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Comparative Studies of Acid and Alkaline Phosphatases from *Bacteroides melaninogenicus*

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Summary

Acid and alkaline phosphatases from an oral strin of *Bacteroides melaninogenicus* were purified to homogeneity from cell extracts and their properties were compared. Molecular weights of acid and alkaline phosphatases were estimated to be 62,000 and 160,000, respectively. Michaelis constant for *p*-nitrophenylphosphate of the acid phosphatase was 0.17 mM and that of the alkaline enzyme was 0.23 mM. The alkaline phosphatase was more stable than the acid phospatase when they were heated at 60℃. The activity of the acid phosphatase was largely reduced by Cu^{2+} or fluoride and the alkaline phosphatase was quite sensitive to inhibition by Zn^{2+}, thiol compounds, or EDTA. The inactivation by EDTA of the alkaline phosphatase was restored with Ca^{2+} or Mg^{2+}.

The acid phosphatase hydrolyzed α-D-glucose 1.6-diphosphate, *p*-nitrophenylphosphate, D-glucose 6-phosphate, and D-fructose 6-phosphate. On the other hand, *p*-nitrophenylphosphate was the most suitable substrate for the alkaline phosphatase. Nucleoside triphosphates were also hydrolyzed by the alkaline phosphatase.

INTRODUCTION

Various microbial acid phosphatases (EC 3. 1. 3. 2) and alkaline phosphatases (EC 3. 1. 3. 1) have been purified and characterized, including those of *Escherichia coli* (10, 24, 28), *Salmonella typhimurium* (36), *Fusarium moniliforme* (38), *Pseudomonas aeruginosa* (4), *Pseudomonas fluorescens* (8), *Bacillus licheniformis* (12), *Micrococcus sodonensis* (11), and *Staphylococcus aureus* (17, 18).

The alkaline phosphatase of *E. coli* is the most extensively studied. The enzyme has a molecular weight of 86,000 (28), contains zinc atoms (31), and is composed of two identical subunits (28). Neu and Heppel (20) demonstrated that the enzyme is localized in the periplasmic space of the cell and it coule be released by osmotic shock.

In genus *Bacteroides,* Porschen and Spaulding demonstrated in the studies of phosphatase activity of various anaerobic bacteria, the production of phosphatase in *B. melaninogenicus* (23). Rudek and Haque also found the production of phosphatase from this organism (25). However, isolation and properties of this enzyme from *B. melaninogenicus* have not been discussed. In *B. thetaiotaomicron,* Salyers and O'brien (26) confirmed that 55 to 65% of alkaline phosphatase was released from the periplasmic space by the treatment of the cells with EDTA and lysozyme in 20%

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sucrose solution. Olsson et al. (22) noted, however, that no alkaline phosphatase activity was liberated by the osmotic shock in *B. fragilis*.

Recently, we applied the osmotic shock technique to *B. melaninogenicus* cells, from which no significant amount of phosphatase was extracted (unpublished observations). However, we found sufficient activities of acid and alkaline phosphatases in the cell extract obtained with ultrasonic treatment. Thus, we undertook to purify acid and alkaline phosphatases and compared their enzymatic properties.

MATERIALS AND METHODS

Bacterial strain and cultivation. Bacteroides melaninogenicus subspecies *melaninogenicus* strain NM-3 was isolated from human gingival crevice deposits (19). Cultures of this organism were grown anaerobically for 5 days at 37°C as described earlier (9, 19). The composition of the medium per liter was as follows; 17g of Trypticase (BBL), 3g of yeast extract (Difco), 5g of NaCl, 2.5g of K_2HPO_4, 0.5g of sodium thioglycollate, 5mg of hemin, and 0.5mg of menadione (27).

Enzyme assay. Acid and alkaline phosphatases activities were estimated by the rate of liberation of *p*-nitrophenol from *p*-nitrophenylphosphate (PNPP).

Reaction mixture consisted of 0.1ml of enzyme, 3ml of 2 mM PNPP dissolved in water and 0.5 ml of 0.72 M acetate buffer, pH 5.5 (for acid phosphatase assay) or 5.5ml of 0.72 M tris (hydroxymethyl) aminomethane (Tris) -hydrochloride buffer (pH 8.5) (for alkaline phosphatase assay). After incubation at 37°C for 20 min, the reaction was stopped by the addition of 1 ml of 0.2 M NaOH.

The liberated *p*-nitrophenol was measured by absorbance at at 410nm. One unit of activity was defined as the amount of enzyme which liberates 1μmole of *p*-nitrophenol per min. The rates of reaction were linear with respect to time and enzyme concentration under these conditions.

Assay of proteins. Protein was estimated by the method of Lowry et al. (16), using bovine serum albumin as a standard.

Assay of inorganic phosphate. In the examination of phosphatases activities toward a number of phosphorylated compounds, the liberated inorganic phosphate was determined according to the method of Fiske and Subbarow (7).

Determination of optimum pH. Acid and alkaline phosphatases activities were assayed at different pH values using appropriate buffers at a final concentration of 0.1 M. The buffers employed were acetate buffer (pH 4.5 to 6.5), Tris-hydrochloride buffer (pH 7.0 to 8.5), and carbonate-bicarbonate buffer (pH 9.0 to 11.0).

Polyacrylamide gel electrophoresis. To examine purity of samples, polyacrylamide gel electrophoresis was carried out using 7.5% acrylamide at pH 9.1 according to the method of Davis (3).

Determination of molecular weights and Stokes radii. The molecular weights of the purified acid and alkaline phosphatases were estimated by gel filtration on a Sephadex G-200 column (2.6 by 100cm). Blue dextran and standard proteins including aldolase, bovine serum albumin, ovalbumin, and cytochrome C were used as reference. The column was eluted with 0.05 M Tris-hydrochloride buffer (pH 8.5) containing 0.15 M NaCl at a flow rate of 16ml/h. From the plot of Kav values against logarithm of the corresponding molecular weights, the molecular weights of both enzymes were determined (6). Stokes radii were estimated by the method of Siegel and Monty (30).

Purification of acid and alkaline phosphatases. All procedures for purification were carried out at 0°C— 4 °C, unless otherwise specified.

(i) Cell extract: From the fully grown cultures (15 liter) of strain NM-3, 90g of the cells wet weight were harvested. The cells suspended in 0.05M Tris-hydrolchloride buffer (pH 8.5) were broken by ultrasonic treatment using a Kubota Insonator, model 200M at 9KHz. for 20 min. A crude extract was obtained by centrifugation of the mixture at 100,000 $\times g$ for 1h.

(ii) Fractionation with ammonium sulfate: Solid ammonium sulfate was added to the extract to give 70% saturation with constant stirring and the mixture was allowed to stir for an aditional 3h. The resultant precipitate was collected by centrifugation at 10,000 $\times g$ for 20 min, dissolved in 0.05 M Tris-hydrochloride buffer (pH 8.5) and dialyzed against this buffer. A small amount of insoluble material in the dialyzed sample was removed by centrifugation at 20,000 $\times g$ for 10 min.

(iii) DEAE-cellulose column chromatography: The dialyzed sample (110 ml) was applied to a DEAE-cellulose column (2.6 by 40cm), previously equilibrated with 0.05 M Tris-hydrochloride buffer (pH 8.5), and the column was washed with 1,000ml of the same buffer. In the washings of the column, acid phosphatase actitvity was detected. The active fractions were combined and concentrated by a rotory evaporator in vacuo at 30°C, and dialyzed against the same buffer containing 0.15 M NaCl. The DEAE-cellulose column was then eluted with a linear gradient of NaCl concentration, produced by mixing 600ml of 0.05 M Tris-hydrochloride buffer (pH 8.5) and 600ml of the same buffer containing 0.7 M NaCl. The alkaline phosphatase eluted at about 0.2 M NaCl. The active fractions were pooled, concentrated, and dialyzed as above. The approximate ratio of the acid phosphatase activity to the alkaline phosphatase recovered in this step was 3 : 1.

(iv) Sephadex G-200 chromatography: The concentrated active fractions of both enzymes from the DEAE-cellulose column were applied separately to gel filtration on a Sephadex G-200 column (2.6 by 100cm), previously equilibrated with 0.05 M Tris-hydrochloride buffer (pH 8.5) containing 0.15 M NaCl, and eluted with this buffered saline at a flow rate of 16 ml/h. Under these conditions of gel filtration, the alkaline phosphatase eluted faster than acid phosphatase. The pooled acid and alkaline phosphatases active fractions were dialyzed against 0.02M acetate buffer (pH 5.1) and against 0.05M acetate buffer (pH 5.9), respectively.

(v) CM-cellulose column chromatography: Acid phosphatase active material from the previous step was applied to a CM-cellulose column (1.6 by 49cm), equilibrated with 0.02 M acetate buffer (pH 5.1). After the column was washed with this buffer until the absorbance at 280 nm of the eluates became less then 0.02, the enzyme was eluted with a linear gradient of NaCl produced by mixing 250ml of 0.02 M acetate buffer (pH 5.1) and 250 ml of the same buffer containing 0.7 M NaCl. Similarly, alkaline phosphatase active fractions were chromatographed on a CM-cellulose column (1.6 by 30cm) with 0.05 M acetate buffer (pH 5.9). In both cases, the enzymes eluted in a single peak of activity. Finally, active fractions were combined and equilibrated with 0.05 M Tris-hydrochloride buffer (pH 8.5) by dialysis.

These preparations were used for the investigation of the biochemical properties of the enzymes.

RESULTS

Purification. Table 1 presents a summary of the results obtained through the succesive steps in the enzyme purification procedures. Acid and alkaline phosphatases from *B. melaninogenicus* NM-3 were purified 204 fold and 229 fold with a recovery of 15% and 11%, respectively. The purified preparations gave single protein bands on polyacrylamide gel electrophoresis (Fig. 1).

Table 1 : Summary of purification of acid and alkaline phosphatase

Prepn	Acid phosphatase				Alkaline phosphatase			
	Protein (mg)	Activity (U)	SP act (a)	Yield (%)	Protein (mg)	Activity (U)	SP act (a)	Yield (%)
Crude extract	1,935	3,430	1.8	100	1,935	2,620	1.4	100
$(NH_4)_2SO_4$ precipitate	1,292	2,850	2.2	83	1,292	2,070	1.6	79
DEAE -cellulose chromatography, concentrate	150	2,340	15.6	68	88	1,550	17.6	59
Sephadex G-200	13.7	2,024	147.7	59	6.8	1,130	166.2	43
CM-cellulose chromatography	1.4	515	367.9	15	0.9	288	320.0	11

(a) activity (U)／total protein (mg)

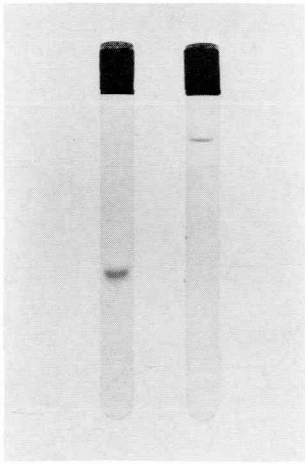

Fig. 1 : Polyacrylamide gel electrophoresis of the purified acid phosphatase (left) and alkaline phosphatase (right). Migration is from top to bottom.

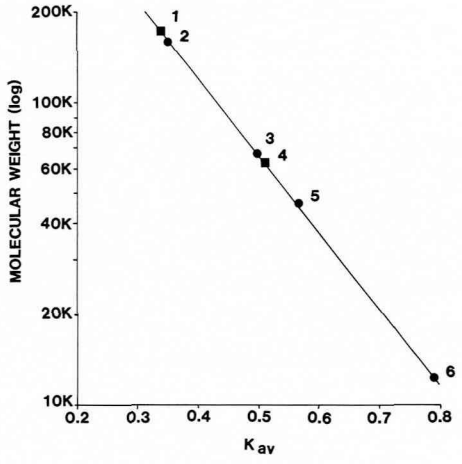

Fig. 2 : Estimation of molecular weights of acid and alkaline phosphatase.
1. alkaline phosphatase, 2. aldolase, 3. bovine serum albumin, 4. acid phosphatase, 5. ovalbumin, 6. cytochrome C

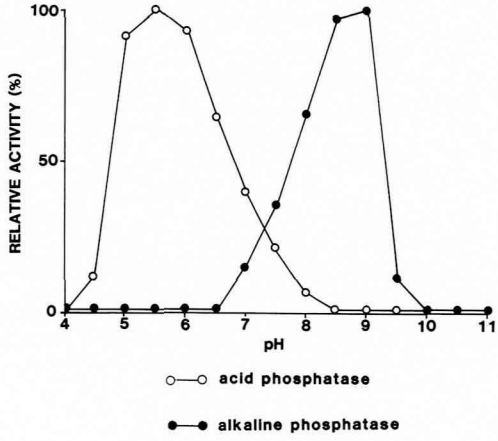

Fig. 3 : Optimum pH

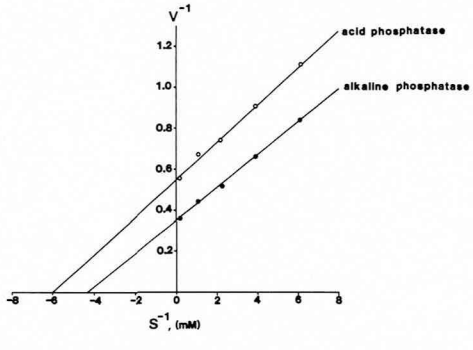

Fig. 4 : Lineweaver and Burk plots of acid and alkaline phosphatase

Molecular weights and Stokes radii. Molecular weights of 62,000 for acid phosphatase and 160,000 for alkaline phosphatase were calculated from Sephadex G-200 gel filtration results (Fig. 2). Stokes radii were estimated to be 3.7 nm and 4.8 nm for the acid and alkaline phosphatases, respectively.

Optimum pH. The pH profiles of enzyme activities of the phosphatases are shown in Fig. 3. The acid phosphatase had a pH optimum between 5.0 to 6.0. The alkaline phosphatase optimum was between 8.5 to 9.0. A remarkable lowering of the activity was noticed at higher pH value than 9.0 in the alkaline enzyme.

Michaelis constants for PNPP. Km values for PNPP were determined from double reciprocal plots of reaction velocity versus concentration of substrate. The acid and alkaline phosphatases had

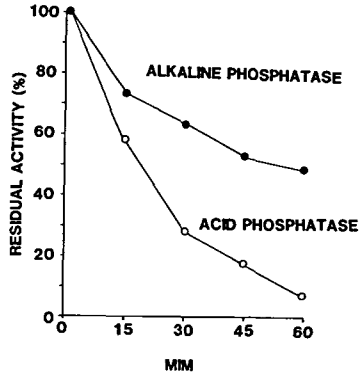

Fig. 5 : Thermostability at 60℃ of acid and alkaline phosphatase

Table 2 : Effect of various chemicals on acid and alkaline phosphatase

Reagents	Concentration (mM)	Acid phosphatase Relative act.	Alkaline phosphatase Ralative act.
None	-	100	100
Co^{2+}	1	94	71
Ca^{2+}	1	94	105
Cu^{2+}	1	16	47
Zn^{2+}	1	83	9
Mg^{2+}	1	123	103
NaF	1	30	102
	10	8	37
KH_2PO_4	1	80	61
	10	72	19
Mercapto-ethanol	1	97	46
	10	102	3
Dithio-threitol	1	87	0
	10	74	0
NaN_3	1	95	104
	10	96	97
EDTA	1	102	0
	10	100	0

Each chemical was added to standard assay components at indicated concentration. The activities are expressed as relative number to contorols. Both enzyme preparations contained 0.17 U/ml enzyme activity.

Table 3 : Recovery of the activity of the alkaline phosphatase from inhibition by EDTA

Materials		Relative Activity
Before EDTA addition		100
After EDTA addition		0
After dialysis		3
After dialysis		
plus Ca^{2+}	1 mM	52
	10 mM	98
plus Mg^{2+}	1 mM	60
	10 mM	96
plus CO^{2+}	1 mM	13
	10 mM	17
plus Cu^{2+}	1 mM	0
plus Zn^{2+}	1 mM	0

EDTA was added to the alkaline phosphatase (0.17 U/ml) at 1 mM, followed by dialysis of this mixture (4 ml) against 2 liter of 0.05 M Tris-hydrochloride buffer (pM 8. 5) for 18 h. After dialysis, each metal ion was added to standard assay components. The activities are relative to control without EDTA.

Km values of 0.17 mM and 0.23 mM, respectively (Fig. 4).

Stability. The enzymatic activities of both phosphatases were adjusted to the same level (0.17 U/ml) and they were heated at 60℃ for 60 min in 0.05 M Tris-hydrochloride buffer (pH 8.5) to compare thermostability. The heated acid phosphatase exhibited only 7% of the activity observed before heating. However, in the alkaline phosphatase, 48% of the original activity remained (Fig. 5). No significant loss of activity of either enzyme was observed on storage at −40℃ for a month in 0.05 M Tris-hydrochloride buffer (pH 8.5).

Effect of various chemicals on the activities. The possible activating and inhibitory effects of various chemicals on the purified preparations of the phosphatases (0.17 U/ml) were investigated. Metal salts (1 mM in final concentration) or other low molecular weight compounds (1 mM and 10 mM, final concentration) were added to the assay components. Results are given in Table 2. In the acid phosphatase, it was slightly activated by Mg^{2+}. $Cu^{2+}$and NaF at 10 mM inhibited strongly. The alkaline phosphatase was quite sensitive to inhibition by Zn^{2+}, thiol reagents, and EDTA. Examination was carried out to determine whether the activity could be restored by dialysis or by addition of some metal ions in EDTA-treated alkaline phosphatase (Table 3). Addition of EDTA to the alkaline phosphatase solution (0.17 U/ml) resulted in complete loss of enzymatic activity. When this mixture was dialyzed against Tris-hydrochloride buffer (pH 8.5), only 3% of the original activity reappeared. However, when Ca^{2+} or Mg^{2+} was added to this dialyzed material in addition to the standard assay components, almost full activity was recovered. No incubation was required for this restoration; enzymatic activity was restored immediately upon the addition of Ca^{2+} or Mg^{2+}. Co^{2+} was slightly effective but Cu^{2+} and Zn^{2+} were ineffective.

Table 4 : Substrate specificity

Phosphorylated compounds	Relative activity	
	Acid phosphatase	Alkaline phosphatase
PNPP	100	100
5′-AMP	30	8
5′-ATP	14	50
5′-GTP	20	48
5′-UTP	25	58
Adenosine 3′,5′-cyclicmonophosphate	0	0
D-Glucose 6-phosphate	90	3
α-D-Glucose 1-phosphate	3	25
α-D-Glucose 1.6-diphosphate	120	20
D-Fructose 6-phosphate	88	5
D-Fructose 1.6-diphosphate	48	15
6-Phosphogluconic acid	26	30
NAD	0	0
NADP	0	18
β-Glycerophosphate	3	2
Phosphoenolpyruvate	1	12
O-Phosphoserine	2	6
Pyrophosphate	65	53

The listed phosphorylated compounds were incubated with acid or alkaline phosphatase (0.17 U/ml). The rate of hydrolysis relative to PNPP was determined. Each compound (2 mM) was used as a substrate component of the assay mixture. Liberated inorganic phosphate was measured by the method of Fiske and Subbarow (7).

Treatment of both phosphatases with periodate at 10 mM reduced the activities to 28% in the acid phosphatase and 43% in the alkaline phosphatase.

Substrate specificity. The relative rates of hydrolysis of a number of phosphorylated compounds by the acid and alkaline phosphatases are documented in Table 4. The relative activities of the enzymes on the listed substrates are shown with respect to PNPP. The acid phosphatase was active toward PNPP, D-glucose 6-phosphate, α-D-glucose 1.6-diphosphate, D-fructose 6-phosphate, and pyrophosphate. In the alkaline phosphatase, the rate of the hydrolysis was highest toward PNPP and moderate activity toward 5′-ATP, 5′-GTP, 5′-UTP and pyrophosphate was observed. Both enzymes exerted little or no hydrolytic activity against the remaining substrates.

DISCUSSION

Bacteroides melaninogenicus appears to play a major role in the pathogenesis of periodontal disease (21, 32, 33). However, relatively little is known about the metabolism and enzymatic properties of these bacteria.

The acid and alkaline phosphatases were separated by chromatography on DEAE-cellulose. The ratio of the activity of each phosphatase was relatively close in this step, and this facilitated the further purification of these enzymes.

In overall purification, either in fold purification or in recovery, no great differences were found between the two preparations. However, the properties of each are quite different, such as molecular sizes, optimum pH, thermostability, sensitivity to the several inhibitors, substrate specificity.

The alkaline phosphatase presented in this communication is quite sensitive to the inihibition by EDTA. This inhibition was reversible with the addition of Ca^{2+} or Mg^{2+}. Several lines of observations like these findings have been reported in another bacterial phosphatases, for example, *Bacillus subtilis* alkaline phosphatase was inhibited by EDTA and the inhibition could be reversed with Zn^{2+} or Co^{2+} (34). In *Micrococcus sodonensis,* alkaline phosphatase was completely inactivated by EDTA and Ca^{2+} was responsible for the restoration, but Mg^{2+} was ineffective and the authors suggested that calcium was an essential component of this enzyme (11). *E. coli* alkaline phosphatase is, too, inhibited almost completely by EDTA and is instantaneously reversed by addition of stoichiometric amounts of Zn^{2+} (35). *E. coli* alkaline phosphatase is generally accepted to contain zinc as a molecular component (31). However, in the case of *Pseudomonas fluorescens* (8) alkaline phosphatase which is completely inactivated by EDTA, the inactivation could never be restored with many metal ions including Mg^{2+} or Ca^{2+}.

The behavior of our phosphatases on DEAE- and CM-cellulose columns resembled that of nonspecific phosphatase of *E. coli,* described by Dvorak et al. (5), namely the latter enzyme which passed through DEAE-cellulose column at pH 7.4 was effectively fractionated by CM-cellulose column chromatography using a gradient elution of NaCl with 0.005M acetate buffer (pH 6.0).

Neither the acid nor the alkaline phosphatase had requirement for divalent ions for the expression of the catalytic activity, only Mg^{2+} stimulated slightly the acid phosphatase. Cu^{2+} and Zn^{2+} were inhibitory to the acid and the alkaline phosphatases, respectively. Yoshida and Tamiya reported in acid phosphatase of *Fusarium moniliforme,* Cu^{2+} and fluoride were inhibitory and EDTA or thiol reagents did not influenced the activity (38). The similar tendency can be found in the acid phosphatase presented here.

The alkaline phosphatase may contain an essential disulfide group for the activity, since it is inactivated readily with the thiol reagents, such as mercaptoethanol or dithiothreitol.

Apparent molecular weight of the acid phosphatase was estimated to be 62,000 on Sephadex G-200 gel filtration. This value is close those of acid phosphatase from *Staphylococcus aureus* (58,000) (17) and *Salmonella typhimurium* (54,000) (37). The latter *Salmonella* enzyme is a dimer of 27,000 subunits. On the other hand, a value of 160,000 for the alkaline phosphatase of *B. melaninogenicus* seems to be rather high. The molecular weights of alkaline phosphatases from several microbial species were reported to be 80,000 to 130,000 (2, 11, 12, 13, 15, 35). However, much higher values, for example, 170,000 in *Blastodiella emersonii* (29) and 154,000 in *Neurospora crassa* (14), were also described. In *E. coli* (1, 28), *P. aeruginosa* (2), *B. licheniformis* (13), *B. subtilis* (39), and *N. crassa* (14), alkaline phosphatases are composed of subunits. The possibility of molecular polymerization in the *B. melaninogenicus* alkaline phosphatase remains to be investigated.

Both phosphatases were inhibited significantly by the treatment with periodate. This suggests that the enzymes contain carbohydrate moieties which are related to the enzymatic activity.

It was reported that some bacterial acid phosphatases exhibited hexose phosphatase activity (5, 37). Our acid phosphatase exerted also this tendency ; it hydrolyzed actively D-glucose 6-phosphate, α-D-glucose 1.6-diphosphate, and D-fructose 6-phosphate, although it acted only quite weakly on α-D-glucose 1-phosphate. As for the alkaline phosphatase of *B. melaninogenicus,* it was more active against nucleoside triphosphates than phosphorylated hexoses. This is the most different event in the substarate specificity between the two enzymes.

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