Purification and Characterization of the Outer Membrane Associated Alkaline Phosphatase of *Lysobacter enzymogenes*

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The alkaline phosphatase (EC 3.1.3.1) associated with the outer membrane of *Lysobacter enzymogenes* was solubilized from a crude membrane preparation with detergent and purified 219-fold using ion-exchange chromatography and gel filtration. The yield of the purified enzyme was 21% and the specific activity was 438 units mg⁻¹. The enzyme was most active at pH 8.5, readily hydrolysed 5'-, 2'- and 3'-ribose and deoxyribose nucleotides, glucose 6-phosphate, glycerophosphates and *p*-nitrophenylphosphate and was strongly inhibited by EDTA and 8-hydroxyquinoline. The EDTA-inhibited enzyme could be reactivated to some extent by CoCl₂ and more effectively by ZnCl₂. The phosphatase was slightly activated by *p*-nitrophenylphosphate and from kinetic studies using this substrate two *K_m* values, 0.56 × 10⁻⁴ M and 3.4 × 10⁻⁴ M, were estimated. The enzyme retained activity in the presence of SDS, unless it was heated, and had an apparent *M_r* of 69 000, as determined by SDS-PAGE. Gel filtration data suggested that the native enzyme might consist of at least two subunits. The properties of the enzyme were consistent with the view that it is held in the outer membrane by hydrophobic interactions.

INTRODUCTION

Species of the genus *Lysobacter* are Gram-negative, non-fruiting, gliding bacteria which share many physiological characteristics with the Myxobacteria (Christensen & Cook, 1978; Reichenbach, 1981; Reichenbach & Dworkin, 1981). *Lysobacter enzymogenes* produces a number of extracellular enzymes (Whitaker, 1970; Christensen & Cook, 1978; von Tigerstrom, 1980, 1981, 1983, 1984), including two phosphatases. One of these phosphatases has been described in detail (von Tigerstrom, 1984). It is produced and excreted into the medium soon after the exponential growth phase. It has an *M_r* of about 25 000, is most active at pH 7.5 and is insensitive to inhibition by EDTA. The other phosphatase is produced during the exponential and early stationary growth phases and remains tightly associated with the cells. This enzyme is hydrophobic, is most active at pH 8.5 and is sensitive to inhibition by chelating agents (von Tigerstrom, 1984). Thus, the two phosphatases are clearly distinct. Recently the cell-associated phosphatase of *L. enzymogenes* was located in the outer membrane of the organism (von Tigerstrom & Stelmashuk, 1985).

It is hoped that an investigation of the properties of the cell-associated phosphatase and of how this enzyme interacts with other outer membrane components will lead to a better understanding of the cell surface of *L. enzymogenes*, and of gliding bacteria in general. This paper describes the purification and characterization of the phosphatase associated with the outer membrane.

METHODS

Chemicals. The sources of most of the chemicals used during the purification and characterization of the phosphatase have been reported (von Tigerstrom, 1984). In addition, DEAE-Sephacel and Sephacryl S-200 were

Abbreviation: pNPP, *p*-nitrophenylphosphate.

obtained from Pharmacia and Zwittergent 3-14 from Calbiochem-Behring. The other detergents, the substrates used for the enzyme assays and the heparin-agarose were from Sigma. The M_r standards used for PAGE were from Pierce, except for phosphorylase *b*, which was purchased from Sigma.

Organism, medium and culture conditions. *Lysobacter enzymogenes* UASM 495 (ATCC 29487) was grown aerobically in 0.8% peptone broth as described previously (von Tigerstrom, 1984). The cells grew to an OD_{600} of about 2.4 and were harvested in the stationary phase by centrifugation at 16300 *g* for 15 min. Tryptone broth, used in previous studies, was not used here since in this medium the growth and the production of some extracellular enzymes were not reproducible.

Enzyme assays. For routine assays the enzyme sources were diluted into 10 mM-Tris/HCl, 50 mM-NaCl, 1.5 mM-MgCl₂, 0.2% Zwittergent 3-14, pH 7.5. The phosphatase assay for the cell-associated enzyme was done as previously described (von Tigerstrom, 1984), except that 4 mM-*p*-nitrophenylphosphate (pNPP) was used. One unit of phosphatase is the amount that hydrolyses 1 μ mol substrate min⁻¹ at 37 °C. For the determination of K_m , 0.05 to 15 mM-pNPP was used. The activity of the enzyme with different substrates was compared by diluting the enzyme without detergent and using assay solutions with 0.15 M-triethanolamine, 0.2% Triton X-100 and 4 mM-substrate, adjusted to pH 8.5 with HCl. Zwittergent 3-14 was replaced by Triton X-100 because it interfered with the phosphate assay. The rates of hydrolysis were estimated from the inorganic phosphate released after 3, 10 and 20 min incubation at 37 °C.

The procedure for the determination of phosphatase activities at different pH values has been described (von Tigerstrom, 1984).

The effect of phosphate on the heat inactivation of the enzyme was determined at 60 °C. The phosphatase was added to 10 mM-Tris/HCl, 50 mM-NaCl, 1.5 mM-MgCl₂, 0.2% Zwittergent 3-14, pH 7.4 with or without 5 mM-potassium phosphate, pH 7.4. At intervals samples were removed and diluted 10-fold for the routine assay of the enzyme. The final phosphate concentration in the enzyme assay was 0.05 mM, which has little or no effect on the phosphatase activity.

The effect of inhibitors on the enzyme activity was determined by incubating the enzyme in the assay solution without pNPP for 10 min at 37 °C and then starting the assay by the addition of the substrate.

The reactivation of enzyme, which had been inhibited by EDTA, was studied by preincubating the enzyme in 10 mM-Tris/HCl, 50 mM-NaCl, 0.2% Zwittergent 3-14, 0.2 mM-EDTA, pH 7.5, for 10 min at 37 °C. Enzyme solution (50 μ l) was then added to 0.45 ml of the assay solution containing the metal ion (0.05 mM final concentration).

Polyacrylamide gel electrophoresis. Conditions for PAGE of the phosphatase have been described in detail (Maizel, 1971; von Tigerstrom, 1984). Samples were prepared under the usual denaturing conditions for the detection of protein (Fairbanks *et al.*, 1971) and prepared in 10 mM-Tris/HCl, 1% SDS, pH 8.0, without heating for the detection of phosphatase activity (von Tigerstrom & Stelmaschuk, 1985).

Analytical methods. Protein concentrations were determined by the Lowry method and by absorbance (Warburg & Christian, 1941). Phosphate concentrations were determined by the method of Ames (1966).

Purification of the enzyme. Trial purifications from a relatively small culture were then scaled up to 6 l and 8 l of culture. These gave very similar results and the purification of the phosphatase from the 8 l culture is described in detail. All operations were done at 0 to 4 °C unless otherwise indicated. The enzyme was concentrated at various stages of the purification by ultrafiltration using an Amicon PM 10 membrane.

Step I. Preparation of cell-free extract. *L. enzymogenes* cells (50 g wet weight) were washed by centrifugation (10 min at 15300 *g*) and resuspension in 20 mM-Tris/HCl, 1 mM-MgCl₂, pH 7.5, resuspended in 225 ml buffer and disrupted in a French pressure cell at 103 MPa. The suspension was diluted to 800 ml with the same buffer and intact cells were removed by centrifugation at 3100 *g* for 15 min to obtain 777 ml cell-free extract.

Step II. Preparation of the 48000 *g* pellet and solubilization. The cell-free extract was centrifuged at 48000 *g* for 60 min, the supernatant was discarded and the pellet was suspended in 10 mM-Tris/HCl, pH 7.5, to yield about 400 ml of suspension. Zwittergent 3-14 was added to a final concentration of 0.2%. The suspension was stirred for 60 min and then left for 18 h. Centrifugation at 27000 *g* for 15 min yielded the solubilized enzyme preparation containing 83% of the phosphatase activity; the pellet was discarded.

Step III. Adsorption to DEAE-Sephacel. Wet DEAE-Sephacel in the acetate form (370 g) was added to the solubilized enzyme. The suspension was stirred for 30 min and then left overnight. The ion-exchanger was packed into a column (5 $\times$ 22 cm), eluted with 200 ml 10 mM-Tris/acetate, pH 7.5, and then with 10 mM-Tris/acetate, 0.3 M-ammonium acetate, 0.05% Zwittergent 3-14, pH 7.5, 17 ml fractions being collected. Three peaks were obtained. The run-off peak contained about 4 % of the enzyme applied and almost half the protein, and the second peak contained most of the enzyme in approx. 500 ml. This was concentrated to 182 ml by ultrafiltration. A third, trailing peak, containing about 24% of the phosphatase activity, was not included for further purification.

Step IV. DEAE-Sephacel chromatography. This step was done at 8 °C. The active fraction obtained above was dialysed against 10 mM-Tris/HCl, 10 mM-NaCl, 1 mM-MgCl₂, 0.05% Zwittergent 3-14, pH 7.5 (starting buffer 1) and applied to a column of DEAE-Sephacel (5 $\times$ 31 cm) pre-equilibrated with the same buffer. The column was then eluted with 280 ml starting buffer 1 and a gradient consisting of 1400 ml starting buffer 1 and 1400 ml 10 mM-

Tris/HCl, 0.6 M-NaCl, 1 mM-MgCl₂, 0.05% Zwittergent 3-14, pH 7.5, 15 ml fractions being collected. The phosphatase activities, the A_{280} values and the conductivities were determined. Fractions 92–107 were pooled and concentrated to 78 ml by ultrafiltration.

Step V. Heparin-agarose chromatography. The phosphatase obtained in the previous step was dialysed against 10 mM-MES, pH 5.5, and applied to a heparin-agarose column (2.5 × 31 cm) equilibrated with 10 mM-MES, 0.05% Zwittergent 3-14, pH 5.5 (starting buffer 2). The column was eluted at 8 °C with 200 ml starting buffer 2 and then with a gradient consisting of 500 ml starting buffer 2 and 500 ml 10 mM-MES, 0.3 M-KCl, 0.05% Zwittergent 3-14, pH 5.5, 11.6 ml fractions being collected. The phosphatase activities, the A_{280} values and the conductivities were determined. Fractions 52–62 were pooled and concentrated to 8 ml by ultrafiltration.

Step VI. Gel filtration. The phosphatase preparation was applied to a Sephacryl S-200 column (2.6 × 96.5 cm) equilibrated with 10 mM-Tris/HCl, 0.1 M-NaCl, 1 mM-MgCl₂, 0.1% Brij 58, pH 7.5, and eluted with the same buffer, 5.4 ml fractions being collected. The phosphatase activities and the A_{280} values were determined. Fractions 36–42 were pooled and concentrated to 12.9 ml.

To ensure the complete removal of the ionic detergent Zwittergent 3-14 from the enzyme preparation, the phosphatase was reapplied, after dialysis against 10 mM-Tris/HCl, 10 mM-NaCl, 1 mM-MgCl₂, pH 7.5, to a smaller DEAE-Sephacel Column (2.5 × 25 cm) and eluted with the buffers described above (step IV), except that the Zwittergent 3-14 was replaced with 0.1% Brij 58 and proportionally smaller buffer volumes were used. Approximately 91% of the phosphatase applied to this column was recovered with only a slight increase in the specific activity.

RESULTS

Purification of the cell-associated phosphatase

The results of the purification procedure described in Methods are summarized in Table 1. After preparation of the cell-free extract the enzyme was sedimented with other particulate components by centrifugation. It then was solubilized with Zwittergent 3-14. Preliminary results had shown that the presence of a detergent was essential for the efficient elution of the enzyme during column chromatography. Only 8% of the enzyme was recovered from the column if the detergent was omitted. Zwittergent 3-14, which was particularly efficient in solubilizing the phosphatase, was therefore present in the buffers used in steps II to V of the procedure.

DEAE-Sephacel was used at two stages, the first time being for batchwise adsorption of the enzyme and elution by increased salt concentration. A considerable amount of activity (24%) was eluted after the main peak. Although the enzyme in this peak was excluded from further purification, subsequent experiments indicated that the two peaks contained the same enzyme. After dialysis of the enzyme from both peaks and reapplication to DEAE-Sephacel the material eluted in a sharp single peak at 0.165 M-NaCl. Furthermore, material from both peaks had identical electrophoretic mobilities in a polyacrylamide gel. Therefore, it appears that the double peak in step III was an artifact of the batchwise chromatography.

A six-fold purification was obtained in step IV, the gradient elution of the enzyme from DEAE-Sephacel (Fig. 1a) and nearly a four-fold purification in step V, the heparin-agarose chromatography (Fig. 1b). Chromatography on heparin-agarose routinely caused a substantial

Table 1. *Purification of the cell-associated phosphatase*

The enzyme was purified as described in Methods. Volumes have been corrected for removal of samples for activity and protein determination.

Purification step	Total protein (mg)	Total phosphatase activity (units)	Specific activity [units (mg protein) ⁻¹]	Purification (fold)
I. Cell-free extract	4700	9522	2	—
II. 48 000 g pellet and solubilization	1817	7862	4.3	2.2
III. DEAE-Sephacel 1	598	4568	7.6	3.8
IV. DEAE-Sephacel 2	92	4309	46.8	23.4
V. Heparin-agarose	12.8	2282	178.3	89.2
VI. Sephacryl S-200	4.5	1973	438.4	219.2

Fig. 1. Purification of the cell-associated phosphatase by DEAE-Sephacel chromatography (a), heparin-agarose chromatography (b) and Sephacryl S-200 gel filtration (c) as described in Methods (purification steps IV, V and VI). The phosphatase activities (●), the A_{280} (○) and the salt concentrations (---) were determined.

loss of the enzyme activity. The low pH may have been a factor, although the enzyme is quite stable when stored at pH 5.5.

The last step of the purification, the gel filtration (Fig. 1c), was done with a buffer, in which Brij 58 replaced the ionic detergent Zwittergent 3-14, which might have interfered with the electrophoresis and further characterization of the phosphatase. Similar results were obtained, however, when Zwittergent 3-14 or deoxycholate was used during gel filtration.

The final product of the purification procedure contained only minor impurities as judged by PAGE. The phosphatase was purified over 200-fold to a specific activity of 438 units (mg protein)⁻¹ and with a 21% yield.

PAGE and M_r determination

Electrophoresis was done in 10% gels containing 0.1% SDS. Enzyme samples were treated in two different ways before application: for the detection of protein bands and the M_r estimation

Fig. 2. Polyacrylamide gel electrophoresis. The following were subjected to electrophoresis in a 10% (w/v) polyacrylamide gel with 0.1% SDS. Lane 1, M_r standards (phosphorylase *b*, serum albumin, ovalbumin, chymotrypsinogen and cytochrome *c*); lanes 2 and 3, 1.03 μ g (0.6 units) purified phosphatase; lanes 4 and 5, 0.021 μ g (0.012 units) purified phosphatase; lane 6, 0.053 μ g (0.03 units) purified phosphatase. Samples in lanes 1, 2 and 5 were heat-denatured in the presence of 1% SDS and 2-mercaptoethanol. Samples in lanes 3, 4 and 6 were treated only with 1% SDS. Lanes 1, 2 and 3 were stained with Coomassie blue and lanes 4, 5 and 6 were subjected to the phosphatase activity stain. The results were very reproducible.

the enzyme was heat-denatured in the usual manner with SDS and 2-mercaptoethanol (Maizel, 1971); whereas for detection of enzymic activity, the enzyme was applied after the addition of only SDS and was not heated (Fig. 2). The heat-denatured preparation showed one major protein band with an apparent M_r of 69 000. Without the heat treatment one major protein band, which coincided with one major activity band of apparent M_r 76 000, was visible if the enzyme was applied at a low concentration. If the amount of enzyme applied was increased five-fold or more, both the protein stain and the activity stain resulted in two closely spaced bands. This may have been due to overloading, the hydrophobic nature of the protein or incomplete denaturation under these conditions.

PAGE without detergent or in the presence of Triton X-100 or Brij 58 was not successful since the protein penetrated the gel very poorly.

An attempt was also made to estimate the native M_r of the enzyme by gel filtration using Sephacryl S-200 with a buffer system containing 0.1% deoxycholate. Blue dextran,

Fig. 3

Fig. 4

Fig. 3. Heat inactivation of the phosphatase. The enzyme was heated at 60 °C in the presence (●) and absence (○) of 5 mM-potassium phosphate as described in Methods and the remaining activities were determined over a 30 min period. The presence of 5 mM-KCl instead of potassium phosphate did not increase the rate of inactivation.

Fig. 4. Determination of kinetic parameters. The purified phosphatase was assayed with pNPP concentrations of 0.05 mM to 15 mM and kinetic parameters were estimated using the double reciprocal plot. The experiment was repeated twice with nearly identical results.

immunoglobulin G, *Escherichia coli* alkaline phosphatase, ovalbumin and RNAase A were used as standards. The *Lysobacter* phosphatase was eluted at a position before the immunoglobulin G, indicating an M_r greater than 150 000.

Stability and solubility

The phosphatase was stable, at least for several weeks, at –22 °C in 10 mM-Tris/HCl, 50 mM-NaCl, 1.5 mM-MgCl₂, 0.2% Zwittergent 3-14, pH 7.4, and for several days at 0 to 4 °C when stored at pH 5.5 to 8.0 in 20 mM-Tris/acetate buffer. The enzyme could be incubated with 1% SDS for several hours at room temperature without significant loss of activity, but appeared to be irreversibly inactivated after heating (97 °C, 10 min) in the presence of 1% SDS and 0.1% 2-mercaptoethanol (Fig. 2). At 60 °C the enzyme was stable for at least 20 min, while only 70% and 2% of the activity remained after heating at 70 °C for 10 min and 20 min, respectively. The rate of inactivation at elevated temperatures was increased significantly by the presence of 5 mM-potassium phosphate (Fig. 3). In a similar experiment 85% of the excreted phosphatase of *L. enzymogenes* was inactivated after 60 min at 65 °C but, in contrast to the cell-associated enzyme, it was completely stabilized at this temperature by the addition of 5 mM-potassium phosphate.

The degree of solubilization of the enzyme by different detergents was determined. The 48 000 g pellet from the cell-free extract was suspended in 10 mM-Tris/HCl, 50 mM-NaCl, 1.5 mM-MgCl₂, pH 7.5, and the detergents were added to a concentration of 0.2%. The preparations were shaken at room temperature (150 r.p.m.) for 60 min. The phosphatase activities were then determined in the preparations and in the supernatants after centrifugation at 27 000 g for 5 min (Table 2). Deoxycholate, Zwittergent 3-14 and Brij 58 were the most effective for solubilizing the enzyme.

pH optimum, K_m and substrate specificities

The activity of the purified phosphatase was determined at different pH values (pH 5 to pH 10). The profile ($V_{\max}$ vs pH) of the purified preparation was essentially the same as that obtained earlier with the crude enzyme (von Tigerstrom & Stelmashuk, 1985). Thus, the cell-

Table 2. *Solubilization of the cell-associated phosphatase with detergents*

The experiment was done once.

Detergent	Percentage of phosphatase solubilized	Detergent	Percentage of phosphatase solubilized
None	22	CHAPS	39
Triton X-305	26	Triton X-45	42
Brij 38	30	Triton X-100	58
Triton X-35	31	Brij 58	68
Octylglucopyranoside	33	Zwittergent 3-14	85
Triton X-57	37	Deoxycholate	86
Cholate	37		

Table 3. *Substrate specificity of the cell-associated phosphatase*

The activities were determined from three assays at 3, 10 and 30 min of a suitably diluted purified enzyme preparation.

Substrate	Phosphate activity	
	U ml ⁻¹	%
pNPP	35.2	100
5'-AMP	48.2	137
2'-AMP	25.0	71
3'-AMP	24.0	68
ADP	30.6	87
ATP	36.6	104
5'-GMP	54.2	154
2',3'-GMP	23.6	67
5'-UMP	50.6	144
2',3'-UMP	30.3	86
5'-CMP	44.0	125
5'-dAMP	51.7	147
5'-dGMP	54.2	154
5'-dTMP	42.9	122
5'-dCMP	44.0	125
α-Glycerophosphate	36.6	104
β-Glycerophosphate	33.9	96
Glucose 6-phosphate	40.8	116

associated phosphatase is most active at pH 8.5. The low activities at pH 9.5 and 10 are in part a reflection of the instability of the enzyme under these conditions.

The K_m value for the enzyme was determined with pNPP as the substrate at concentrations from 0.05 mM to 15 mM. The activity rose sharply until the concentration was about 2 mM and then increased slowly and nearly linearly up to 15 mM-pNPP. Higher substrate concentrations were not used. Two K_m values, 0.56×10^{-4} M and 3.4×10^{-4} M, were estimated from the reciprocal plot (Fig. 4). It appears that the enzyme continues to be slightly activated by increasing substrate up to very high concentrations. Attempts to overcome this effect by adding divalent metal ions and detergent to the assay solutions were not successful. Routine assays were done with 4 mM-pNPP. At this concentration the phosphatase was only 13% less active than with 15 mM-pNPP.

The phosphatase was routinely assayed in Tris/HCl buffer with pNPP as the substrate. To determine the substrate specificity of the enzyme it was assayed with other phosphomonoesters in the presence of triethanolamine to avoid possible transphosphorylation reactions. The activities obtained with 18 different substrates were calculated from a linear rate of inorganic phosphate release (Table 3). The results indicated that the enzyme is non-specific with respect to

Table 4. *Effect of inhibitors on phosphatase activity*

Inhibitor	Concn (mM)	Inhibition (%)
Potassium phosphate	1.0	30
	4.0	48
Sodium arsenate	1.0	41
	4.0	73
<i>o</i> -Phenanthroline	0.05	7
	0.5	58
8-Hydroxyquinoline	0.05	26
	0.5	99
EDTA	0.01	27
	0.02	57
	0.05	97

the substrate used. In general, all ribo- and deoxyribonucleoside 5'-phosphates were hydrolysed faster than pNPP. Ribonucleoside 2'- or 3'-phosphates were hydrolysed more slowly than pNPP. Glucose 6-phosphate and α - and β -glycerophosphates were hydrolysed at rates similar to pNPP.

Inhibition and reactivation

The enzyme was relatively insensitive to inorganic phosphate and arsenate but some chelating agents, especially EDTA and 8-hydroxyquinoline, were strongly inhibitory (Table 4). However, the addition of metal ions to the assay solution was not required for maximum activity unless the enzyme was first treated with a chelating agent.

It was, therefore, of interest to determine which metal ions could reactivate EDTA-inhibited phosphatase. To ensure maximum inhibition the enzyme was dissolved in a solution containing 0.2 mM-EDTA and then assayed in a solution containing the divalent metal ions at 0.05 mM. No activation was obtained with NiCl_2 , FeSO_4 or BaCl_2 . The addition of MgCl_2 , CaCl_2 , MnCl_2 , CoCl_2 and ZnCl_2 caused reactivation of 4.6%, 7%, 14.5%, 24% and 66% of the enzyme activity, respectively. These results are in general agreement with earlier observations (von Tigerstrom & Stelmashuk, 1985) and suggest that the cell-associated phosphatase is a Zn^{2+} -containing enzyme.

DISCUSSION

The cell-associated enzyme from *L. enzymogenes* resembles many other alkaline phosphatases in that it hydrolyses a number of phosphomonoesters, is most active at pH 8.5 and is probably a Zn^{2+} -containing metalloenzyme (McComb *et al.*, 1979). The enzyme has a relatively high K_m for pNPP and is unusual in that it is a hydrophobic protein.

According to the results of SDS-PAGE, the M_r of the phosphatase or its active subunit is about 69000. The fact that gel filtration indicated a much higher M_r could mean that the native enzyme is composed of subunits. However, the higher apparent M_r might be due to the presence of detergent during the gel filtration.

Although many bacterial phosphatases have been described (McComb *et al.*, 1979), the cell-associated phosphatase of *L. enzymogenes* is of particular interest because of its hydrophobic nature and its tight binding to the outer membrane of the organism (von Tigerstrom & Stelmashuk, 1985). Very few enzymes are located in the outer membrane of bacteria (Lugtenberg & Van Alphen, 1983). However, the phosphatases of *Capnocytophaga ochracea* and related species of anaerobic gliding bacteria have also been found to be partially associated with the outer membrane (Poirier & Holt, 1983). The phosphatase from *C. ochracea* appears to be less hydrophobic than the enzyme from *L. enzymogenes*, though, since it can be eluted from ion-exchange chromatography columns without detergent. When the *L. enzymogenes* enzyme was chromatographed without detergent only 8% of the phosphatase was recovered.

The *L. enzymogenes* phosphatase described here has a much higher specific activity than other bacterial phosphatases (McComb *et al.*, 1979). The specific activity is 1.6 times higher than that of the phosphatase from *L. enzymogenes* which is excreted into the medium (von Tigerstrom, 1984) and 60 times higher than the specific activity of the *C. ochracea* alkaline phosphatase (Poirier & Holt, 1983).

Many of the characteristics of the cell-associated enzyme, such as its hydrophobicity, substrate specificity and M_r , support the earlier conclusion that the enzyme is distinct from the excreted phosphatase of this organism (von Tigerstrom, 1984). Whether the two enzymes are related in amino acid composition or antigenicity remains to be determined.

Since the phosphatase is located outside the cytoplasmic membrane it probably has a nutritional function and may work in cooperation with the excreted phosphatase to degrade phosphomonoesters in the environment. The enzyme is probably held in the outer membrane by hydrophobic interaction with other envelope components. However, it is more readily released from the outer membrane by detergent treatment than are a number of major outer membrane proteins (R. G. von Tigerstrom & T. A. Solleveld, unpublished).

The substrates do not have free access to the active site of the enzyme from the exterior of the organism, since intact cells have low phosphatase activity. However, treatment of the cells with toluene, an agent which disrupts membrane function but does not solubilize the enzyme, leads to nearly full expression of the enzyme activity (von Tigerstrom, 1984). Thus, the active site may face the periplasm or the enzyme may be partially covered by slime or capsular material. We have now raised antibodies to the enzyme and with these the position of the phosphatase in the outer membrane and its interaction with other outer membrane components can be investigated in more detail.

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