Isolation and Properties of a Small Manganese-Ion-Stimulated Bacterial Alkaline Phosphatase

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1. An alkaline phosphatase was partially purified from extracts of *Halobacterium cutirubrum*. 2. The enzyme has a mol.wt. of 15500 and is therefore less than one-quarter of the size of other known bacterial alkaline phosphatases. 3. It is stimulated up to ten-fold by Mn^{2+} , but not by Ca^{2+} or Mg^{2+} . 4. The activities with and without Mn^{2+} cannot be separated by gel filtration and have similar restricted substrate specificities. 5. The only substrates for the enzyme that have so far been found are *p*-nitrophenyl phosphate, 5'-dATP, 5'-dTTP and 5'-dTTP.

Alkaline phosphatases have been isolated from a wide variety of animal and microbial sources (Stadtman, 1961; Fernley, 1971; Reid & Wilson, 1971). They have molecular weights in the range 70000–200000 and appear to be dimers. The *Escherichia coli* enzyme has been particularly well studied (Reid & Wilson, 1971), since the discovery by Horiuchi *et al.* (1959) and Torriani (1960) that its synthesis was repressed by orthophosphate. Its molecular weight was originally reported at 75000–80000 (Garen & Levinthal, 1960), but subsequent studies gave values from 67000 to 110000, depending on the experimental conditions (Reid & Wilson, 1971). The molecular weight of *Bacillus subtilis* alkaline phosphatase was found to be 69000 by Glenn & Mandelstam (1971), and Bhatti (1974) reported a value of 100000 for the *Serratia marcescens* enzyme, both within the range observed for the *E. coli* phosphatase. Available evidence suggests that both the animal and bacterial enzymes contain bound Zn^{2+} as an essential cofactor that can only be replaced partially by Co^{2+} and, even less effectively, by Mg^{2+} (Fernley, 1971; Reid & Wilson, 1971). They also appear to be essentially non-specific phosphatases that catalyse the hydrolysis of a wide variety of phosphorylated substrates, including *p*-nitrophenyl phosphate, the standard substrate used in alkaline phosphatase assays since its introduction for this purpose by Garen & Levinthal (1960).

Nucleotide phosphomonoesterases, or nucleotidases, are a more specific group of phosphatases that are also widely distributed among animals and microorganisms (Drummond & Yamamoto, 1971). The molecular weights of the known bacterial 5'-nucleotidases range from 44000 to 137000, and they all hydrolyse a wide range of ribo- and deoxyribonucleotides and sugar nucleotides. However, *p*-nitrophenyl phosphate does not appear to be a substrate. Many of these enzymes require a bivalent

cation for activity and are active below pH7, but there are exceptions.

During studies of nucleic acid metabolism in the extreme halophile *Halobacterium cutirubrum*, we observed an alkaline phosphatase that was both unusually small and stimulated by Mn^{2+} . The enzyme hydrolyses *p*-nitrophenyl phosphate, but the only other substrates found for it so far are a limited number of deoxyribonucleotides. In the present paper, we describe the partial purification and properties of this enzyme.

Experimental

Materials

Chemicals and substrates were from the following suppliers: *p*-nitrophenyl phosphate (disodium salt), BDH Chemicals, Toronto, Ont., Canada; tris-(hydroxymethyl)methylaminopropanesulphonic acid Calbiochem, La Jolla, CA, U.S.A.; Blue Dextran, Sephadex G-100 and Sepharose 6B, Pharmacia (Canada), Dorval, P.Q., Canada; nucleotides, P-L Biochemicals Inc., Milwaukee, WI, U.S.A., or Sigma Chemical Co., St Louis, MO, U.S.A.; crystalline bovine serum albumin, ovalbumin grade V, α -chymotrypsinogen A type II (ox pancreas), cytochrome *c* type VI (horse heart), sugar phosphates and Tris (Trizma base), Sigma Chemical Co. All other chemicals were from Fisher Scientific Co., Ottawa, Ont., Canada.

Buffers. The pH of buffers refers to the value at 25°C. High-salt buffer had the following composition; 2.5 M-KCl/1 M-NaCl/10 mM-Tris/HCl, pH 8.6

Cell culture

H. cutirubrum N.R.C. 34001 cells were grown in Gochnauer & Kushner's (1969) complex medium as

previously described (Fitt *et al.*, 1975; Peterkin & Fitt, 1971). They were harvested in mid-exponential phase after 48 h of growth, when the culture has an E_{660} of approx. 1.98 (with the complex medium as blank) and the number of viable cells was approx. 2.3×10^9 /ml.

Isolation of *H. cutirubrum* alkaline phosphatase

All operations were done at 0–5°C. Concentrations of $(\text{NH}_4)_2\text{SO}_4$ were calculated by the method of Green & Hughes (1955).

The bacteria (12 g wet wt./litre of growth medium) were harvested by centrifugation at $27000g_{\text{max}}$ for 20 min. They were carefully dispersed in high-salt buffer (3 ml/g wet wt.) and stirred magnetically to give a smooth suspension as described by Louis & Fitt (1972). The suspension was sonicated for 1 min with a Bronwill Biosonik II sonic disintegrator (large probe, setting 60). The homogenate was centrifuged at $45000g_{\text{max}}$ for 20 min to give the crude extract and a pellet that was discarded. The crude extract was dialysed for a total of 2 h against two changes of 60 vol. of 10 mM-Tris/HCl, pH 8.6. The dialysed extract was fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation and the protein insoluble between 40% and 80% saturation was dissolved in high-salt buffer and dialysed overnight against two changes of 50 vol. of high-salt buffer to give the $(\text{NH}_4)_2\text{SO}_4$ fraction. Samples (6 ml) of the latter were passed through a column (2.5 cm $\times$ 90 cm) of Sephadex G-100 prepared and run by upward flow as described previously for Sephadex G-200 gel filtration in high-salt buffer (Louis *et al.*, 1971). The active fractions emerged in a single peak with a K_{av} of 0.60. They were pooled and concentrated against an Amicon UM10 membrane filter (Amicon Corp., Lexington, MA, U.S.A.) to give the Sephadex G-100 fraction.

Enzyme assays

H. cutirubrum alkaline phosphatase was assayed by measuring the rate of hydrolysis of *p*-nitrophenyl phosphate in its presence (Garen & Levinthal, 1960). The standard assay mixture (final volume 0.5 ml) contained: 30 mM-Tris/HCl, pH 8.6; 8 mM-*p*-nitrophenyl phosphate (disodium salt); 1.5 M-KCl; 0.6 M-NaCl; 1 mM-disodium EDTA; enzyme. Incubation was at 37°C for 15 min, 0.05 M-NaOH (0.5 ml) was added and the E_{400} of the mixture was then measured spectrophotometrically by using a control lacking enzyme as a blank. A unit of alkaline phosphatase activity was defined as the amount of enzyme catalysing the liberation of 1 nmol of *p*-nitrophenol/min. The ϵ_{400} of *p*-nitrophenol was taken to be 18300 (Dawson *et al.*, 1969). For assay of the Mn^{2+} -stimulated activity, 5 mM- MnCl_2 was included in the assay medium. After incubation at 37°C, the reaction was

stopped by heating at 100°C for 1 min, an equal volume of 10 mM-Tris/HCl, pH 8.6, was added and the E_{400} was determined immediately. [NaOH cannot be used in the presence of Mn^{2+} , because it causes the precipitation of $\text{Mn}(\text{OH})_2$.]

Alternatively, the reactions in the absence and presence of Mn^{2+} were followed directly with a Zeiss PMQ II spectrophotometer fitted with an automatic zero, a transmission-extinction converter, a recorder and a thermostatically controlled cell holder.

Orthophosphate assays

Orthophosphate was determined by the methods of Fiske & SubbaRow (1925) or Marsh (1959) scaled down to a total volume of 1 ml.

Protein assay

Protein was determined spectrophotometrically (Warburg & Christian, 1942).

Results and Discussion

Table 1 summarizes the results of a typical isolation of *H. cutirubrum* alkaline phosphatase. The purifications have been limited to about 20-fold by the instability of the enzyme in the conditions tried so far.

The molecular weight of the partially purified enzyme was determined by analytical gel filtration on Sephadex G-100 and found to be 15500 (Table 2). The peaks of Mn^{2+} -independent and Mn^{2+} -stimulated activity (see below) corresponded exactly. The enzyme is therefore much smaller than any known bacterial or eukaryote alkaline phosphatase and is the fourth unusually small enzyme to be isolated from *H. cutirubrum*, the other three being polynucleotide phosphorylase, DNA-dependent RNA polymerase and RNA-dependent RNA polymerase (Fitt *et al.*, 1975; Louis & Fitt, 1971*a,b*, 1972; Louis *et al.*, 1971; Peterkin & Fitt, 1971). Further work will be necessary to establish if a significant proportion of *H. cutirubrum* enzymes are of low molecular weight compared with their non-halophile counterparts. Aitken & Brown (1972) have reported a value of 70800 for *H. cutirubrum* isocitrate dehydrogenase at low ionic strength and 135000 in the presence of 4 M-KCl, and our results show that both the adenine phosphoribosyltransferase and L-alanine dehydrogenase of this organism have molecular weights in the range 70000–85000 at high ionic strength (P.S. Fitt, E. K.-K. Kim & P. I. Peterkin, unpublished work), so small size is not necessarily characteristic of enzymes from extreme halophiles. However, the fact that four out of the few *H. cutirubrum* enzymes so far studied in any detail have this property is of great interest.

H. cutirubrum alkaline phosphatase is also unusual

Table 1. *Isolation of H. cutirubrum alkaline phosphatase*

The enzyme was isolated from *H. cutirubrum* (12g wet wt.) and its activity measured in the standard assay as described in the Experimental section.

Fraction	Vol. (ml)	Activity (units)	Sp. activity (units/mg)	Relative purification	Yield (%)
Crude extract	32	2912	4.1	1.0	100
(NH ₄) ₂ SO ₄ fraction	12.8	1635	12.4	3.0	56
Sephadex G-100 fraction	10.0	700	92.1	22.5	24

Table 2. *Molecular weight of H. cutirubrum alkaline phosphatase*

The molecular weight of *H. cutirubrum* alkaline phosphatase was determined by analytical gel filtration on columns (2.5 cm × approx. 90 cm) of Sephadex G-100 that were calibrated with the standard proteins indicated. The gel was swollen and the columns run in high-salt buffer as described for Sephadex G-200 columns by Louis *et al.* (1971). K_{av} values were calculated from the void volumes (V_0 , determined with Blue Dextran), the calculated bed volumes (V_t) and the measured elution volumes (V_e) by the standard method (Fischer, 1969). In Expt. 1, $V_0 = 160$ ml and $V_t = 439$ ml. In Expt. 2, $V_0 = 172$ ml and $V_t = 452$ ml. The amount of *H. cutirubrum* alkaline phosphatase applied to the columns was 766 units in Expt. 1 and 306 units in Expt. 2, based on the standard assay without Mn²⁺. The molecular weights of the standard proteins are those given by Determann (1968) and Fish *et al.* (1969).

Protein	Expt. 1		Expt. 2		mol.wt.
	V_e (ml)	K_{av}	V_e (ml)	K_{av}	
Cytochrome <i>c</i>	336	0.63	354	0.65	12400
α -Chymotrypsinogen A	300	0.50	318	0.52	25000
Ovalbumin	260	0.36	278	0.38	45000
Bovine serum albumin	220	0.22	—	—	67000
<i>H. cutirubrum</i> alkaline phosphatase					
(a) Mn ²⁺ -independent	328	0.60	344	0.62	15500 (Expt. 1) 15500 (Expt. 2)
(b) Mn ²⁺ -stimulated	—	—	344	0.62	15500

Table 3. *Effect of bivalent cations on the activity of H. cutirubrum alkaline phosphatase*

The activity of *H. cutirubrum* alkaline phosphatase was measured in the standard assay conditions with the indicated concentrations of bivalent cations. The P_i released in the presence of 5 mM-Mn²⁺ was assayed by the method of Marsh (1959) and found to be 5.6 nmol/min.

Bivalent cation concn. (mM)	Cation . . .	<i>p</i> -Nitrophenol released (nmol/min)				
		Ca ²⁺	Co ²⁺	Mg ²⁺	Mn ²⁺	Zn ²⁺
0		0.50	0.50	0.50	0.50	0.50
1.25		—	—	—	0.70	—
2.5		—	—	—	2.0	—
5		0.49	0.77	0.47	5.5	0
20		0.46	0.72	0.43	4.5	0
50		0.46	0.75	0.44	1.2	0

in comparison with similar enzymes (Stadtman, 1961; Reid & Wilson, 1971; Fernley, 1971) in being stimulated up to tenfold by Mn²⁺ with *p*-nitrophenyl phosphate as substrate (Table 3). Ca²⁺ and Mg²⁺ had little or no effect on the rate of the reaction when present at the optimum concentration for Mn²⁺, but inhibited

the enzyme slightly at higher concentrations, whereas Zn²⁺ inhibited *p*-nitrophenyl phosphate hydrolysis completely. Co²⁺ stimulated the enzyme by about 50%, but was ineffective compared with Mn²⁺ even when used at ten times the optimum concentration of the latter (5 mM). In the presence of 5 mM-Mn²⁺,

Fig. 1. Gel filtration of *H. cutirubrum* alkaline phosphatase on a Sepharose 6B column

H. cutirubrum alkaline phosphatase ($(\text{NH}_4)_2\text{SO}_4$ fraction (Table 1) was passed through a column (2.5 cm $\times$ 88 cm) of Sepharose 6B agarose gel prepared and run in high-salt buffer as described by Louis *et al.* (1971) for Sephadex G-200, except that it was not necessary to swell the agarose gel (supplied in pre-swollen form by the manufacturers) in high-salt buffer in order to obtain reproducible results. The void and bed volumes were 172 ml and 432.5 ml respectively, and 4 ml fractions were collected. The E_{280} and the alkaline phosphatase activity in the standard assay with and without Mn^{2+} were determined for every third fraction. $\bullet$, E_{280} ; Δ , enzyme activity without Mn^{2+} ; $\square$, enzyme activity with Mn^{2+} . (Note the difference in scales for activity with and without Mn^{2+} .)

Table 4. Stability of *H. cutirubrum* alkaline phosphatase at high and low ionic strength

A portion of the Sephadex G-100 fraction (Table 1) in high-salt buffer was dialysed for a total of 2 h against two changes of 100 vol. each of 10 mM-Tris/HCl, pH 8.6. The salt-free enzyme and an undialysed sample of the Sephadex G-100 fraction were then stored at 4°C and assayed at intervals in the conditions of the standard assay (i.e. with 1.5 M-KCl/0.6 M-NaCl with and without 5 mM-MnCl₂). After 24 h, the salt-free enzyme was dialysed for 72 h against several changes of 4.3 M-NaCl/10 mM-Tris/HCl, pH 8.6, and re-assayed with and without Mn^{2+} .

Time (days)	Residual activity (%)			
	High ionic strength		Low ionic strength	
	-Mn ²⁺	+Mn ²⁺	-Mn ²⁺	+Mn ²⁺
0	100	100	100	100
1	93	52	7.8	5.6
2	82	44	—	—
7	71	41	—	—
After dialysis	—	—	5.3	5.6

5.6 nmol of P_i /min, were released, in excellent agreement with the rate (5.5 nmol/min) of *p*-nitrophenol formation in the same reaction mixture.

A portion of the $(\text{NH}_4)_2\text{SO}_4$ fraction was passed through a calibrated Sepharose 6B agarose gel filtration column (Fig. 1) to see if any alkaline phosphatases of higher molecular weight were present. It can be seen that all the phosphatase active against

p-nitrophenyl phosphate emerged in a single peak near the total volume of the column and that once again the activities with and without Mn^{2+} travelled together. Although the alkaline phosphatase was too highly retarded to be in the region of proportionality between K_{av} and $\log(\text{mol. wt.})$ with Sepharose 6B, it was eluted shortly before cytochrome *c*, as predicted from its behaviour on Sephadex G-100 columns.

The results in Table 4 show that the Mn^{2+} -stimulated activity was significantly less stable than the Mn^{2+} -independent one at high ionic strength, although both were irreversibly denatured after 24 h at low ionic strength. Indeed, we have occasionally observed as much as a 30-fold stimulation by Mn^{2+} of the alkaline phosphatase activity of crude extracts, but it has rapidly declined to tenfold or less during storage and purification. This instability accounts for variable degrees of Mn^{2+} -stimulation reported in subsequent Tables.

EDTA eliminates the Mn^{2+} -stimulated activity, as expected, but even at a concentration of 10 mM had comparatively little effect on the activity in the absence of the cation (Table 5). Thus if any other bivalent cation is necessary for activity of the *H. cutirubrum* phosphatase, it must be very tightly bound.

In the conditions of the standard assay, the reaction was linear with time for 15 min with up to 20 units and was unaffected by changes in Tris concentration in the range 0.01–0.3 M. The rate of hydrolysis of *p*-nitrophenyl phosphate in the absence of Mn^{2+} was constant in the pH range 7.0–9.0 in Tris/HCl, imidazole/HCl, Tris(hydroxymethyl)methylaminopropanesulphonic

Table 5. Effect of EDTA concentration on the activity of *H. cutirubrum* alkaline phosphatase

The activity of the enzyme (2.7 units, without Mn^{2+}) was measured in the standard assay with and without 5 mM- $MnCl_2$ at the indicated EDTA concentrations.

EDTA concn. (mM)	<i>p</i> -Nitrophenol released (nmol/min)	
	- Mn^{2+}	+ Mn^{2+}
0	2.51	9.90
1 (Standard assay)	2.65	10.67
2	2.88	9.72
5	2.82	2.83
10	2.34	2.26

Table 6. Effect of NaCl concentration on the activity of *H. cutirubrum* alkaline phosphatase

The activity of the enzyme (2.62 units without Mn^{2+}) was measured in the standard assay conditions with and without 5 mM- $MnCl_2$ at the NaCl concentrations indicated. The enzyme solution (Sephadex G-100 fraction) was first dialysed twice against 10 mM-Tris/HCl, pH 8.6 (100 vol.), for a total of 2 h to remove salt. The required NaCl concentrations were obtained by evaporating an appropriate amount of a concentrated NaCl solution to dryness in the tubes and carefully redissolving the solid in the assay reagents before addition of the enzyme.

NaCl concn. (M)	<i>p</i> -Nitrophenol released (nmol/min)	
	- Mn^{2+}	+ Mn^{2+}
0	0.85	6.11
0.5	2.56	13.38
1	2.96	12.21
2	2.62	10.43
3	2.60	8.68
4	2.39	7.15

acid/NaOH and veronal buffers. It fell steadily on either side of the plateau to zero at pH 5 (acetate buffer) and pH 10 (glycine/NaOH buffer). In the presence of Mn^{2+} , the effect of pH was essentially the same, except that the rapid oxidation of Mn^{2+} made measurements difficult at pH 9 and above. Thus the enzyme is clearly an alkaline phosphatase.

H. cutirubrum alkaline phosphatase not only requires a high ionic strength for stability, but is also most active at NaCl concentrations of 0.5 M and above (Table 6). However, it still has appreciable activity in the absence of NaCl (or KCl) and in the presence of Mn^{2+} there is a well-defined optimum salt concentration of about 0.5 M, although the enzyme is still stimulated threefold by Mn^{2+} in the presence of 4 M-NaCl. In the absence of Mn^{2+} , there is a broad plateau of activity with 0.5–4 M-NaCl, so that the

Table 7. Substrate specificity of *H. cutirubrum* alkaline phosphatase

The activity of the enzyme was determined in the conditions of the standard assay with and without 5 mM- $MnCl_2$. The concentration of each substrate was 8 mM. The reactions were stopped by addition of an equal vol. of 7% (w/v) $HClO_4$ and the orthophosphate formed was determined by the method of Fiske & SubbaRow (1925). The controls were complete assay mixtures lacking enzyme. No detectable release of P_i over the control values was found with the following compounds; 3'- and 5'-AMP, 5'-ATP, 5'-dAMP, phosphoenolpyruvate, creatine phosphate, ribose 5-phosphate, glucose 6-phosphate, 6-phosphogluconic acid and sodium pyrophosphate.

Substrate	P_i release (nmol/min)	
	- Mn^{2+}	+ Mn^{2+}
<i>p</i> -Nitrophenyl phosphate	5.3	13.3
5'-dATP	1.9	3.3
5'-dTTP	8.0	2.1
5'-dTMP	6.7	1.6

phosphatase is already fully active at an ionic strength about one-quarter to one-half that usually necessary with enzymes from extreme halophiles (Larsen, 1967; Kushner, 1968; Louis & Fitt, 1972; Peterkin & Fitt, 1971).

Table 7 shows the substrate specificity of *H. cutirubrum* alkaline phosphatase. Two conclusions are evident. First, it is not the usual non-specific alkaline phosphatase found in most cells and typified by the *E. coli* enzyme (Reid & Wilson, 1971); of the substrates tried so far, only dTMP and dTTP were as good as *p*-nitrophenyl phosphate although there was also some limited activity with dATP. Secondly, no case was found where a substance was a substrate in the presence of Mn^{2+} and not in its absence, or vice versa, even though the bivalent cation was an activator with *p*-nitrophenyl phosphate and dATP and an inhibitor with the two thymidine nucleotides. (These variations may merely be related to the solubilities or ease of dissociation of the Mn^{2+} salts of the compounds concerned.) It can be seen (Table 8) that dTTP was a classical competitive inhibitor of the *p*-nitrophenyl phosphatase activity in the absence of Mn^{2+} . In the presence of the bivalent cation, the inhibition was of a mixed type (probably because of the binding of Mn^{2+} by dTTP), but there was nevertheless a large effect on the K_m for *p*-nitrophenyl phosphate. Thus these two substrates compete for the same active site. Further, the thermal stability of the activities towards the two substrates, assayed either in the presence or absence of Mn^{2+} , were similar (Table 9). These results, together with those from the analytical gel filtrations (Table 2), provide strong evidence that the activities towards *p*-nitrophenyl

Table 8. *Effect of 5'-dTTP on the hydrolysis of p-nitrophenyl phosphate*

K_m and V values of *H. cutirubrum* alkaline phosphatase were determined by varying the p-nitrophenyl phosphate concentration in the absence and presence of 8 mM-5'-dTTP. The other reaction conditions were those of the standard assays with and without 5 mM-Mn²⁺. The kinetic constants were obtained from plots of [S]/ v versus [S].

Mn ²⁺ concn. (mM)	5'-dTTP concn. (mM)	K_m (mM)	V (nmol/min per mg of protein)
0	0	1.8	72
0	8	2.3	72
5	0	0.5	424
5	8	1.3	164

Table 9. *Thermal stability of H. cutirubrum alkaline phosphatase*

The enzyme in high-salt buffer was incubated at the indicated temperatures for 5 min. The samples were then cooled in ice and assayed with and without 5 mM-Mn²⁺ by using either 8 mM-p-nitrophenyl phosphate (standard spectrophotometric assay) or 8 mM-5'-dTTP (orthophosphate release, Table 7) as substrates. The residual activity is expressed as a percentage of the activity of the unheated enzyme assayed in the same conditions.

Incubation temp. (°C)	Residual activity (%)			
	<i>p</i> -Nitrophenyl phosphate		5'-dTTP	
	-Mn ²⁺	+Mn ²⁺	-Mn ²⁺	+Mn ²⁺
40	87	95	93	69
50	73	90	77	60
60	32	45	35	31

phosphate and dTTP either with or without Mn²⁺ are due to a single enzyme, but final proof will require extensive purification of the protein concerned.

Although Mn²⁺-stimulated nucleotide phosphomonoesterases are well known (Drummond & Yamamoto, 1971), these enzymes are nearly all inhibited by EDTA, generally have pH optima below 7 and are inactive against *p*-nitrophenyl phosphate. Further, they usually hydrolyse a wide range of ribo- and deoxyribo-nucleotides. On the basis of our present evidence, it does not appear that the *H. cutirubrum* enzyme resembles any of these known nucleotidases (Drummond & Yamamoto, 1971) and further study will be necessary to establish the nature of its true intracellular substrate. Nevertheless, in view of its ability to catalyse the hydrolysis of *p*-nitrophenyl phosphate at alkaline pH, we believe it can justifiably be considered as an unusual alkaline phosphatase.

In conclusion, the results presented above show that *H. cutirubrum* contains an alkaline phosphatase with a restricted substrate specificity. It is unusual in being stimulated by Mn²⁺ and in having a significantly lower molecular weight than other similar bacterial enzymes.

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