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A SOLUBLE ALKALINE PHOSPHATASE FROM *BACILLUS LICHENIFORMIS* MC14

HISTOCHEMICAL LOCALIZATION, PURIFICATION, CHARACTERIZATION AND COMPARISON WITH THE MEMBRANE-ASSOCIATED ALKALINE PHOSPHATASE

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Summary

Growth conditions affect the quantity and distribution of alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) in *Bacillus licheniformis* MC14. The soluble alkaline phosphatase, which has been found in biochemical localization studies between the cell wall and cell membrane (Glynn, J.A., Schaffel, S.D., McNicholas, J.M. and Hulett, F.M. (1977) J. Bacteriol. 129, 1010—1019), was localized via electron microscope histochemistry in cells cultured under conditions which result in increased quantities of this activity. This soluble alkaline phosphatase was stabilized with 20% glycerol and purified to homogeneity as determined by sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis. The purified enzyme is soluble in dilute buffer. This soluble alkaline phosphatase has been characterized and compared to the membrane-associated alkaline phosphatase from this organism.

Introduction

Membrane-associated alkaline phosphatase from *Bacillus licheniformis* MC-14 has been purified and characterized as previously [1]. Alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) extracted with 1.0 M Mg^{2+} and heated for 5 min at 80°C remained soluble after removal of Mg^{2+} by extensive dialysis and centrifugation at

105 000 × *g* for 60 min. This enzyme will be referred to as the heat-salt solubilized alkaline phosphatase. The enzyme remains insoluble after Mg^{2+} is removed if the heat step is omitted [1]. The membrane alkaline phosphatase has also been purified without the drastic heat step. This enzyme requires 0.2 M Mg^{2+} for solubility [2] and will be referred to as the salt-dependent alkaline phosphatase.

McNicholas and Hulett [3] reported that cells grown to mid-logarithmic phase in 2% Neopeptone (Difco) contained alkaline phosphatase which was localized histochemically on the inner surface of the cell membrane.

Soluble alkaline phosphatase has been found in late logarithmic cells when this organism is grown in 1% Neopeptone. This enzyme, which is very unstable in dilute buffers, is released upon protoplast formation and remains soluble after extensive dialysis and high speed centrifugation (100 000 × *g*, 60 min) [4].

It was necessary to isolate and characterize this soluble alkaline phosphatase to establish whether it is similar to the membrane-associated alkaline phosphatase or perhaps is an entirely different protein. This paper reports a method of stabilization, purification and a partial characterization of the soluble alkaline phosphatase. We also report the histochemical localizations of the soluble alkaline phosphatase in cells grown under conditions which result in the increased production of the soluble form of alkaline phosphatase.

Materials and Methods

The organism used in this study in the facultative thermophile *B. licheniformis* MC14 [1]. Stocks were maintained on slants of 2% tryptone (Difco) and 2% agar (Difco) (pH 7.2) at 4°C.

Cells from tryptone plates which had been incubated overnight were used to inoculate liquid medium. The inoculum for fermentor growth was grown in four 1000-ml flanks containing 400 ml 1.0% Neopeptone broth and 0.1% fructose on a water bath shaker at 55°C, until the absorbance at 540 nm was 0.6—0.8 (1.0 cm path length). At this time, the 1600 ml culture was used to inoculate a 14 l New Brunswick fermentor which contained 12 l 1.0% Neopeptone and 0.1% fructose (agitation at 400 rev./min and aeration of 10 l/min at 55°C). Cells were harvested at peak enzyme production, usually at an absorbance (540 nm) of 1.2—1.4, by centrifugation in a Sharples centrifuge and frozen until used.

Assays. Alkaline phosphatase was assayed by adding enzyme to *p*-nitrophenyl phosphate (1.0 mM in 1.0 M Tris-acetate, pH 8.0/1 mM magnesium acetate) at 55°C. The reaction was stopped by the addition of 0.5 ml 13% K_2HPO_4 to 2.5 ml assay mixture. For all of the assays except those performed in the purification, the initial velocity of the hydrolysis of *p*-nitrophenyl phosphate was determined by the use of a Gilford recording spectrophotometer with a constant temperature cuvette chamber. 1 unit of alkaline phosphatase activity is defined as that amount of enzyme that liberates 1 μmol *p*-nitrophenol/min under the defined conditions.

Inorganic phosphate was determined by the method of Fiske and SubbaRow [5].

Histochemistry. The histochemical techniques used by McNicholas and Hulett [3] were used with slight modifications which are outlined in the Results section.

Purification of soluble alkaline phosphatase. Cells were converted into protoplasts as described previously [4] with the exception that protoplast-forming solution contained 25.0 mM $MgCl_2$/5 mg/ml lysozyme. 1 g cells was added to each 2 ml of protoplast-forming solution. Purification of soluble alkaline phosphatase involved converting cells to protoplasts which were removed by centrifugation at 10 000 × *g* for 30 min. Membrane fragments or remaining protoplasts were removed from the supernatant fraction by a second centrifugation at 157 000 × *g* for 2 h. The supernatant fraction was then subjected to a 70% $(NH_4)_2SO_4$ precipitation. The enzyme-containing supernatant fraction from this step was dialyzed overnight at 4°C in 0.01 M Tris-acetate buffer, pH 7.3/0.1 mM $CoCl_2$/20% glycerol (buffer 1). The dialysate was centrifuged at 40 000 × *g* for 1 h. The supernatant fraction from this step was applied to a 2.0 × 40 cm CM-Sephadex column equilibrated in buffer 1. A 160 ml 0.05—0.4 M $MgCl_2$ linear gradient was used to elute the enzyme. Fractions of high specific activity were pooled, dialyzed against buffer 1 and applied to a second CM-Sephadex column similar to the first column with the exception that a 400 ml 0.05—0.4 M Mg^{2+} gradient was used. Fractions of high specific activity from this step were soluble in dilute buffer (0.01 M Tris-acetate, pH 7.3) when subjected to high speed centrifugation.

SDS-polyacrylamide gels. Electrophoresis through polyacrylamide gels (7%) in the presence of SDS was done according to the procedure of Laemmli [6]. Marker proteins included bovine serum albumin, ovalbumin, chymotrypsinogen and sperm whale myoglobin. Markers and alkaline phosphatase samples were prepared by the method of Fairbanks et al. [7].

pH Optimum. The pH optimum for the soluble alkaline phosphatase was determined by the same procedure reported for alkaline phosphatase solubilized from the membrane [2]. 1.54 μg of pure soluble alkaline phosphatase was used per assay.

Amino acid composition. The techniques for the determination of the amino acid compositions have been described previously [2]. 18 nmol of the purified soluble alkaline phosphatase were used per analysis. Tryptophan was determined by the method of Edelhoch [8].

Double diffusion analysis. The techniques described by Glynn et al. were used [4].

10% SDS-polyacrylamide slab gel electrophoresis. Samples (1.0 ml) of the salt-dependent or periplasmic alkaline phosphatase containing 2% SDS and 0.04 M mercaptoethanol were boiled for 5 min. The boiled samples were dialyzed for 2 h at room temperature against 150 ml electrophoresis running buffer (28.8 g glycine/6 g Trisma base/21) containing 2% SDS. Samples were prepared for electrophoresis using the method of Fairbanks et al. [7]. 15 μg alkaline phosphatase were included in each sample mix. Gel preparation, electrophoresis, staining and destaining were carried out according to the method of Weber and Osborn [9]. The slab gel was dried on filter paper prior to being photographed.

Results

The biochemical localization of alkaline phosphatase in *B. licheniformis* MC14 has been previously reported [2,4]. These studies have shown that an insoluble alkaline phosphatase is associated with the membrane fraction and that a soluble alkaline phosphatase is located in the region between the cell membrane and the cell wall. Histochemical studies [3], however, localized only the insoluble membrane-bound alkaline phosphatase in sites along the inner side of the membrane. The following experiments were done to determine why the histochemical studies showed only the insoluble, membrane-bound enzyme, whereas biochemical evidence indicated that a soluble alkaline phosphatase is released upon protoplast formation.

Effect of medium concentration on alkaline phosphatase synthesis and distribution. The cells which were used in the previous histochemical localizations were grown in 2% Neopeptone containing 0.1% fructose [3]. In the biochemical studies, however, cells were grown in 1% Neopeptone containing 0.1% fructose. Glynn et al. [4] have shown that when cells are grown in 1% Neopeptone, the percentage of soluble alkaline phosphatase increases from approx. 20% during early- and mid-logarithmic phase growth, to 59 and 79% during late-logarithmic and stationary phase growth, respectively. A comparative study was carried out to determine if the concentration of the medium affected the percentage distribution of the soluble and membrane-associated alkaline phosphatase.

Two cultures of cells were grown to the late-logarithmic, early-stationary phase of growth; one in 1% Neopeptone, the other in 2% Neopeptone. At the time of harvest, the cells grown in 1% or 2% Neopeptone had 0.2 and 0.1 unit alkaline phosphatase activity/ml of culture, respectively. The cells were then converted to protoplasts, releasing the soluble alkaline phosphatase. The soluble enzyme was separated from the protoplasts by centrifugation at $4000 \times g$ for 15 min. After the protoplasts were lysed by dilution in buffer 1 (without glycerol), both the lysed protoplast sample and the soluble material released on protoplast formation were centifuged at $157\,000 \times g$ for 1 h. 80% of the total alkaline phosphatase was released as a soluble enzyme upon protoplast formation of cells grown in 1% Neopeptone. Only 5—20% was released when cells grown in 2% Neopeptone were treated similarly. The remaining activity was found associated with the cell membrane fraction in both cases.

We have previously reported a thorough histochemical investigation of the distribution of alkaline phosphatase grown in 2% Neopeptone [3]. These studies were carried out on mid- or late-logarithmic phase cells and neither showed any evidence of lead phosphate deposits outside the cytoplasmic membrane. One of the reasons is that as little as 5% of the total cellular alkaline phosphatase may be released upon protoplast formation in cells grown to late-logarithmic phase growth and less than 1% in cells grown to mid-logarithmic phase growth in 2% Neopeptone. Also cells grown in 2% medium make one-half the total amount of enzyme/ml culture of that produced in 1% Neopeptone medium.

Stability of the soluble alkaline phosphatase. Glynn et al. [4] discovered that the soluble alkaline phosphatase was quite unstable when the sucrose in the protoplast-forming solution was removed by dialysis against Tris-acetate buffer. We have found that including 20% glycerol in any buffer used results in the stabilization of the enzyme if solutions are kept at 4°C.

Histochemical localization. The histochemical localization of the alkaline phosphatase(s) in late-logarithmic, early-stationary phase cells grown in 1% Neopeptone was examined using the procedures of McNicholas and Hulett [3] with certain modifications. The cells were grown to a later stage of growth because the greatest amount of soluble enzyme is formed during stationary growth in 1% Neopeptone. Glycerol (20%) was included in all buffers to stabilize the soluble alkaline phosphatase. All other procedures were identical to those previously reported.

Inhibition by lead or glutaraldehyde of alkaline phosphatase activity in cells grown in 1% Neopeptone did not differ from studies reported for cells grown in 2% Neopeptone [3].

Fig. 1 shows a control cell which was subjected to the histochemical mix, minus the substrate, *p*-nitrophenyl phosphate. Figs. 2 and 3 show examples of cells subjected to the complete histochemical mixture. Sites of alkaline phosphatase activity are distributed along the inner side of the cytoplasmic membrane, associated with the mesosomes and in the periplasmic region between the cell wall and cell membrane. The glycerol in the buffer may have caused a slight plasmolysis of the cells which accentuates the visualization of the deposits between the cell wall and the cell membrane.

Purification of soluble alkaline phosphatase. Glynn et al. [4] reported that enzymatic assays of the soluble proteins released upon protoplast formation indicated the presence of phosphatase activity, and that this mixture of released soluble proteins contained a component which showed immunological identity with the salt-extractable membrane alkaline phosphatase from *B. licheniformis* MC14.

Table I gives a summary of a typical purification of this soluble alkaline phosphatase using 15 g frozen cells. This procedure resulted in a 325-fold purification and a specific activity of 162.5. SDS-polyacrylamide gel electro-

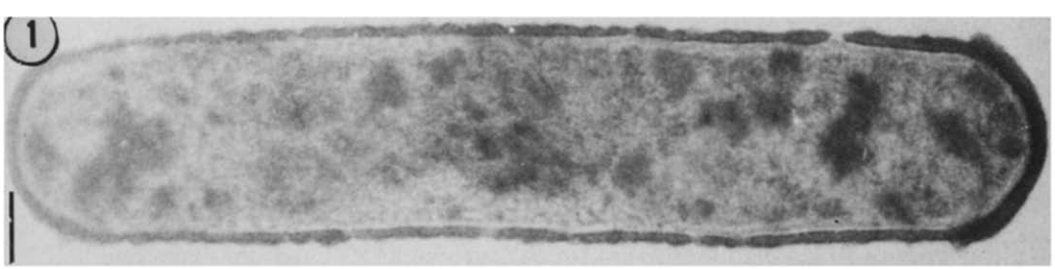
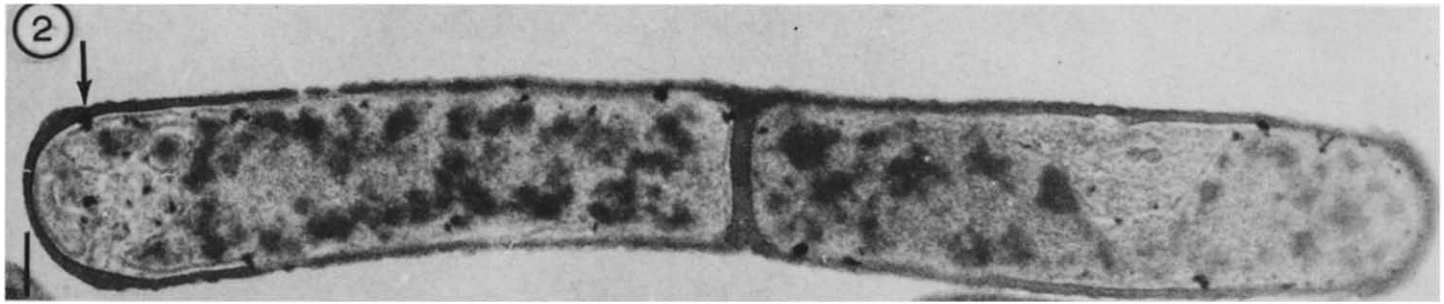


Fig. 1. *B. licheniformis* cultured at 55°C in 1% Neopeptone and harvested at the late logarithmic-early stationary phase of growth. Cells were incubated in histochemical mixture containing $Pb(NO_3)_2$ but lacking *p*-nitrophenyl phosphate. Bar indicates 0.1 μm.



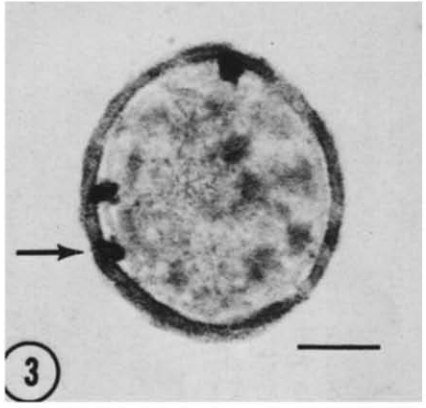


Fig. 2. A longitudinal section of *B. licheniformis* cultured at 55°C in 1% Neopeptone medium, harvested at the late logarithmic-early stationary phase of growth, and reacted in the histochemical mixture containing 0.06 mM *p*-nitrophenyl phosphate and 3.0 mM $Pb(NO_3)_2$. Lead phosphate deposits can be seen. Bar represents 0.1 μm.

Fig. 3. A cross-section of *B. licheniformis* cultured at 55°C in 1% Neopeptone medium, harvested at the late logarithmic-early stationary phase of growth, and reacted in the histochemical mixture containing 0.06 mM *p*-nitrophenyl phosphate and 3.0 mM $Pb(NO_3)_2$. Lead phosphate deposits can be seen. Bar represents 0.1 μm.

TABLE I

PURIFICATION OF SOLUBLE ALKALINE PHOSPHATASE FROM *BACILLUS LICHENIFORMIS* MC14

	Volume (ml)	Protein (mg)	Units of enzyme activity	Specific activity	Recovery (%)	Purification (fold)
1. Protoplasts in sucrose	45	4590	2295	0.5	100	1.0
2. Supernatant fraction from 1	38	418	1368	3.3	60	6.6
3. Supernatant fraction from 70% $(NH_4)_2SO_4$	45	180	1215	6.8	53	13.6
4. Dialysis and centrifugation	66	66	1254	19.0	55	38.0
5. CM-Sephadex	44	18	1188	66.0	52	132.0
6. Dialysis	47	18	1222	67.9	53	135.0
7. Second CM-Sephadex	42	2	336	168.0	15	336.0
8. Dialysis	55	2	325	162.5	14	325.0

phoresis of this protein gave a single sharp band indicating a pure enzyme. The more than 3.75-fold decrease in total units recovered from the second CM-Sephadex column was the result of conservative selection of pooled fractions in order to insure purity of the enzyme.

Determination of subunit molecular weight. The subunit molecular weights of the soluble and salt-dependent alkaline phosphatase were compared using SDS-polyacrylamide slab gel electrophoresis.

The apparent subunit molecular weights for the soluble and the salt-dependent alkaline phosphatase were 59 700 and 60 000, respectively. The two alkaline phosphatase(s) appear as a single band when both are run in the same lane (data not shown).

Determination of native molecular weight. The molecular weight for the native soluble alkaline phosphatase was determined by two methods: the sedimentation equilibrium method of Yphantis [18] and gel-exclusion chromatography. The data from the sedimentation experiment were graphed as $[Y_r - Y_0]$ vs. r^2 where r is equal to the distance from the center of rotation, Y_r is the y value (vertical fringe displacement) at any r, and Y_0 is the value of y at the meniscus. The straight line slope, which suggested homogeneity of the sample, was used to calculate an approximate molecular weight of 127 000 ± 4000. The density of the solvent was determined pycnometrically and the partial specific volume was determined from the amino acid composition. The gel exclusion data yielded a calculated molecular weight of 12 000. The heat-salt and salt-dependent alkaline phosphatase native molecular weights (Yphantis method) have been reported as 121 000 ± 3000 [10] and 124 000 ± 3000, respectively [2].

Amino acid composition. The amino acid composition of the soluble alkaline phosphatase and the membrane-associated alkaline phosphatase [1,2] are shown in Table II. The number of residues per subunit for all three purifications are listed. Differences in the amino acid compositions of the membrane alkaline phosphatase(s) purified by the two procedures [10,2] have been

TABLE II

AMINO ACID COMPOSITION OF ALKALINE PHOSPHATASE FROM *B. LICHENIFORMIS* MC14

Amino acid analysis was done as described previously.

Amino Acid	Average Resid./Subunit Mol. Wt.		
	Soluble alkaline phosphatase	Membrane alkaline phosphatase [a]	Membrane alkaline phosphatase [b]
Lysine	89	66	64
Histidine	14	11	8
Arginine	5	7	17
Asx	53	50	65
Threonine[c]	26	31	34
Serine[c]	49	34	40
Glx	41	51	54
Glycine	42	43	54
Alanine	32	47	52
Valine	8	39	55
Methionine	+[e]	18	16
Isoleucine	26	21	24
Leucine	37	36	35
Tyrosine	15	29	16
Phenylalanine	13	17	15
Tryptophan[d]	3	9	4
Cysteine	0	0	0
Proline	1	0	22

[a] From Ref. 10.
[b] From Ref. 2.
[c] Values were determined by extrapolation to zero time.
[d] Determined by the method of Edelhoch [5].
[e] Labeled with [^{35}S]methionine.

previously discussed [2]. The composition of the soluble alkaline phosphatase compared to the membrane-associated enzyme showed many similarities. Significant differences were noted in the following residues: lysine, valine, methionine and alanine.

Repeated analysis of the soluble alkaline phosphatase from different purifications were carried out in an attempt to recover methionine in a non-oxidized state. The correct amounts of methionine were recovered for control proteins of known amino acid sequences or membrane alkaline phosphatase subjected to the same hydrolysis procedures. When the soluble alkaline phosphatase from cells grown in [^{35}S] methionine was isolated, it had approximately the same specific activity as the membrane alkaline phosphatase isolated from the same culture. Therefore, we conclude that methionine is present in both the soluble and membrane alkaline phosphatase in approximately the same amounts. The decreases in the nonpolar amino acids and the apparent increase in the lysine residues in the soluble alkaline phosphatase are consistent with the increased solubility of this enzyme as compared to the membrane associated alkaline phosphatase.

K_m determination. The K_m and K_i values for the soluble alkaline phosphatase were determined by the procedures reported for the membrane alkaline phos-

TABLE III

SUBSTRATE SPECIFICITIES OF SOLUBLE ALKALINE PHOSPHATASES

Substrate specificity of soluble alkaline phosphatase involved reaction of the enzyme (1.54 μg/assay) with various substrates (saturating substrate concentration: $1 \cdot 10^{-2}$ M) in a 1.0 M Tris-acetate buffer, pH 8.0. The reactions were allowed to proceed for 1, 3 and 5 min at 55° C and stopped with 20% trichloracetic acid. Inorganic phosphate was determined [6]. Blanks contained all components of the reaction mixture except the enzyme. PNPP, *p*-nitrophenyl phosphate.

Substrate	Relative Activity (% PNPP)		
	Soluble alkaline phosphatase	Membrane alkaline phosphatase [a]	Membrane alkaline phosphatase [a]
PNPP	100	100	100
5'AMP	73	112	87
3'AMP	53	84	62
5'CMP	94	87	77
5'IMP	96	107	93
5'UMP	125	94	81
5'ATP	13	8	11.3
3-PGA	29	13	28.3
D-Glucose 6-phosphate	17	16	30.0
Glycerol 2-phosphate	20	38	36.0
PP_i	20	1.7	66.6
PNP-sulfate	0	0	0.0
Bis PNPP	0	—	—

[a] From Ref. 2.

phatase [2]. The K_m for *p*-nitrophenyl phosphate was $4.0 \cdot 10^{-4}$ M. This compared to a K_m of $2.52 \cdot 10^{-4}$ M for the salt-dependent alkaline phosphatase and $4.0 \cdot 10^{-4}$ M for the heat-salt alkaline phosphatase.

The apparent K_i for inhibition of inorganic phosphate was $1.25 \cdot 10^{-4}$ M. This compares to a K_i of $1.1 \cdot 10^{-2}$ M for the salt-dependent membrane alkaline phosphatase and $2.5 \cdot 10^{-3}$ M for the heat-salt alkaline phosphatase.

pH Optimum. The procedure was carried out as described previously [2]. The soluble alkaline phosphatase has an optimum pH of 9.75. The reported pH for both the salt-dependent and heat-salt membrane alkaline phosphatase [10] was 10.1. The salt-dependent alkaline phosphatase is active over a greater pH range and retains 34% of its maximal activity at pH 11.2, whereas the heat-salt alkaline phosphatase and the soluble alkaline phosphatase retain no activity at pH 11.0.

Hydrolysis of substrates by soluble and membrane-associated alkaline phosphatase. The rate of hydrolysis of various substrates by the soluble enzyme and the membrane associated enzyme [7,10] are listed in Table III. This table shows that both enzymes hydrolyze nucleotide monophosphates most effectively. Lower rates of hydrolysis were observed when 3 phosphoglyceric acid, glucose 6-phosphate, 5'ATP or glycerol 2-phosphate were used as substrates.

Double-diffusion analysis. Crude extracts containing soluble alkaline phosphatase activity have been shown to possess a component which exhibits

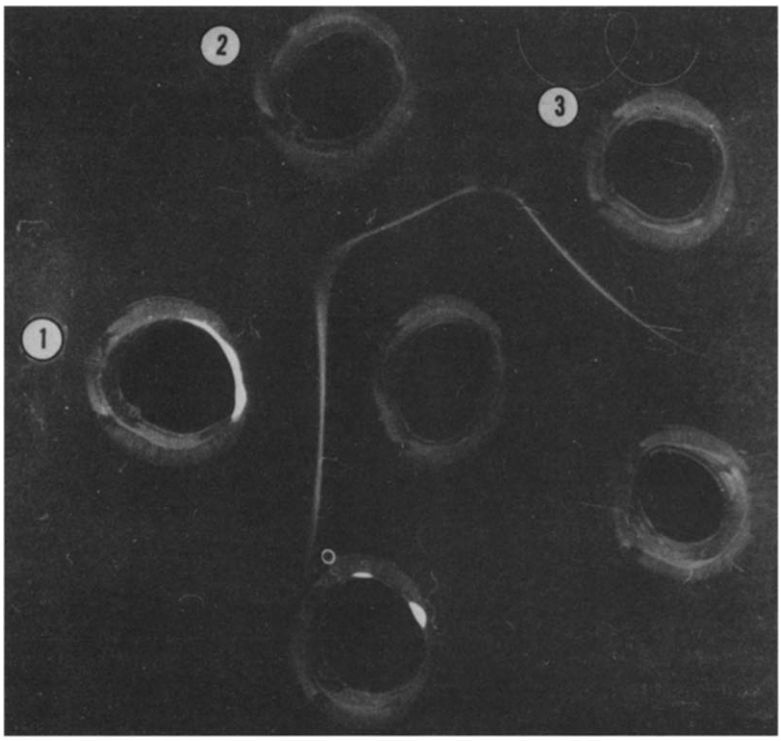


Fig. 4. Ouchterlony double-diffusion precipitation reactions of antisera against the membrane-associated alkaline phosphatase (center well) vs. soluble alkaline phosphatase (wells 1 and 3) or the membrane-associated enzyme (well 2). Soluble alkaline phosphatase (20λ of 0.2 mg/ml) Membrane alkaline phosphatase (20λ of 0.18 mg/ml).

immunological identity in Ouchterlony double-diffusion analysis [7]. To verify that the observed precipitin line was due to the soluble alkaline phosphatase, it was necessary to use purified soluble alkaline phosphatase in the analysis (Fig. 4). The precipitin lines indicate immunological identity between the membrane associated alkaline phosphatase and the soluble alkaline phosphatase.

Discussion

We have shown that changes in the concentration of the growth medium cause an alteration of the quantity of alkaline phosphatase produced and in the distribution of this enzyme in *B. licheniformis* MC14. When this organism is cultured in 1% Neopeptone, twice as much total enzyme is synthesized and 10—30-times more soluble enzyme is released upon protoplast formation than when the organism is grown in 2% Neopeptone. These data, coupled with the incorporation of procedures which stabilized the soluble enzyme, made it possible to confirm its location as determined by biochemical studies [4] via electron microscopy histochemistry. Culture conditions have also been shown to affect the distribution of alkaline phosphatase in *B. licheniformis* 749 C [11].

The soluble alkaline phosphatase, purified to homogeneity, had a specific activity somewhat less (163) than that obtained for purified membrane phosphatase (purified heat-salt solubilized and salt-dependent membrane alkaline phosphatase had specific activities of 228 and 198, respectively). It is likely that more inactive soluble alkaline phosphatase co-purifies with the soluble enzyme than the more stable membrane alkaline phosphatase.

The purified soluble alkaline phosphatase is quite similar to the form which is associated with the membrane. Total immunological identity with membrane alkaline phosphatase, indicates that the two forms are closely related. Subunit molecular weight analysis indicates that the difference in molecular weight is no greater than a few hundred daltons. These data indicate that the two forms differ only slightly. The formation of isozymes differing in only the amino terminal amino acid (arginine) has been reported previously in *E. coli* alkaline phosphatase [12] and was the result of growth conditions.

Certain characteristics of the soluble alkaline phosphatase are very different from the membrane-associated enzyme. The greatest differences were in stability and solubility of this form of the enzyme in aqueous solution. It is important to note that the soluble form of the enzyme is found to be released upon protoplast formation or upon lysis of cells [4]. This indicates that lysing of cells alone does not cause a nonspecific association of the soluble form of the enzyme with the membrane. It is also much more sensitive to phosphate inhibition than the salt-dependent alkaline phosphatase. While the pH optima for both membrane and soluble alkaline phosphatase are similar, the decrease in activity after pH 10 is much more abrupt for the soluble alkaline phosphatase than for the salt-dependent alkaline phosphatase. It is interesting to note that the salt-extracted membrane alkaline phosphatase which was solubilized by the heat step (and remained soluble in dilute buffers) is more sensitive to inorganic phosphate inhibition and is also less stable at higher pH values than the salt-dependent purified enzyme. Thus, both the soluble forms (one which is released upon protoplast formation and one which loses the salt-dependency for solubility during a rather drastic 80°C, 5 min heat step) show greater sensitivity to inorganic phosphate inhibition and higher pH environments.

In summary, we have shown that growth conditions affect the distribution of alkaline phosphatase in *B. licheniformis* MC14. The soluble enzymatic activity which was released upon protoplast formation [4] has been shown to be very similar to the membrane-associated salt-extractable alkaline phosphatase and is likely the product of the same gene.

Recently the location of alkaline phosphatase on the cytoplasmic membrane surfaces of *B. licheniformis* has been re-examined. Lactoperoxidase ^{125}I labeling experiments indicate that the active, salt-extractable alkaline phosphatase is asymmetrically localized in a 70 : 30 distribution between the inner and outer surfaces on the cell membrane, respectively [13].

These findings would support both the previous histochemical localization reported by McNicholas and Hulett and the localization presented here. This is a unique distribution for an enzyme which is also secreted through the cytoplasmic membrane [14,15,16,17] and raises questions with respect to the mechanism of secretion and the possibility of the membrane-associated alkaline phosphatase (from either location) being a precursor to the secreted enzyme.

Preliminary pulse-chase experiments indicate that a portion of the salt-extractable membrane alkaline phosphatase is a precursor to the secreted alkaline phosphatase. Further experiments are in progress to explore these preliminary data in relation to currently hypothesized mechanisms of protein secretion.

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