Purification and characterization of alkaline phosphatase containing phosphotyrosyl phosphatase activity from the bacterium *Prevotella intermedia*

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Abstract A novel alkaline phosphatase, designated PiALP, has been purified and characterized from *Prevotella intermedia* ATCC 25611, an anaerobe implicated in progressive periodontal disease. The enzyme was a homodimer of apparently identical subunits of M_r 54 kDa. Thiol-reducing agents completely inhibited the purified enzyme. The enzyme was highly stable even at 80°C. It exhibited substantial activity against tyrosine-phosphate-containing Raytide. The phosphatase activity was sensitive to orthovanadate and Zn^{2+} but highly resistant to okadaic acid. The amino acid sequence of peptides derived from PiALP showed a high degree of identity (65%) with alkaline phosphatases from *Zymomonas mobilis* and *Synechococcus*. The present results imply that PiALP might represent a new family of alkaline phosphotyrosyl phosphatases which has not been described previously.

Key words: Alkaline phosphatase; Thermostable; Phosphotyrosyl phosphatase; *Prevotella intermedia*

1. Introduction

Prevotella intermedia, an anaerobic, Gram-negative, rod-shaped bacterium, is isolated from lesions of rapidly aggressive periodontitis and is strongly implicated as a periodontal pathogen [1]. While the detailed mechanism of pathogenesis of this organism remains unknown, it produces a variety of potential virulence factors, including high alkaline phosphatase (ALPase) activity [2,3]. However, only limited information is currently available regarding the phosphatase activities of the periodontopathic bacteria. While studying the properties of the ALPase from *P. intermedia*, we found that the purified enzyme exhibited substantial activities against *O*-phospho-L-tyrosine, a compound that acts as substrate for phosphotyrosyl phosphatase (PTPase). In the present study, we report the purification and characterization of a novel ALPase containing PTPase activity from the bacterium *P. intermedia*. In addition, the internal amino acids of the protein sequences were obtained and compared with the other phosphatases.

2. Materials and methods

2.1. Bacterial strain and growth conditions

P. intermedia ATCC 25611 was kindly provided by T. Koga, Kyushu University, Fukuoka, Japan. *P. intermedia* ATCC 25611 was grown anaerobically [5% (v/v) CO_2, 10% (v/v) H_2, 85% (v/v) N_2] at 37°C for 2 days in brain heart infusion broth (Difco) supplemented with yeast extract (0.5%), hemin (5 μg/ml) and menadione (0.5 μg/ml).

2.2. Enzyme assay

Standard phosphatase reactions were carried out in 50 mM Tris-HCl, pH 7.8/50 mM NaCl/5% glycerol at 37°C for 30 min. The following substrates were used. (i) *p*NPP: 1 ml reaction mixtures contained 10 μmol of *p*NPP; absorption at 420 nm was measured. One unit of phosphatase activity corresponded to 1 μmol of substrate hydrolyzed per min at 37°C. (ii) Raytide (Oncogene Science): 40 nmol of Raytide was phosphorylated by $p60^{c-src}$ (Oncogene Science) in the presence of [γ-^{32}P]ATP as described by Barik [4]. Unincorporated ATP was then removed by passing the reaction mixture through a Sephadex G-50 quick-spin column. (iii) Phosphorylase kinase: 5 μg of phosphorylase kinase (Sigma) was phosphorylated in vitro by cAMP-dependent protein kinase (PKA) and [γ-^{32}P]ATP as described by Barik [4]. ^{32}P-labeled Raytide and phosphorylase kinase were analyzed by denaturing 40% and 20% polyacrylamide gels, respectively, essentially as described [5]. The radioactivities were quantitated using densitometric scanning apparatus, Bio-Image Analyzer BAS 2000 (Fuji film) [6].

The optimal pH was determined using 0.1 M acetate buffer (pH 5.0–6.5), 0.1 M Tris-HCl buffer (pH 6.5–8.5), and 0.1 M glycine-NaOH buffer (pH 8.5–10.0).

2.3. Purification

All procedures were carried out at 4°C. Bacterial cells (20 g) were harvested from 8 l culture of *P. intermedia* ATCC 25611 by centrifugation at 10 000×*g* for 20 min. The cells were washed twice with 0.1 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl (TBS buffer), suspended in 50 mM Tris-HCl (pH 7.5), and broken by sonic disruption using six 1-min pulses of a sonic disruptor. The resulting sonicate was stirred in the same buffer containing 1% Triton X-114 for 3 h at 4°C, and then was centrifuged at 100 000×*g* for 40 min. The supernatant fraction was collected as the crude enzyme extract and applied to a DEAE Bio-Gel (Bio-Rad) column (6×20 cm) that had been equilibrated with 50 mM Tris-HCl buffer (pH 8.4). The column was washed with the equilibration buffer until no protein was detected in the effluent by measurement of A_{280}, and then developed with a linear gradient of NaCl (0–0.5 M) in the buffer. The active fractions were pooled and concentrated by filtration through a Centricon-30 ultrafiltration device (Amicon). The concentrated solution was applied to a CM Bio-Gel (Bio-Rad) column (1×30 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The enzyme was eluted with a linear gradient of NaCl (0–0.5 M) in the same buffer. The active fractions were pooled and dialyzed against 100 mM potassium phosphate buffer (pH 7.5) containing 100 mM NaCl. The dialyzed enzyme solution was applied to a column of Sephacryl S-300 (Pharmacia) (1×90 cm) equilibrated with the same buffer as for the dialysis. The active fractions were pooled and concentrated as described above. The concentrated enzyme solution was applied to a column of HiTrap Chelating (Pharmacia) (bed volume 1 ml) equilibrated with 50 mM potassium phosphate buffer (pH 7.2) containing 0.5 M NaCl connected to a FPLC system (Pharmacia). The enzyme was eluted with the equilibration buffer containing 1 M glycine. This procedure was repeated four times and the pooled active fractions were concentrated 5-fold with Centri-

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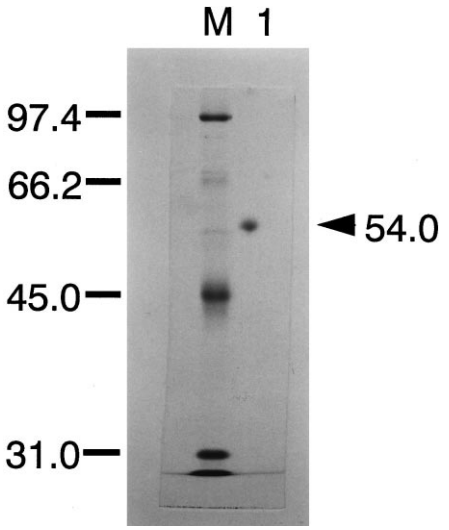


Fig. 1. Purification of PiALP. SDS-PAGE analysis was performed on 14% gels. The gel was stained with Coomassie brilliant blue. Lane 1 contains the purified PiALP (3 μg), and lane M contains molecular weight markers. The positions and molecular weights of standard proteins are indicated in the left lane.

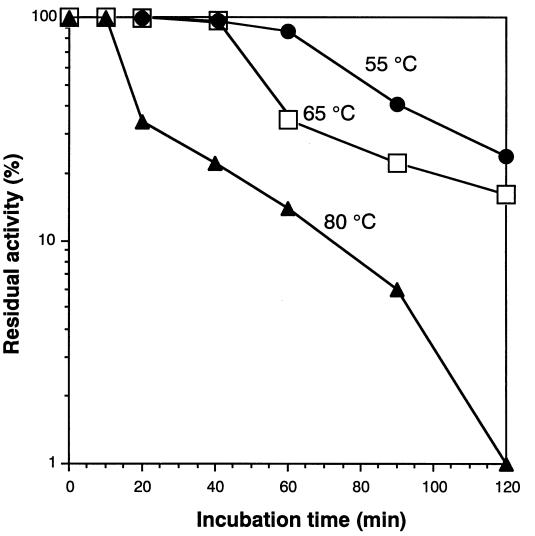


Fig. 2. Thermostability of PiALP. Enzyme solution at a concentration of 10 μg/ml was incubated at various temperatures and cooled on ice. The residual activity was measured at 37°C with an enzyme concentration of 1 μg/ml.

con-30. The concentrated enzyme solution was subjected to FPLC using Superdex 200HR 10/30 pre-equilibrated with 200 mM Tris-HCl (pH 7.5), which was also used for determining the molecular mass of PiALP. For molecular weight standards, the MW-Marker kit was used (Oriental Yeast). The purified enzyme was frozen and stored at −80°C.

2.4. Peptide microsequencing

Initial attempts to sequence PiALP were unsuccessful and indicated that the N-terminal region of the protein was blocked. The purified protein was thus digested with *Staphylococcus aureus* V8 protease. The peptides generated were separated on SDS-PAGE on 20% gels and electrophoretically transferred to Immobilon-P. Microsequencing (Takara Shuzo) was performed on an automatic gas phase sequencer, HP G1005A (Hewlett-Packard, CA, USA).

2.5. Thermostability studies

The enzyme was incubated at various temperatures in 50 mM Tris-HCl (pH 9.0) for different periods of time. Thermal inactivation was stopped by cooling the aliquots on ice, and then the residual phosphatase activity was measured at 37°C.

2.6. Other methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the molecular mass of the subunits by the method of Laemmli [7]. Molecular weight markers for SDS-PAGE were from Bio-Rad. Protein concentrations were routinely estimated according to Bradford [8] with bovine serum albumin as the standard.

3. Results

3.1. Purification of PiALP

The purification of PiALP from Triton X-114 extracts is summarized in Table 1. The PiALP was purified 109-fold with a final specific activity of 356.3 U/mg, and the overall yield of the activity was 4.1%. The most notable property observed in the course of purification was that the enzyme bound on both anion- and cation-exchange chromatographic matrices. This enabled us to successfully separate the enzyme from other *P. intermedia* proteins.

The molecular mass of native enzyme from *P. intermedia* was estimated by gel filtration to be ∼108 kDa. On SDS-PAGE, the purified enzyme revealed a single band with a molecular mass of ∼54 kDa (Fig. 1). These results suggested that the enzyme was a homodimer of identical 54-kDa subunits.

3.2. Temperature and pH optima of PiALP

The optimal temperature and pH for PiALP were determined in vitro by using *p*NPP as a substrate; essentially similar results were obtained by using a phosphorylated peptide such as Raytide (data not shown). The purified enzyme had the highest activity at around 65°C for *p*NPP as substrate. It was found to be extremely stable to heating. No loss of ac-

Table 1
Purification of ALPase from *P. intermedia* ATCC 25611

Preparation	Total activity (U)[a]	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Triton X-114 extract	701.6	214.15	3.28	100	1
DEAE Bio-Gel agarose	197.5	17.60	11.22	28.1	3.4
CM Bio-Gel agarose	79.6	2.40	33.17	11.3	10.1
Sephacryl S-300	46.4	0.58	80.00	6.6	24.4
HiTrap chelating	35.3	0.15	235.30	5.0	71.7
Superdex 200Hr 10/30	28.5	0.08	356.25	4.1	108.6

[a]Phosphatase activity was measured with *p*NPP as substrate.

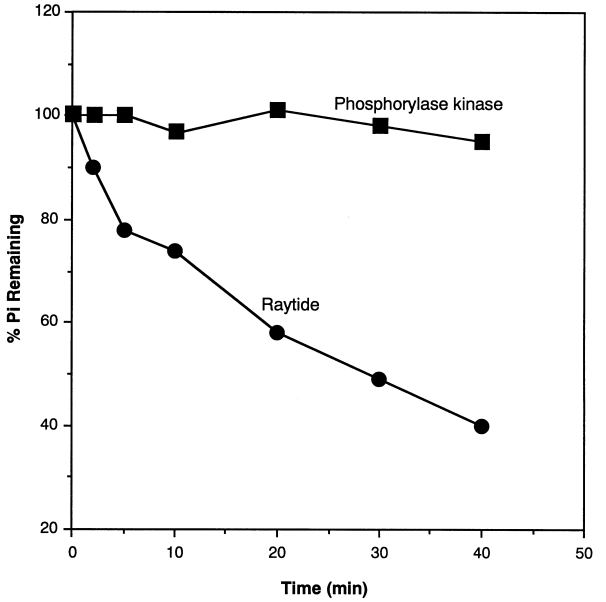


Fig. 3. Substrate specificity of PiALP. Purified PiALP (1 μl, containing 0.5 μg of protein) was assayed for activity toward a variety of phosphoprotein substrates. Standard assay conditions were employed as described in Section 2. At various times, 5 μl portions of the reaction mixture were taken out and analyzed by SDS-PAGE, followed by direct autoradiography and densitometric scanning of the autoradiograph. Shown are the percentages of phosphate remaining versus time of incubation for Raytide (●) and phosphorylase kinase (■) as substrates.

tivity was observed after 10 min incubation at 80°C. The time required for 50% loss of activity was about 90 min at 55°C (Fig. 2). The optimum pH value for the activity was approximately 8.4.

3.3. Inactivation of PiALP by DTT

PiALP was quite sensitive to inhibition by thiol-reducing agents such as DTT and 2-mercaptoethanol (Table 2). However, this inactivation was reversible, since the activity of the DTT-treated PiALP was almost completely recovered after removal of the DTT by dialysis.

3.4. Effect of inhibitors on PiALP activity

To characterize PiALP further, the effect of inhibitors on the activity of the enzyme was investigated. As shown in Table 3, sodium orthovanadate, sodium molybdate and *N*-ethylmaleimide, which are known inhibitors of PTPases, were also found to inhibit the activity of PiALP. On the other hand, the activity was not inhibited by okadaic acid, which is known to be a specific inhibitor of serine/threonine protein phosphatases. Other compounds, including EDTA and Zn^{2+}, also inhibited the activity.

Table 2
Effect of thiol-reducing agents on the activity of PiALP

Compound	Concentration (mM)	% of activity
None		100
Dithiothreitol	1	10
	10	0
2-Mercaptoethanol	1	71
	10	5

*p*NPP (10 mM) was used as substrate under standard assay conditions as described.

3.5. Substrate specificity of PiALP

As shown in Fig. 3, Raytide, a synthetic peptide containing tyrosine as the only phosphorylated residue, was found to be an efficient substrate for PiALP. Angiotensin II, phosphorylated tyrosine residue by $p60^{c-src}$, was dephosphorylated by PiALP (data not shown). However, it exhibited no detectable phosphatase activity toward phosphorylase kinase.

3.6. Determination of the internal amino acid sequence of the purified PiALP

The N-terminal sequence of the protein could not be sequenced, since its amino-terminus appeared to be blocked. Another sample of electrophoretically purified PiALP was subjected to partial digestion with *S. aureus* V8 protease. The resulting peptides were separated by SDS-PAGE, electroblotted onto PVDF membranes, and visualized with Coomassie blue. Portions of the membranes containing the most visually prominent bands were excised and subjected to 20 cycles of automated gas-phase Edman sequence analysis. As shown in Table 4, a distinct sequence was obtained, Thr-Asp-Met-Leu-Ala-Val-Ser-Val-Ser-Ser-Thr-Asp-Ala-Ile-Gly-His-Lys-Tyr-Gly-Thr. The amino acid sequence had 65% identity with two ALPases, PhoD from *Zymomonas mobilis* [9] and PhoV from *Synechococcus* [10]. Interestingly, a sequence homologous to the putative phosphorylation site [10], Leu-Ala-Val-Ser-Leu-Ser-Ser-Thr-Asp-Ala-Val, was found.

4. Discussion

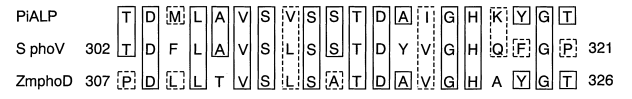
In the present study, a novel alkaline phosphatase, PiALP, was purified to homogeneity from *P. intermedia* and we discovered that it contained PTPase activity. Phosphatase activities have been observed in several periodontopathogenic bacteria, including *P. intermedia*. However, limited information is known about these phosphatases. Only two phosphatases from oral bacteria have been reported [11,12]. Concerning ALPases from oral bacteria, only one ALPase, from *Porphyromonas gingivalis* (PgALP), has been purified and characterized so far. PgALP has been shown to be a homodimer of identical subunits of M_r 61 kDa and have phosphoseryl phosphatase activity [12]. The result of SDS-PAGE (Fig. 1) and HPLC analysis of PiALP showed that it was a homodimer having a molecular weight of about 54 kDa. The thermostability of PiALP was evidenced by its high temperature optimum of 65°C and its ability to retain 50% activity even after 15 min exposure at 80°C (Fig. 2). The extreme thermal stability of an enzyme from a human oral bacterium is without

Table 3
Effect of various chemicals on the activity of PiALP

Chemical	Concentration	% of activity
None		100
Sodium molybdate	1 mM	31
Sodium orthovanadate	1 mM	1
N-Ethylmaleimide	5 mM	66
Okadaic acid	1 μM	113
$ZnCl_2$	2 mM	6
EDTA	5 mM	0

*p*NPP (10 mM) was used as substrate under standard assay conditions as described.

Table 4
Comparison of the amino acid sequences of peptides derived from PiALP with other phosphatases



Amino acids which are identical in PiALP and one or both of SphoV and ZmphoD are shown in solid-line boxes, with conservative amino acid changes in dashed-lined boxes. SphoV: PhoV of *Synechococcus*; ZmphoD: PhoD of *Z. mobilis*.

precedence and defies explanation at present. It is tempting to speculate that an ancestral relatedness between *P. intermedia* and the oldest phylogenetic kingdom in nature, such as the hyperthermophilic archaebacteria, exists, raising fundamental questions about the origin and the role of tyrosine phosphorylation.

The 20 internal amino acid sequences of PiALP were compared with all the protein sequences in the SwissProt database and BLAST program. A significant high sequence similarity was found between PiALP and the ALPases of *Z. mobilis* (PhoD) and the cyanobacterium *Synechococcus* (PhoV). The PiALP contained a putative phosphorylation site (LAV-SLSSTD(Y/A)V) that is conserved between PhoD and PhoV, although the involvement of this site in regulation of activity remains speculative [9,10]. These results strongly indicated that the PiALP would belong to the same family with PhoD from *Z. mobilis* and PhoV from *Synechococcus*. On the other hand, in previous studies [9,10], PTPase activities in these two ALPases were not investigated. Unfortunately, no information is available about PTPase activities of PhoD and PhoV at present. *Escherichia coli* ALPase also has PTPase activity [13], but it did not appear to show significant homology with PiALP, PhoD and PhoV. Furthermore, from the standpoint of its substrate specificity for phosphorylase kinase and the sensitivity to dithiothreitol and divalent ion for activity, PiALP would be distinct from *E. coli* ALPase [14]. On the other hand, when reviewing the complete sequences of PhoD and PhoV, we were not able to find the highly conservative motif HCXAGXGR (both Cys and Arg are essential for activity) in the active domain of PTPases [15]. PiALP also shared no overall sequence with the SA (small, acidic) PTPases, which are the prokaryotic PTPases without this motif [16]. However, PiALP was inhibited by sodium orthovanadate, sodium molybdate and Zn^{2+}, which are known inhibitors of PTPases [17]. Furthermore, it did exhibit substantial activity against tyrosine-phosphates, since it could dephosphorylate Raytide (a gastrin derivative) as well as angiotensin II. These results suggest that this ALPase group (i.e. PhoD, PhoV and PiALP) might constitute a novel family of alkaline PTPases significantly different from other known PTPases.

The dissociation of the PiALP dimer by thiol-reducing agents and simultaneous loss of enzymatic activity strongly suggests that the two subunits are jointed by disulfide bond(s), and that the dimer is the active form. All PTPs reported to date contain a signature sequence, HCXAGXGR, at their active site. Catalysis has been shown to proceed through the formation of a covalent phospho-thio intermediate involving the Cys residue of the signature sequence [18]. The fact that PiALP not only does not require thiol-reducing agents for activity, but these agents actually inhibit its activity, suggests that the mechanism of PiALP action must be different from that of the known PTPases.

In conclusion, we have purified a novel prokaryotic ALPase containing a PTPase activity. Cloning and complete sequencing of the PiALP gene and further studies of its structure and function will help clarify the mechanism behind its unique biochemical properties as described here.

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