EVOLUTION OF CELL-SURFACE ACID PHOSPHATASE OF *BURKHOLDERIA PSEUDOMALLEI*

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Abstract. Acid phosphatase active fractions were obtained from cell-free extract, outermembrane fraction and culture filtrate of *Burkholderia pseudomallei* by column chromatography with sepharose 6B and DEAE cellulose. The comparison of the elution patterns of protein, sugar and enzymatic activity among these three components suggested that the enzyme is a glycoprotein evolving from premature proteins through glycosylation and that the enzyme is translocated during glycosylation from the cytoplasm to the outer membrane and finally excreted into the environment. When tunicamycin, a glycosylation inhibitor, was added to the culture, the peaks of sugar and enzymatic activity were lowered concomitantly leaving the protein peak unchanged in the elution pattern of the culture filtrate. The affinity of the bacterial surface to antienzyme sera was demonstrated by immuno-fluorescence microscopy.

INTRODUCTION

In our previous study on acid phosphatase activity of *Burkholderia pseudomallei*, a complex nature of the enzymatic activity was revealed in pH-activity pattern, heat-stability, and membrane association (Kondo *et al*, 1991a; Dejsirilert *et al*, 1989). Further studies showed that the activity was concentrated in the antigenic glycoprotein fraction obtained from culture filtrate. The fraction reacted to the sera from melioidosis patients (Kondo *et al*, 1991b, 1994a). Interesting was an observation that tyrosine phosphate was the most effective substrate, producing even higher reaction than the commonly used screening substrate, p-nitrophenyl phosphate (Kanai and Kondo, 1991; Kondo *et al*, 1994a).

As for the complex nature of the enzymatic activity of the whole cells, we postulated two possibilities, the presence of multiple acid phosphatases or the developmental stages of a predominant single enzyme. Our preliminary study on this question was made by examining the effects of tunicamycin, a glycosylation inhibitor, on the enzymatic activity of whole *B. pseudomallei* cells. The results suggested strongly that the latter possibility is more acceptable (Kondo *et al*, 1994b).

In the present report, we present the data comparing the fractionation patterns of the enzymatic activities among cell-free extract, triton-solubilized membrane fraction, and culture filtrate. By following the elution feature of column chromatography in regard to protein, sugar and enzymatic activity, evolution and translocation of the glycoprotein acid phosphatase were suggested. Tunicamycin experiments and immunofluorescence microscopy with antienzyme sera gave the additional support to this view.

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MATERIALS AND METHODS

Microorganisms

Local strains (UB12, UB16, UB18, UB19, UB20, UB26) of *B. pseudomallei* were employed in this study. They had been isolated from melioidosis patients admitted in Sappasitthiprasong Ubon Hospital and identified on the basis of growth on 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*, 1991c). The further confirmation was made by immunofluorescence assay using antibodies against the protein fraction and endotoxin of *B. pseudomallei* (Naigowit *et al*, 1993; Petkanchanapong *et al*, 1992).

Culture medium and cultivation of micro-organisms

The stock culture has been maintained on stock culture soft agar (DIFCO) at room temperature or in 10% skim milk at -20° to -70°C. For the purpose of obtaining the culture filtrate, tryptone soy broth (TSB) was employed. Meanwhile, tryptone glucose extract (TGE) agar was preferably used to harvest a massive amount of cells. In this case, one hundred plates of 9 cm diameter dispensed with TGE agar were usually arranged. In the experiments to see the effects of tunicamycin, Mueller-Hinton (MH) liquid medium was our choice.

Culture filtrate

To harvest the culture filtrate for analytical fractionation, tripton soy broth (TSB) was employed. Six bottles each dispensed with 100 ml of TSB were inoculated with UB26 strain and incubated at 37°C for 2 days. Sterilization of these cultures was made by adding 1.5 ml of 37% formaline solution to each bottle and left to stand overnight. The completion of sterilization was comfirmed using trypticase soy agar (TSA). Then, the cultures were centrifuged at 8,000 rpm for 30 minutes. The resulting supernatant was dialysed against water for 3 days and concentrated by Ficol (Pharmacia). This material was subjected to the fractionation by gel-filtration.

Cell disruption

The cells harvested from the growth on TGE agar were suspended in 100 ml of 0.01M EDTA solution in 0.1M Tris buffer pH 7.2. The suspension was subjected more than 15 cycles of freezing and thawing in the temperature rang between -20°C and 35°C. Thus treated suspension was then sonicated for 3 minutes to promot cell disruption and dispersion.

Separation of membrane fraction and cell-free extract from the disrupted-cell homogenate

The cell homogenate 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 here 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 precipitate 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 to be used as membrane fraction. The supernant was taken as cell-free extract to be subjected to further fractionation by column chromatography.

Fractionation of cell components by column chromatography

The three cell components (condensed culture filtrate, triton X-100 solubilized membrane fraction, cell-free extract) were fractionated by column chromatography with sepharoses 6B, sephadex G-75, or DEAE cellulose. The elution patterns were followed by the determination of protein content, sugar content and acid phosphatase activity. The column conditions of each experiments are described in the Results section.

Determination of protein and sugar content

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 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 mµ. Glucose was used as sugar standard. Anthrone test was also employed for sugar determination in some experiments.

Preparation of antisera

Antisera to phosphatase-active glycoprotein frac-

tion obtained by column-chromatography with DEAE-cellulose were prepared by immunizing five guinea pigs and 3 rabbits. Guinea pigs weighing around 250 g were injected intramuscularly 3 times at one week intervals, each time employing 200 μg of MH-a or LPS (Naigowit *et al*, 1993) emulsified with Freund complete adjuvant. Rabbits weighing around 2,000 g were immunized intramuscularly with 2 mg of the antigens in the same way as above. Three weeks after the final immunization, the animals were exsanguinated to separate the sera. The sera were pooled and inactivated at 56°C for 30 minutes.

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 phosphate solution (0.2%). For pH range from 3.19 to 6.22, acetate buffer solutions (0.1M) were prepared. For pH range higher than 6.5, 0.1M Tris-HCl buffer 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 mμ. To the blank tube NaOH solution was added at zero time.

Indirect immunofluorescence microscopy

Colonies of *B. pseudomallei* on tryptic soy agar were suspended in PBS to a concentration of approximately 10^9 cells per ml. Tris suspension density was adjusted by turbidity comparison with McFarland standard tube. It was then treated with 0.35% formalin to kill the cells. One drop of the suspension was placed on a glass-slide and air-dried. The cells were fixed with acetone for 15 minutes. The fixed smears were covered with a 1:8 dilution of the pooled antisera and incubated in a moist chamber at 37°C for 45 minutes. The smears were washed 3 times with fresh PBS each-times, and air-dried. Finally, the smear was treated with 20 μl of a 1:20 dilution of fluorescein isothiocyanate-conjugated anti-IgG of homologous species (ICN Immunobiologicals, IL, USA) and incubated in a moist chamber at 37°C for 45 minutes. Fluorescence microscopy was conducted with a flouresscence microscope (Olympus BH).

Pathologic specimens kindly supplied from Sappasitthiprasong Ubon Hospital were also subjected to this immunofluorescent microscopy in the same procedure.

RESULTS

Comparison of the sephadex G-75 elution patterns of ammonium sulfate-precipitate fracions of 4-day and 14 day culture filtrates

A preliminary observation on the development of protein and sugar molecules in the culture filtrates was made comparing the two culture filtrates in incubation days 4 and 14. In this experiment, the culture was inoculated with the mixture suspension consisting of five local strains of *B. pseudomallei* (UB12, UB16, UB18, UB19, UB20). Culture filtrate was obtained by centrifugation 30,000 × g, for 30 minutes, after 120°C, 10 minute-heating for bacterial killing. The condensation of the filtrates was made by ammonium precipitation, dialysis against water and Ficol (Pharmacia).

The results are shown in Fig 1. It is evident that

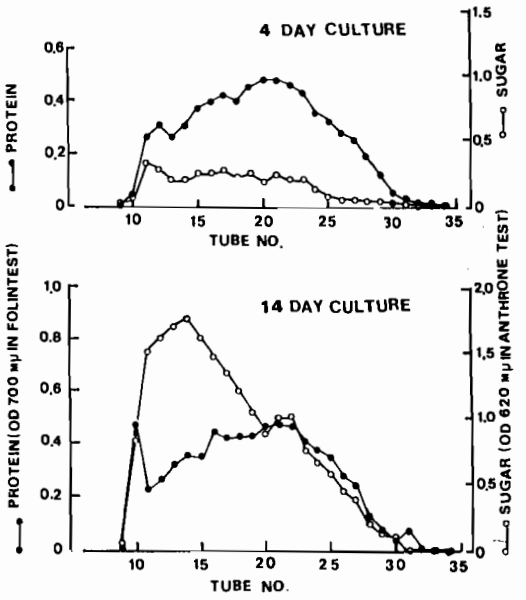

Fig 1–Gel filtration patterns in sephadex G-75 column (2 × 30 cm) loaded with ammoniumsulfate-precipetated fraction of the 4-day and 14-day culture filtrates of *B. pseudomallei*.

high molecular sugar components increased greatly in the old culture of 14 days, though protein content did not change markedly.

Comparison of the elution patterns of gel-filtration with Sepharose 6B among cell-free extract, outermembrane fraction solubilized by triton x-100 treatment and the culture filtrate of *B. pseudomallei*

The culture filtrate of a 2 day growth of *B. pseudomallei* (UB26) in TSB was sterilized with formaldehyde (0.5%) and centrifuged at 8,000 rpm. The supernatant was condensed and dialysed against water, then fractionated by column chromatography with Sepharose 6B (2.5 × 35 cm). Elution was made with water, and the eluates were collected in 8 g amounts. Determination of protein, sugar and acid phosphatase was conducted with aliquots of each eluate fraction. The same type of experiment was conducted with the cell-free extract (100,000 × g: one hour supernatant) prepared from the disrupted cell homogenate of the same strain grown on TSA, and also with the membrane fraction solublized by Triton X-100 (2% in 0.2M KCl) treatment.

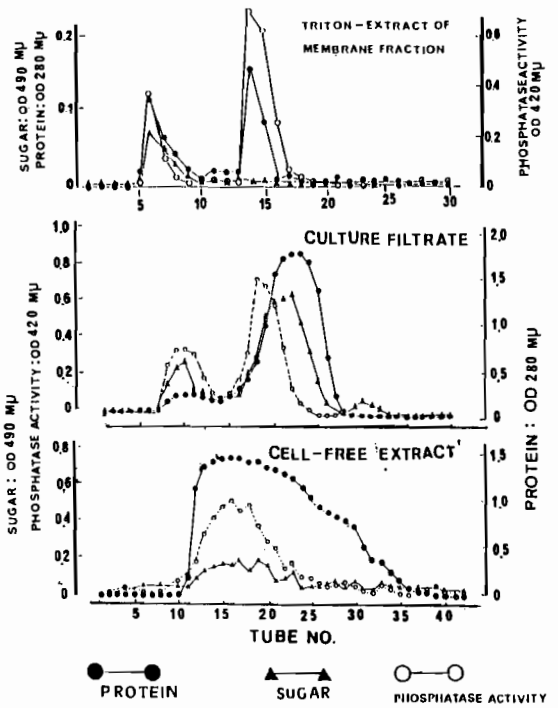

Fig 2–Gel-filtration (sepharose 6B) patterns of cell-free extract, membrane fraction, and culture filtrate obtained from *B. pseudomallei.*

The results are shown in Fig 2. First of all, we notice in this figure that the elution patterns of the culture filtrate and the triton-extract of the membrane fraction are similar but distinct from that of the cell-free extract of the whole cells. In the cell-free extract (the cytoplasm), the elution patterns regarding any of the three components (protein, sugar, and the enzymatic activity) showed a diffuse distribution of molecular size. No characteristic feature is observed in the elution curves. In the other two materials, however, the elution patterns consisted of two clearly separated peaks. Proteins, sugar and enzymatic activity were in the higher molecular peaks, apparently being associated with each other. In the lower molecular peaks of the culture filtrate, proteins, sugar and the enzymatic activity appear to be present independently if not all.

The effects of tunicamycin (glycosylation inhibitor) on the level of proteins sugar and acid phosphatase activity in the culture filtrate

With a view that acid phosphatase of *B. pseudomallei* may be a glycoprotein, an experiment was undertaken to examine the effects of tunicamycin on the level of proteins, sugars, and acid phosphatase activity at pH 5.5. A single colony of *B. pseudomallei* (UB16) on Ashdown plate was inoculated to TSB, then subcultured to Mueller Hinton liquid medium (MH). Two separate lots of 300 ml MH were arranged therefrom, and one of them was added with tunicamycin to a concentration of 25 μg/ml and the other was left as it is control culture. After 2 days incubation at 37°C, the filtrate of each lot were harvested, sterilized with formaldehyde (0.5%) overnight, centrifuged to get supernatant, condensed, dialysed, and subjected to column chromatography with Sepharose 6B in the same way as the preceding experiment. The results shown in Fig 3 indicate that, though the protein level did not change substantially in the presence of tunicamycin, the level of sugar and acid phosphatase activity were reduced significantly.

In this experiment, the eluates of tubes No. 4 to 8 and of tubes No. 9 to 15 in both columns loaded with the filtrates with or without tunicamycin were each pooled, condenced and lyophilized to be weighed. Their dry weight is indicated in Fig 3. Tunicamycin decreased selectively the actual yield

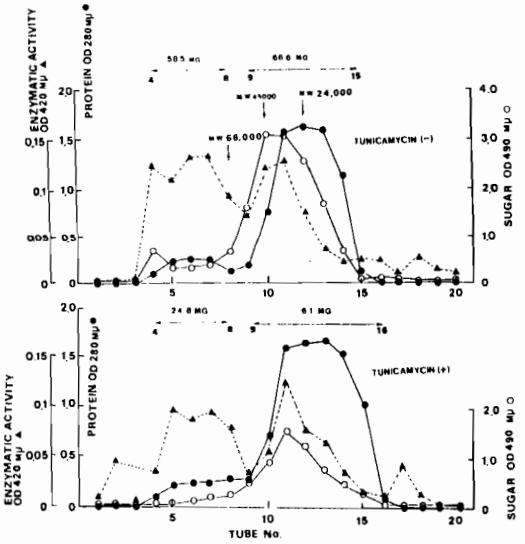

Fig 3–Gel-filtration patterns by sepharose 6B column chromatography of the culture filtrates of *B. pseudomallei* grown in the presence or absence tunicamycin.

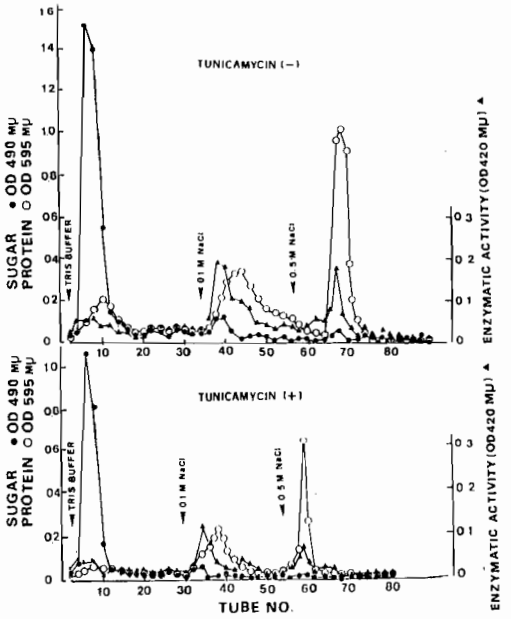

Fig 4–Separation by DEAE-cellulose column (1.5 × 10 cm) of acid phosphatase-active fractions as glycoproteins from culture filtrates of *B. pseudomallei* grown in the presence or absence of tunicamycin (glycosylation inhibitor).

of the high molecular fraction having the enzymati cactivity. To the column, molecular size markers (Trypsinogen, 24,000; egg albumin, 45,000; bovine albumin, 66,000) were also applied to estimate the molecular size of the eluted fractions by comparison. From the eluted location of the markers, the molecular size of the first peak was estimated to be around 100,000 and the size of second peak was around 33,000-45,000 daltons.

This sort of tunicamycin experiment was repeated in the same procedure but using DEAE-cellulose for column chromatography. The filtrate concentrated to 12 ml was loaded to a column of 1.5 × 10 cm and eluted stepwise with 0.025 M Tris-HCl buffer, then 0.1 M NaCl and finally with 0.5 M NaCl. The size of the eluates was 4 ml of which protein content, sugar content and the enzymatic activity were determined (Fig 4). In both cases with or without tunicamycin, three distinct peaks associated with protein, sugar and the enzymatic activity were obtained in accordance with the three steps of elution. However, the enzymatic activity and sugar appears more closely associated in the elution pattern, suggesting the nature of glycoproteins of the enzyme. Again in this experiment, the overall reduction of protein, sugar and the enzymatic activity was noted in the tunicamycin-exposed culture.

Staining of *B. pseudomallei* by indirect immunofluorescence assay with antienzyme sera

We have already reported about the successful identification of *B. pseudomallei* isolates on agar plates (Naigowit *et al*, 1993) by the indirect immunofluorescent method as described in Materials and Methods.

In the later study, the method was applied to clinical specimens of melioidosis patients including pus, sputum, urine, and others. More than 80% of the clinically diagnosed melioidosis specimens showed positive staining reaction. In Fig 5A is the staining of *B. pseudomallei* isolate on agar media and B is the direct staining of pus from a melioidosis patient.

DISCUSSION

We have been concerned with acid phosphatase activity of *Pseudomonas pseudomallei* for the past decade since this species together with *P. cepacia*

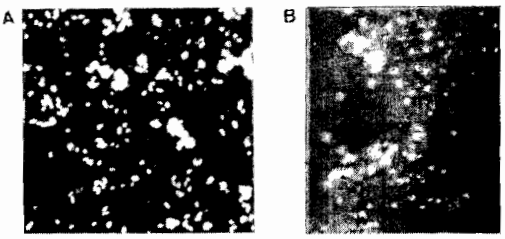

Fig 5–Indirect immunofluorescence microscopy for identification of *B. pseudomallei* in isolates on culture media (A) and in smears of pus (B), with the antibodies against acid phosphatase-active fraction obtained from the culture filtrate of the homologous microorganisms.

and *P. mallei* was found to have a much stronger activity and different pH pattern from those of the other *Pseudomonas* species. In agreement with this distinction, those three species are now classified into a newly established genus *Burkholderia* separated from *Pseudomonas* on the basis of genetic and chemical characterization (Yabuuchi *et al*, 1992).

During the course of our study along various lines a suggestion was made that the enzyme must be a glycoprotein and located on the cell surface as protein tyrosine phosphatase, and that the complex nature of the enzymatic activity regarding pH-activity pattern and thermostability may be the reflection of the evolutional stage of the enzyme.

In the present study, our first observation (Fig 1) was the increase of sugar to protein ratio in the old culture filtrate of *B. pseudomallei*. This finding supports the well-known feature of this species that the growing cells produce a high amount of extracellular polysacchrides (Jayanetra, 1989; Kaliev *et al*, 1990; Vorachit *et al*, 1993).

An assumption was made therefore that such extra-cellular polysaccharides consist not only free forms of highly polymerized saccarides but also of sugar moieties of cell-surface lipopolysaccharides and glycoproteins, and that they all together constitute the so-called glycocalyx. The data in the present study are in favor of this idea.

The data shown in Fig 2 suggest strongly that acid phosphatase may be synthesized and secreted through successive steps within the cells of *B. pseudomallei*. At first, it is synthesized as an unglycosylated thermo-labile precursor. Such precursor proteins will be increasingly glycosylated and translocated into the outer membrane to become cell-surface enzymes. The glycosylation may be a prerequisite for this efficient transport through the intracellular millieu, including the organelles corresponding to the endoplasmic reticulum and Golgi apparatus.

In this study, gel-filtration was successful for the intended purpose. This method does not have specific chemical selectivity other than molecular size, so there would be little possibility to lose any components contained in the loaded material. This property has an advantage in the comparative observation of the fractionation patterns among different materials. The high molecular glycoprotein fraction accompanied with acid phosphatase was separated only from the culture filtrate and the outermembrane but not from the cell-free extract (cytoplasm).

Glycosylation of proteins is thought to confer the increased surface activity to the conjugated matrial (Kato *et al*, 1988) so that it may be more easily integrated in to the membrane structure. Glycosylation can affects protein folding (Readarer and Hinnen, 1991) and increase thermostability (Marsh *et al*, 1977).

This report may well explain our previous observation that there are two acid phosphatases in *B. pseudomallei* different in optimum pH and thermostability, and that the enzyme of the higher optimum pH and thermostability locates in the outermembrane and the enzymatic activity at lower pH are thermolabile and presumably locating in the cytoplasm.

The glycoprotein nature of the enzyme was also suggested from the observation that tunicamycin in proper concentration decrease the sugar content and the enzymatic activity in the culture filtrate without leaving protein content unchanged. This may suggest that glycosylation is related with the enzymatic function (Kuo and Lampen, 1974). Actually we had already found that tunicamycin in the culture medium alter the pH activity pattern of the enzyme in the growing cells of *B. pseudomallei* (Kondo *et al*, 1994b).

The surface location of the glycoprotein acid phosphatase was further indicated by the immunofluorescence staining with the antisera against the phosphatase-active fraction.

The enzymatic activity is particulalerly strong

when thyrosine phosphate is employed as substrate (Kanai and Kondo, 1991; Kondo *et al*, 1994). This may suggest that the acid phosphatase activity as determined with the screening substrate p-nitrophenolphosphate represents protein tyrosine phosphatase as a component of signal transfer system in the cell surface (Walton and Dixon, 1993). In the kingdom of microorganisms, yeast and *Yersinia* are known to have this system (Vogel and Hinnen, 1990; Schwaniger *et al*, 1990; Guan and Dixon, 1990; Bliska *et al*, 1991). We have already reviewed this problem in a previous publication (Kanai and Kondo, 1994). We suspect that such glycoprotein acid phosphatase location on the cell surcface may function as a receptor to receive stimuli from the environment. This assumption will be examined in another paper.

REFERENCES

Bliska JB, Guan K, Dixon JE, *et al*. Tyrosine phosphate hydrolyses of host proteins by an essential *Yersinia* virulence determinant. *Proc Natl Acad Sci USA* 1991; 88 : 1183-91.

Dejsirilert S, Butraporn Jr, Chiewsilp D, *et al*. High activity of acid phosphatase of *Pseudomonas pseudomallei* as a possible attribute relating to its pathogenicity. *Jpn J Med Sci Biol* 1989; 42 : 39-49.

Guan K, Dixon JE. Protein tyrosine phosphatase activity of an essential virulence determinant in *Yersinia*. *Science* 1990; 249 : 553-5.

Jayanetra P. Melioidosis in Thailand-update, microbiological aspect of melioidosis. Proc 1st Nat Cong Pathol 1989; March 22-24: Chiang Mai Univ Thailand.

Kanai K, Kondo E. Substrate response in acid phosphatase activity of *Pseudomonas pseudomallei* and *Pseudomonas cepacia*, with special reference to tyrosine phosphatase. *Jpn J Med Sci Biol* 1991; 44 : 225-37.

Kanai K, Kondo E. Review. Recent advances in biomedical sciences of *Burkholderia pseudomallei* (Basonym: *Pseudomonas pseudomallei*). *Jpn J Med Sci Biol* 1994; 47 : 1-45.

Kapliev VI, Denisov II, Kurilov Y. Morphological characterization of *Pseudomonas pseudomallei* mucoid variants. *Abtopob* 1990; 579 : 41-5.

Kato A, Murata K, Kobayashi K. Preparation and characterization of ovalalbumin-dextran conjugate having excellent emulsifying properties. *J Agric Food Chem* 1988; 36 : 421-5.

Kondo E, Dejsirilert S, Wojprasit N, *et al*. Heat-stable and heat-labile components of nonspecific acid phosphatase detected in *Pseudomonas pseudomallei*. *Jpn J Med Sci Biol* 1991a; 44 : 51-62.

Kondo E, Naigowit P, Phanichruttiwong P, *et al*. Fatty acid profile and acid phosphatase activity of fresh isolates of *Pseudomonas pseudomallei*. *Jpn J Med Sci Biol* 1991c; 44 : 195-211.

Kondo E, Petkanchanapong V, Naigowit P, *et al*. Demonstration of acid phosphatase activity in antigenic glycoproteins obtained from the culture filtrate of *Pseudomonas pseudomallei*. *Jpn J Med Sci Biol* 1991b; 44 : 214-24.

Kondo E, Wangroonsaub P, Kanai K. Effects of tunicamycin on the pH-activity pattern of acid phosphatase in *Pseudomonas pseudomallei*. *Southeast Asian J Trop Med Public Health* 1994b; 25 : 144-50.

Kondo E, Wangroonsub P, Naigowit P, *et al*. Separation of antigenic glycoprotein fractions from cell-free homogenate of *Pseudomonas Pseudomallei* and characterization as tyrosine phosphatase. *Southeast Asian J Trop Med Public Health* 1994a; 25 : 437-42.

Kuo S-C, Lampen JO. Tunicamycin-An inhibitor of yeast glycoprotein synthesis. *Biochem Biophys Res Commun* 1974; 58 : 287-95.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193 : 265-75.

Marsh JW, Dennis J, Wriston JC Jr. Glycosylation of *Escherichia coli* L-asparaginase. *J Biol Chem* 1977; 252 : 7678-84.

Naigowit P, Kurata T, Wangroongsub P, *et al*. Application of indirect immunoflurescence microscopy to colony identification of *Pseudomonas pseudomallei*. *Asian Pacific J Allerg Immunol* 1993; 11 : 149-54.

Petkanjanapong V, Naigowit P, Kondo E, *et al*. Use of endotoxin antigens in enzyme-linked immunosorbent assay for the diagnosis of *P. pseudomallei* infections (melioidosis). *Asian Pacific J Allerg Immunol* 1992; 10 : 145-50.

Readarer MA, Hinnen A. Removal of N-glycosylation of the yeast acid phosphatase severely affects protein folding. *J Bacteriol* 1991; 173 : 3539-46.

Schwaniger R, Dumermauth E, Schwringruber ME. Effects of seven different mutations in the pho 1 gene on enzymatic activity, glycosylation and secretion of acid phosphatase in *Shizosaccharomyces pombe*. *Mol Gen Genetics* 1990; 221 : 403-10.

Vogel K, Hinen A. The yeast phosphatase system. *Mol Microbiol* 1990; 4 : 2013-7.

Vorachit M, Lam K, Jayanetra P, Costerton JW. Resistance of *Pseudomonas pseudomallei* growing as a biofilm on silostic discs to ceftazidime and cotrimoxazol. *Antimicrob Agents Chemother* 1993; 37 : 2000-2.

Walton KM, Dixon JE. Protein tyrosine phosphatases. *Ann Rev Biochem* 1993; 62 : 101-20.

Yabuuchi E, Kosako Y, Oyaizu H, *et al.* Proposal of *Burkholderia* gen nov. and transfer of seven species of the genus *Psudomonas* homology group II to the new genus, with the type species of *Burkholderia cepacia* (Palleroni and Holmes, 1981) comb nov.