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SUBSTRATE RESPONSE IN ACID PHOSPHATASE ACTIVITY OF *PSEUDOMONAS PSEUDOMALLEI* AND *PSEUDOMONAS CEPACIA*, WITH SPECIAL REFERENCE TO TYROSINE PHOSPHATASE

Koomi KANAI and Eiko KONDO

National Institute of Health, Department of Medical Sciences, 88/7 Soi Bamrasnaradura Hospital, Tivanon Road, Nonthaburi 11000, Thailand

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SUMMARY: The substrate response in acid phosphatase activity of *Pseudomonas pseudomallei* and *Pseudomonas cepacia* was examined with different phosphate esters including hexose phosphates and phosphoaminoacids in a whole cell assay system. The enzymatic activity against each substrate was evaluated in terms of percent activity to that against para-nitrophenyl phosphate set as 100. A remarkable finding was that the phosphatase reaction was the highest with phosphotyrosine or phosphoserine as substrate showing 180% activity. This tyrosine phosphatase activity was resistant to heating at 60 C for 20 min and inhibited greatly by 0.1% ZnCl₂. *Pseudomonas cepacia* showed the same pattern of substrate response and the same characteristics of tyrosine phosphatase activity.

INTRODUCTION

The present authors have been studying on acid phosphatase activity of *Pseudomonas pseudomallei*, since this species together with *P. cepacia* was distinct from other species of *Pseudomonas* by the high enzymatic activity (1,2). The activity was examined in a whole cell assay system with *p*-nitrophenyl phosphate (PNPP) as substrate. PNPP is a nonspecific substrate which can be used effectively to screen for phosphatase activity in general. The nonspecific acid phosphatase may or may not imply one enzyme of wide specificity.

PNPP has been employed as a convenient tool in the genetical and biochemical studies in microbiology. Because of the nonspecificity, however, the

enzymatic activity measured with PNPP does not always provide enough information on the physiological role of the enzyme. During the course of our studies (1,2) on acid phosphatase activity of *P. pseudomallei* with PNPP, a question was raised concerning the biological significance of the enzyme, especially in connection with the high adaptability of this species of bacteria to the environmental changes (3).

An important suggestion came from the recent development of the knowledges about protein tyrosine phosphatase in cell growth and differentiation (4), and also from the first demonstration of the presence of such an enzyme in *Yersinia* (5) as an essential virulence determinant.

Being encouraged by these and other related literatures (6-9), we proceeded to the preliminary study on the substrate response of acid phosphatase in *P. pseudomallei*. The experiments were rewarded with a noteworthy observation that tyrosine phosphatase showed the highest activity in *P. pseudomallei* and *P. cepacia*.

MATERIALS AND METHODS

Microorganisms: The strains of *P. pseudomallei* and *P. cepacia* employed in this study were the isolates from melioidosis patients admitted to Sappasitthiprasong Ubon Hospital in Ubon Ratchathani. As *P. pseudomallei*, UB3 was the strain isolated from the blood in 1989. UB48, UB54, and UB52 were the fresh isolates in 1991, the former two from the blood and the latter one from the sputum. As *P. cepacia*, four strains were employed; UB77 of blood origin, UB76 and UB78 each of pus origin, and UB80 of urine origin. All these strains were identified by routine laboratory tests in the hospital, and further confirmed by ourselves on the basis of fatty acid analysis by gas-liquid chromatography, pH-activity patterns of acid phosphatase (10), and immunofluorescence microscopical assay (IFA) as will be reported elsewhere. IFA was a reliable test to differentiate the two species.

Cell cultures: To harvest the cell mass for the enzymatic study, the strains were grown as follows. Each stock culture was transferred to blood agar, which was incubated overnight at 37 C. One colony on the plate was then picked up and inoculated to nutrient broth to be incubated overnight. This broth culture was spread with a cotton swab onto a plate of tripton glucose extract agar (TGE), which was incubated overnight at 37 C. The growth developed was then suspended in 3 ml of 0.9% saline using a spreader. A 1:20 dilution of this suspension was prepared and subjected to the determination of optical density at

420 nm with a Coleman spectrophotometer. From the optical density obtained, further adjustment was made to prepare a suspension of OD:5 or OD:4.

Chemicals: Phosphate esters were obtained from Sigma Chemical Co. (St. Louis, MO). The medium ingredients were Difco Products (Detroit, MI). For phosphate determination, ammonium molybdate, sodium sulfit, sodium disulfit, and 1-amino-2-naphthol-4 sulfonic acid were obtained from Merck Co. (Darmstadt, F. R. G.). Zinc chloride was purchased from Sigma Chemical Co. Para-nitrophenyl phosphate was obtained from Wako Junyaku (Osaka).

Enzyme assay: The reaction mixture (0.6 ml) in a pyrex tube of 16 × 100 mm contained 0.4 ml of 0.1 M sodium acetate buffer or Tris-HCl buffer of a given pH, 0.1 ml of 10 mM substrate solution, and 0.1 ml of a bacterial suspension of the standard density. After incubation at 37 C for 60 min, the amount of phosphate released was determined according to King's method (11).

RESULTS

Substrate Response of P. pseudomallei and P. cepacia in the Hydrolysis Experiments with Various Phosphoesters

The mode of substrate response in acid phosphatase activity of both species of *Pseudomonas* was surveyed with various phosphoesters; phospho-L-serine, phospho-L-threonine, phospho-L-tyrosine, glucose 6-phosphate, fructose 1, 6-di-phosphate, adenosine 5'-monophosphate, adenosine 2'-3'-cyclic monophosphate, 6-benzoyl-2 naphthylphosphate, naphthylphosphate, and phenolphthalein phosphate. The reactions at five different pH values of the bacterial suspensions of OD:5 were recorded in 60 min of incubation. The results are shown in Fig. 1 (*P. pseudomallei*, UB54) and Fig. 2 (*P. cepacia*, UB77).

The highest reactions were obtained when phospho-L-serine and phospho-L-tyrosine were employed as the substrate at pH 5 to 7, but no reaction to phospho-L-threonine. Glucose 6-phosphate and Fructose 1, 6-di-phosphate were also an effective substrates in both species of *Pseudomallei* but adenosine 5' monophosphate and adenosine 2'-3'-cyclic monophosphate were poor in this respect, especially the latter was not hydrolyzed at all. 6-Benzoyl-2 naphthylphosphate and naphthyl phosphate were each a good substrate but phenolphthalein phosphate was much less responsive. Generally speaking, there was no notable difference between *P. pseudomallei* and *P. cepacia* in the above-stated mode of substrate response.

Fig. 1. Substrate-response in acid phosphatase activity of *P. pseudomallei*, strain UB54.

Fig. 2. Substrate-response in acid phosphatase activity of *P. cepacia*, strain UB77.

Fig. 3. Substrate-response in acid phosphatase activity of *P. pseudomallei*, strain UB54. Relative activity on each substrate to a value of 100 assigned to the reaction on para-nitrophenyl phosphate.

An additional experiment was then conducted in the same manner, with a OD:4 suspension of *P. pseudomallei* (UB54) at pH 6.0 to compare the reaction intensity to each substrate with that to PNPP. The reactions for 60 min were read, and the inorganic phosphate released was determined to calculate the relative (%) activity to each substrate by assigning the value of 100 to the activity against PNPP. The results shown in Fig. 3 are the averages of three tubes. As is clear in this figure, tyrosine phosphatase activity of 180% was the highest response so far tested.

Tyrosine Phosphatase Activity in Other Strains of P. pseudomallei (UB3 and UB52) and P. cepacia (UB76 and UB78)

Tyrosine phosphatase activity of the two species of *Pseudomonas* as detected in the preceding experiments was subjected to a confirmation test with the same (UB54) and other two strains (UB52 and UB3) of *P. pseudomallei* and additional two strains (UB76 and UB78) of *P. cepacia*.

In the tests, the pH-activity pattern was examined in a wider range with more different pH points. The results are shown in Figs. 4 and 5. The pH-pattern

Fig 4. pH-Activity patterns of tyrosine phosphatase in three strains of *P. pseudomallei*.

obtained here is not different from those we observed with PNPP, showing a two to three peaks or shoulders (1,2). The highest peaks were observed at pH 6 to 6.5 in most cases.

Heat-resistance of Tyrosine Phosphatase

Saline suspensions (OD:5) of *P. pseudomallei* (UB48) and *P. cepacia* (UB80) were placed in water baths kept at 60 C and 80 C for 20 min, respectively. After termination of heating, the suspensions were transferred immediately to ice-water. Their pH activity patterns of tyrosine phosphatase were then examined in

Fig. 5. pH-Activity patterns of tyrosine phosphatase in two strains of *P. cepacia*.

comparison with the unheated control suspensions. The results (Fig. 6) showed that, though their tyrosine phosphate activity was completely abolished by heating at 80 C, the activity still remained considerably after heating at 60 C for 20 min. In *P. cepacia*, the enzymatic activity at pH higher than 6 appears to be enhanced by treatment at 60 C.

Inhibition of Tyrosine Phosphatase by ZnCl₂

Acid phosphatase is generally inhibited by molybdate, vanadate, ZnCl₂, and NaF. Experiments were conducted to examine whether or not ZnCl₂ can inhibit tyrosine phosphatase of *P. pseudomallei* (UB54) and that of *P. cepacia* (UB78). ZnCl₂ was added to incubation mixtures of pH 6.2 in the final concentrations of 0.1% and 1.0% at the start of assay. The determination of phosphate released was made in 20, 40, and 60 min in comparison with the control tube without Zn Cl₂.

Fig. 6. Effects of heat-treatment on tyrosine phosphatase activities of *P. pseudomallei* and *P. cepacia*.

The enzymatic activity of both species was inhibited almost completely by 1% ZnCl_2 and about 60% inhibition was observed by 0.1% (Fig. 7).

Fig. 7. Inhibition of tyrosine phosphatase activities of *P. pseudomallei* and *P. cepacia* by ZnCl_2 .

DISCUSSION

In the present study on substrate response of acid phosphatase, it has become clear that tyrosine phosphate and serine phosphate are the most efficient substrates in *P. pseudomallei* and *P. cepacia* releasing a much larger amount of phosphate than PNPP. This tyrosine phosphatase activity was very similar to PNPPase in the pH-activity pattern and also in the mode of heat-resistance. These observations may suggest a good possibility that what we have observed

hitherto (1,2) in terms of acid phosphatase is tyrosine phosphatase and/or serine phosphatase.

Besides, we know that protein tyrosine phosphatases have been isolated and purified from various source materials with PNPPase activity as an indicator (12-14). From an inhibition experiment with okadaic acid, Takai and Mieskes (15) concluded that PNPPase activity is intrinsic to protein tyrosine phosphatase. This information may suggest that the PNPPase and tyrosine phosphatase activities of *P. pseudomallei* and *P. cepacia* represent protein tyrosine phosphatase. The bacterial protein tyrosine phosphatase as such was recently demonstrated in *Yersinia* as an essential virulence factor (5).

Our study on acid phosphatase activity of *P. pseudomallei* was initially started on an assumption that it may be one of the attributes relating to its pathogenicity (1). Such examples have already been shown in *Salmonella typhimurium* (7-9). A regulator gene *phoP* controls the expression of the genes of *pag* loci including the locus for nonspecific acid phosphatase (*pag N*) and the other locus needed for the survival within macrophages. "Survival" may be understood as the environmental adaptation of bacteria in the host cell. Increasing amounts of evidences have now been accumulated to show that protein phosphorylation and dephosphorylation on the cell surface are of the vital importance in such adaptive response of bacteria (16,17). This would be one aspect of microbial pathogenicity, if not all.

In our previous paper (2), we reported the presence of heat-stable and heat-labile components of acid phosphatase in *P. pseudomallei* which were different in pH optimum and heat-stability. The heat-stable one was a membrane-associated enzyme. We speculated that heat-resistance would result from the progress of glycosylation of the enzyme protein. This idea prompted us to an attempt to isolate and purify acid phosphatase from 0.35% formalin-treated culture filtrates of *P. pseudomallei*, and the separated glycoprotein fractions showed the enzymatic activity (18). Glycoprotein acid phosphatase have been isolated by other authors from *Saccharomyces* (19,20) and *Leishmania* (21-23) as a cell-surface enzyme which is eventually released into the surrounding environment.

From these literatures together with our own experiences including ZnCl_2 inhibition and heat-stability, we assume that tyrosine phosphatase may be a cell-surface enzyme in *P. pseudomallei*, and that it may be protein tyrosine phosphatase of glycoprotein nature.

Meanwhile, *P. pseudomallei* has a high adaptability to the in vitro growth environment (3). Unlike *P. cepacia* and *P. aeruginosa*, *P. pseudomallei* grew in

heart infusion broth of initial pH 4.5 under both aerated and unaerated conditions and survived keeping a high level of viable counts for as long as 30 days. The bacilli can persist in the lesions of melioidosis patients, and the relapse of the disease is common (24). Though we are suspecting that such high adaptability of *P. pseudomallei* to changing environments in vitro and in vivo is associated with the high activity of protein tyrosine phosphatase as a cell-surface signaling mechanism, the situation is not so simple, because *P. cepacia* had a high activity of tyrosine phosphatase as well. On the other hand, *P. aeruginosa* did not reveal the enzymatic activity in our preliminary study. Other virulence determinants must be considered simultaneously. An attempt is now under ways to compare tyrosine phosphatase activity between the species of intracellular and nonintracellular parasites.

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