccharide preparation of the bacilli and the mouse sera to a glycoprotein fraction obtained from the culture filtrate (Kondo *et al,* 1991b).

All of the four fractions gave a most strong positive reaction to both patient sera. To the animal sera, however, only fraction D and F reacted strongly. Fraction G was moderate in intensity and fraction H without the enzymatic activity did not react at all.

Then, an attempt was made to examine whether or not the animal antisera may inhibit the enzymatic activity of fractions D, F, and G. The fractions treated by incubation with the sera were assayed for their p-nitrophenyl phosphatase activity at pH 5.8. However, no inhibition was observed in the employed conditions. These results are shown in Table 1.

DISCUSSION

We have previously demonstrated the presence of glycoproteins with acid phosphatase activity in the culture filtrate of *P. pseudomallei.* The glycoproteins were separated by ammonium sulfate precipitation, gel-filtration with Sephadex G-75, and column chromatography with DEAE-cellulose. They reacted to the sera from melioidosis patient in gel-diffusion precipitation assay (Kondo *et al,* 1991b).

The acid phosphatase activity was then examined with various substrates. Interesting was an observation that tyrosine phosphate and serine phosphate were the most effective substrates. An assumption

was made from this finding that the glycoproteins may be cell-surface receptor-like enzymes such as protein tyrosine phosphatase which plays a signal transfer role in response to environmental stimuli (Kanai and Kondo, 1991).

The present study confirmed the presence of glycoproteins similar to the above and suggests that they are synthesized within the cell and excreted into the environmental medium after they are translocated onto the cell-surface.

Our fractionation results did not mean, however, that the acid phosphatase as glycoproteins was purified as a single molecule. On the contrary, it appears more probable that there are the enzymes of various molecular weight due to the different extent of glycosylation. Another line of study is now going on to analyse this aspect in our laboratory.

* All the fractions obtained in the present fractionation reacted strongly with the sera of melioidosis patients in IFA. However, when the animal antisera against the glycoprotein fraction from the culture filtrate or LPS preparation were employed, their antigenicity was revealed differently from one fraction to another. Especially, fraction H, with no enzymatic activity, did not give positive reactions to both antisera in IFA. This observation suggests that fraction H may be a cytoplasmic component which is not excreted into the outside environment or does not have antigenicity common with that of antigens obtained from the culture filtrate. At the same time, it supports an idea that the acid phosphatase molecules are an important part of the antigenicity of *P. pseudomallei,* and that fraction H may represent nonfunctional or premature molecules of acid phosphatase, still or a completely different protein component.

One puzzling observation was that fractions D, F, and G crossreacted with both antisera against LPS and the glycoproteins of the culture filtrate. There would be two possibilities: one is LPS contamination of the glycoprotein fractions another is common antigenicity between the polysaccharide moiety of LPS and the glycoproteins. The latter possibility could not be excluded in view of a similar example shown by Menz *et al* (1991) that lipophosphogly can and a secreted glycoprotein acid phosphatase of the promatigotes of *Leishmania mexicana* share common antigenicity.

Our attempt to inhibit the acid phosphatase activity of the separated fractions by treatment with animal antisera was not successful, at least under the conditions employed. No conclusion could be derived from this negative result, however, until we repeat such experiment with high titer antibodies.

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