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Purification of *Escherichia coli* Alkaline Phosphatase

Improved Growth Conditions for the Bacteria, Modified Methods of Preparation of the Enzyme

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An improved method for the growth of *E. coli* bacteria and subsequent purification of alkaline phosphatase is described. The growth medium has been varied in order to obtain maximal production of the enzyme.

The enzyme has been released from the bacteria by converting the cells into spheroplasts. The purification procedure involves chromatography on DEAE-cellulose, dialysis against "activation buffer", gel filtration, and in some cases zone electrophoresis.

Alkaline phosphatase, purified by the procedure described in this paper, has a somewhat higher specific activity than reported earlier, sediments as a single symmetrical boundary in the ultracentrifuge, and migrates as a single peak when subjected to column electrophoresis. Disc electrophoresis experiments, however, indicate the existence of several isoenzymes.

The main physical and chemical properties of the purified enzyme are similar to the alkaline phosphatase prepared from *E. coli* cells with other methods.

Alkaline phosphatases are present in a variety of biological materials.[1,2] However, the alkaline phosphatase from *Escherichia coli* (orthophosphoric monoester phosphohydrolyase EC 3.1.3.1) has been most extensively studied. This enzyme has been identified by Horiuchi *et al.*[3] and by Torriani[4] and first prepared and characterized by Garen and Levinthal.[5] The enzymatically active *E. coli* alkaline phosphatase contains zinc[6] and is a dimer[7] or, as recently reported, a tetramer of identical subunits.[8] The specific zinc binding properties of the enzyme have been studied in great detail.[9,10] Specificity and kinetics have been investigated by several groups and the mechanism of the catalytic action has been delineated.[11–23]

Various methods of purification of alkaline phosphatase from *E. coli* have been published,[5–7,11,24–30] and in most of them almost identical growth

media were used. The original material is either broken cells, extracts of acetone powders prepared from whole cells, or spheroplasts. The reported purification procedures consist of chromatography on DEAE-cellulose, either separately or in combination with crystallization. The preparation method by Malamy and Horecker [26] gives a crystalline enzyme with high specific activity. However, crystallization of alkaline phosphatase results in a rather low yield of purified material.

In order to obtain a higher degree of enzyme synthesis, attempts have been made to change the culturing conditions of *E. coli* bacteria. In addition, the methods for enzyme purification have been modified and alkaline phosphatase with high specific activity can be obtained in good yield. The improvements achieved in the growth conditions and in the procedure for enzyme preparation are described in the present communication.

EXPERIMENTAL AND RESULTS

Bacterial strain. Escherichia coli, strain C_4F_1, derived from K-12, was a generous gift from Dr. A. Torriani, Biological Laboratories, Harvard University, Cambridge, Mass., U.S.A. With respect to alkaline phosphatase this strain is constitutive. Stock cultures of these cells were maintained on nutrient agar in tubes.

Reagents. Reagent grade chemicals were used without further purification. Tris-(hydroxymethyl aminomethane): "Sigma 121" was used. Bactopeptone and Bactoagar were Difco products. Inosine was from Koch-Light Laboratories Ltd. Lysozyme and *p*-nitrophenyl phosphate disodium tetrahydrate were purchased from Sigma, and 2-mercaptoethanol from Eastman Organic Chemicals. The DEAE-cellulose was a Whatman product and the cellulose powder for zone electrophoresis came from Grycksbo Pappersbruk AB, Sweden. Sephadex was a product of Pharmacia Fine Chemicals, Uppsala, Sweden.

Growth conditions. Medium A contained: 0.12 M Tris, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl, 0.003 M Na_2SO_4, 0.001 M $MgCl_2$, 2.0×10^{-4} M $CaCl_2$, 2.0×10^{-6} M $ZnCl_2$, 0.5 % glucose, and 0.5 % Difco Bactopeptone. The medium was adjusted to a final pH of 7.4 with HCl. A glucose solution was prepared separately as a sterile 20 % solution and added to the autoclaved medium at the time of incubation.

Medium B contained: 0.12 M Tris, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl, 0.003 M Na_2SO_4, 0.001 M $MgCl_2$, 2.0×10^{-4} M $CaCl_2$, 4.0×10^{-5} M $ZnCl_2$, 0.5 % glucose, 0.45 % Difco Bactopeptone, and 1.0×10^{-5} M inosine. The medium was adjusted to a final pH of 7.8 with HCl. A glucose solution was prepared separately as a sterile 20 % solution and added to the medium at the time of incubation.

Turbidity measurements. For the purpose of estimating growth of cells, turbidity measurements were carried out at 590 nm in a Zeiss PMQ II spectrophotometer.

Determination of protein concentration. The protein concentration was measured spectrophotometrically in a Zeiss PMQ II spectrophotometer at 278 nm according to the method by Malamy and Horecker.[26] A value of 0.72 was employed as the absorbance of a 1 mg/ml solution of the enzyme. This extinction coefficient was confirmed with measurements of protein dry weight, following precipitation by trichloroacetic acid.

Enzyme activity measurements. The enzyme activity measurements were carried out as described previously,[26] in a Zeiss PMQ II spectrophotometer. The unit of enzyme activity and the specific enzyme activity was defined according to Malamy and Horecker.[26]

Ultracentrifugation. Ultracentrifugation experiments were performed at 20°C and 59 780 rpm with a Spinco model E ultracentrifuge.

Optical absorption measurements. The optical absorption spectra were recorded at 25°C in a Zeiss M4Q II or a Cary 15 recording spectrophotometer using quartz cuvettes with an optical path of 1 cm.

Amino acid analyses. Amino acids were analyzed after hydrolysis of the protein during different times, at 110°C, in 6 M HCl in evacuated sealed tubes. The analyses were performed with a Beckman 120 B automatic amino acid analyzer, according to the

standard procedure.[31] Determinations of tryptophan were performed on native enzyme samples by the method of Spies and Chambers.[32] Half-cystine and methionine were determined by the method of Moore.[33] Results have been calculated on the basis of a molecular weight for *E. coli* alkaline phosphatase of 80 000.

Zinc analyses. Determination of zinc in the enzyme solutions was performed with the dithizone method,[34] and by emission spectrography using a Perkin-Elmer atomic absorption spectrometer 303. Prior to zinc analyses, the samples were dialysed against four changes of 100-fold volume excesses of deionised water for 24 h, or in some cases against 1.0×10^{-3} M metal-free Tris-HCl buffer, pH 8.0. A water or buffer blank was run parallel to all determinations without detecting zinc in these blanks. Metal-free buffer was prepared through extraction with dithizone in CCl_4 as described elsewhere.[34] Distilled water which had been passed through a cation exchange column was utilized. All glassware was acid washed according to Thiers.[35]

Concentration of dilute proteins. Dilute solutions of protein were concentrated by means of ultrafiltration through dialysis tubing under reduced pressure, or with lyophilization.

Changes in growth conditions

The composition of medium A and its pH were varied in order to obtain both abundant cell growth and maximal alkaline phosphatase synthesis.

Effect of inosine. The synthesis of alkaline phosphatase in *E. coli* is repressed by the presence of inorganic phosphate in the growth medium.[4] Since use of bactopeptone introduces some phosphate into the culturing solution, the enzyme synthesis is consequently lowered. Bowne *et al.*[36] have shown that inosine is an effective phosphate acceptor. Experiments were therefore devised, in which inosine was added to the growth medium to elucidate the effect on the rate of synthesis of alkaline phosphatase in *E. coli*. The bacteria were grown in (a) 1 l of medium A + inosine or (b) 1 l of medium A with no inosine added. The solutions, in 3 l flasks, were shaken on a rotary shaker at 37°C for 24 h. Aliquots of the bacterial suspension were withdrawn at various times and turbidity and enzyme activity measurements were carried out.

The growth and enzyme synthesis curves, compared in Fig. 1, clearly indicate that the enzyme production is 10 % higher in the medium containing inosine. The results

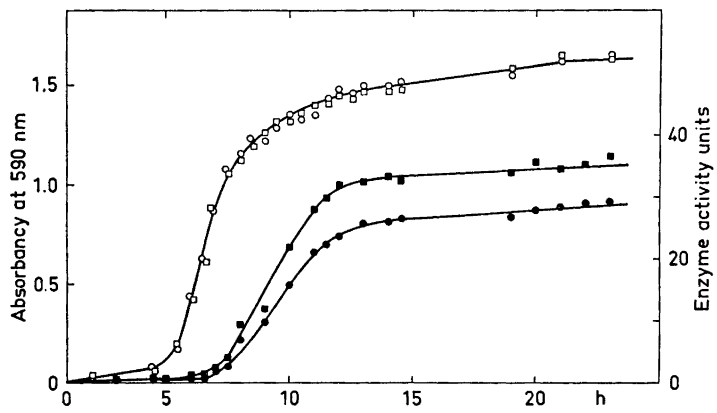

Fig. 1. Effect of inosine concentration on the growth of *E. coli* cells and on the alkaline phosphatase synthesis. ○, cell growth in the absence of inosine; □, cell growth in the presence of 5.0×10^{-4}M inosine; ●, enzyme synthesis in the absence of inosine; ■, enzyme synthesis in the presence of 5.0×10^{-4} M inosine.

show that even with this constitutive bacterial strain, the formation of alkaline phosphatase is increased when cells are grown in a medium with very low phosphate concentration.

Effect of changes in zinc concentration. The growth of cells and the production of the enzyme were examined with various concentrations of zinc in the culture medium, between 1.0×10^{-6} M and 6.0×10^{-5} M. These experiments were performed with cultures containing 1 l of medium. The solutions were treated as described above. Turbidity and enzyme activity were measured. Actually, no effects were detected in the growth of bacteria at different zinc concentrations. However, striking differences were observed in the amount of alkaline phosphatase activity. In general, the results obtained suggested that 4.0×10^{-5} M $ZnCl_2$ is the concentration of zinc giving the highest enzyme activity.

Coleman *et al.*[29] have found that zinc deficiency does not cause any inhibition of alkaline phosphatase synthesis in the *E. coli* C 90 strain. Under metal-free conditions this strain synthesizes a zinc-free inactive apoenzyme, that can be completely activated by the addition of zinc *in vitro*. Similar results have been obtained with the *E. coli* strain utilized in the present study (unpublished results).

Effect of pH. It was noticed that a decrease of pH during the growth process apparently affects the growth rate and the production of enzyme. In order to study this effect in detail, nine different 1 l solutions of medium A, of different pH values, were treated as described earlier. The turbidity and the alkaline phosphatase activity were measured. Fig. 2 shows that the increase of pH to 7.8 gave higher gains, as compared to other pH values.

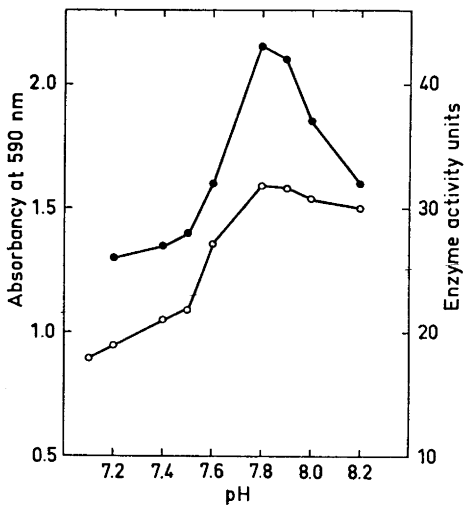

Fig. 2. Effect of pH on the growth of *E. coli* cells and on the synthesis of alkaline phosphatase. ○, cell growth; ●, enzyme synthesis.

Effect of other factors. It should be mentioned that during the course of variation of the growth media, we observed that the increase of the Na_2SO_4 concentration to 4.5×10^{-3} M resulted in intensified cell growth. However, the properties of the organism were altered. Thus, the substrate, *p*-nitrophenyl phosphate, was no longer hydrolysed by the intact cells. Furthermore, after treatment with EDTA and lysozyme according to Malamy and Horecker,[26] no alkaline phosphatase activity was detected in the sucrose medium. It is not clear whether these findings represent depressed synthesis of enzyme, alternation in cell permeability, a change in the location of the alkaline phosphatase, or the presence of an internal inhibitor in the local environment of the enzyme. The possibility that these culturing conditions favour the growth of a mutant strain has not been ruled out.

Preparation and purification of the enzyme

Bacteria for these preparations were grown in medium B (see section *Changes in growth conditions*).

A typical preparation of the enzyme is described below, and the results are summarized in Table 1. As indicated in Table 1, the starting material was the cells from a 5 l batch of culture medium. Sometimes growth was also carried out in cultures of 10 – 20 l and 50 l, with proportionately the same results.

Table 1. Summary of preparation and purification of *E. coli* alkaline phosphatase.

Fraction	Total enzyme activity (units) [20]	Protein (mg)	Specific activity (units/mg protein)
Whole cells (grown in 5 l culture media)	210 000		
After lysozyme treatment	416 000	744	560
After DEAE-cellulose chromatography	363 000	224	1620
After dialysis against activation buffer	362 000	176	2060
After gel filtration	358 000	108	3300

Preparation of cell extract. Five 3 l flasks containing 1 l of medium B were inoculated with 20 ml of the *E. coli* K-12 C_4F_1 cultures. The flasks were incubated at 37°C on a rotary shaker until the solutions reached a final turbidity of 1.60. The intact bacteria contained 40.0 units of alkaline phosphatase activity per ml of culture. Harvesting of cells from the cultures was carried out by centrifugation in an International Serum Centrifuge, at 2000 *g* for 60 min, at 15°C. After centrifugation at 2300 *g* for 45 min in a Sorwall RC-2 Centrifuge, at 4°C, the pellets were washed with 0.01 M Tris-HCl buffer, pH 8.0.

The cells were suspended in 1 l of 0.033 M Tris-HCl buffer, pH 8.0, containing 20 % sucrose. Then the cells were treated with lysozyme according to Malamy and Horecker.[26] The suspension was centrifuged at 8000 *g* for 20 min in a Sorwall RC-2 Centrifuge, at 4°C. The supernatant solution, containing the active enzyme, was immediately applied to the chromatographic column, at 4°C.

DEAE-cellulose chromatography. DEAE-cellulose was suspended in 0.01 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl. A column with the dimensions 2.0 × 30.0 cm was packed and equilibrium with the buffer salt solution. The alkaline phosphatase solution was then filtered through at a flow rate of 90 ml/h. After the adsorption of the enzyme, the column was washed twice with 25 ml of 0.01 M Tris-HCl buffer, pH 8.0, + 0.05 M NaCl. The enzyme was eluted with the same buffer, containing 0.125 M NaCl. The flow rate was 1 ml per min and fractions of 6 ml were taken. Among the several protein peaks in the chromatogram, only one contained alkaline phosphatase activity.

The fractions containing active enzyme were pooled and dialysed as described below.

Dialysis against "activation buffer". After the DEAE-cellulose chromatography, the enzyme was dialysed against 300 ml of "activation buffer", consisting of 6 mM mercaptoethanol, 2 mM $ZnCl_2$ in 0.001 M Tris-HCl buffer, pH 8.0, at 4°C for 12 h. During dialysis a white precipitate usually formed which was centrifuged at 4000 *g* for 10 min at 4°C. The precipitate was discarded and the enzyme solution was dialysed against three changes of 50-fold volume excess of 0.001 M Tris-HCl buffer, pH 8.0, for 24 h at 4°C. The enzyme solution was concentrated by ultrafiltration to a final concentration of 10 mg of protein per ml and subjected to gel filtration at 4°C.

Gel filtration on Sephadex G-150. The dialysed and concentrated enzyme solution was filtered through a Sephadex G-150 column (3.0 × 120 cm) which had been equilibrated

with 0.01 M Tris-HCl buffer, pH 8.0. The chromatogram was developed with the same buffer.

The column effluents were collected in 7.0 ml portions at a rate of about 20 ml/h. The absorbance at 278 nm was recorded and the elution pattern showed two protein peaks, one of which contained alkaline phosphatase activity. Fractions of high specific activity were purified further with zone electrophoresis.

Preparative zone electrophoresis. An electrophoresis column (4 × 100 cm) was packed with ethanolysed cellulose powder. The packing and treatment of the column and the whole electrophoresis were performed according to the standard procedure given by Porath.[37] 100 mg of alkaline phosphatase in a volume of 10 ml was dialysed against 0.01 M Tris-HCl buffer, pH 8.0, for 10 h at 4°C, and applied to the column and washed in with 10 ml of the buffer. The electrophoresis was stopped after 24 h at 500 V, 2 mA and the protein was eluted with the same Tris-HCl buffer. Fractions of 7 ml were collected, flow rate 2 ml/min. Usually two protein peaks were detected, one of which contained the bulk of alkaline phosphatase activity.

Crystallization. When crystallization was desired, the procedure described by Malamy and Horecker [26] was employed.

Storing of the enzyme. Three different methods were utilized for enzyme storage: (1) lyophilization; (2) in 61 % ammonium sulfate, pH 8.0, containing 1.0×10^{-4} M $ZnSO_4$; or (3) in 1.0 M NaCl solution, pH 8.0, containing 1.0×10^{-5} M $ZnCl_2$; all in a cold room, 4°C.

Criteria of purity and properties of enzyme

Rechromatography on DEAE-cellulose. The purity of the solution after gel filtration was tested on a DEAE-cellulose column (1.3 × 18 cm). The enzyme solution was dialysed against 0.01 M Tris-HCl buffer, pH 7.5, at 4°C, and the column was equilibrated with the same buffer. The dialysed solution (2 ml), containing 3.5 mg of protein per ml, was applied to the column, at 4°C and was then eluted by gradient elution which started

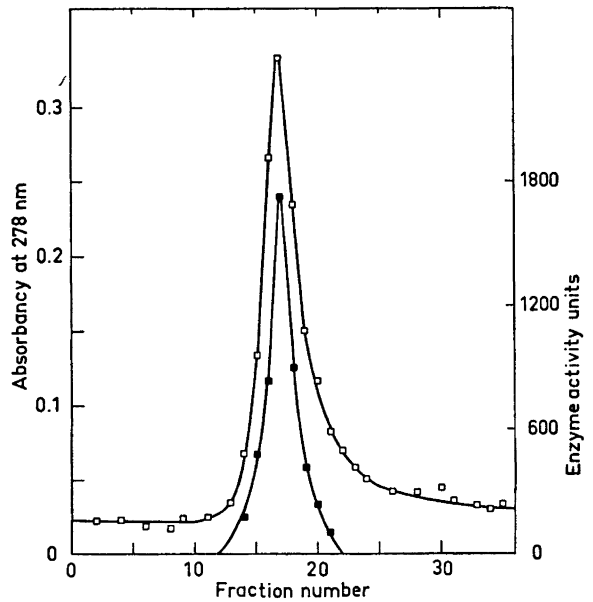

Fig. 3. Rechromatography of the purified *E. coli* alkaline phosphatase. For experimental details, see the text. □, absorbancy at 278 nm; ■, alkaline phosphatase activity units.

with 160 ml of 0.01 M Tris-HCl buffer, pH 7.5, in the closed mixing chamber and 0.20 M Tris-HAc, pH 7.5, + 0.20 M sodium acetate in the reservoir flask. The flow rate was 1 ml/min and fractions of 4 ml were taken. The elution pattern shown in Fig. 3 gave one single symmetrical protein peak with alkaline phosphatase activity.

Sedimentation analysis. As shown in Fig. 4, ultracentrifugation experiments with the purified enzyme gave only on symmetrical sedimentation boundary. At pH 8.0 the sedimentation coefficient was 6.1×10^{-13} sec^{-1}.

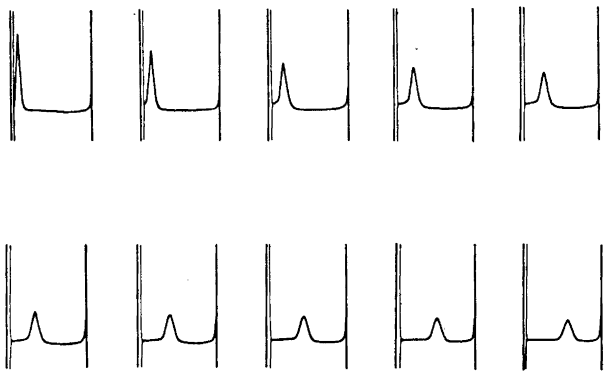

Fig. 4. Ultracentrifugation of the purified *E. coli* alkaline phosphatase. The photograph was taken at 8 min intervals after reaching speed, at a phase angle of 55°. The protein concentration is 1.0×10^{-4} M, in 0.01 M Tris-HCl buffer, pH 8.0.

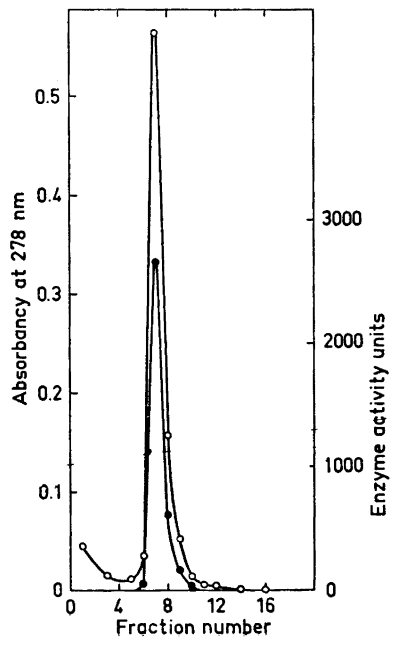

Fig. 5. Zone electrophoresis of the purified *E. coli* alkaline phosphatase. For experimental conditions, see the text. ○, absorbancy at 278 nm; ●, alkaline phosphatase activity.

Zone electrophoresis. The electrophoresis was performed mainly as described above. The enzyme solution was dialysed against 0.01 M Tris-HCl buffer, pH 8.0, for 10 h, at 4°C. 1 ml of the solution was applied to the column (1 × 80 cm) and was washed in by 3 ml of the buffer. The electrophoresis was stopped after 22 h at 500 V and 2 A. The protein was eluted and fractions of 6 ml were collected. The rate of elution was about 1.5 ml/min. Fig. 5 shows the elution diagram of a typical experiment.

Table 2. Zinc content of purified *E. coli* alkaline phosphatase.[a]

Method of zinc analysis	Enzyme sample	Zinc[b] (mol/l)	Protein (mol/l)	Zinc atoms per protein molecule	Enzyme activity (units[20]/mg)
Dithizone	After gel filtration	9.1×10^{-5}	3.0×10^{-5}	3.03	3300
Atomic absorption	After gel filtration	8.9×10^{-5}	3.0×10^{-5}	2.97	3300
Dithizone	After lyophilization	6.8×10^{-5}	2.2×10^{-5}	3.08	3250
Atomic absorption	After lyophilization	6.7×10^{-5}	2.2×10^{-5}	3.05	3250

[a] This table shows the zinc analysis on a single preparation. Zinc analysis on several preparations gave a mean value of 2.7–3.3 zinc/protein molecule.
[b] The results are the averages of triplicate analyses on each sample.

Table 3. Amino acid composition of *E. coli* alkaline phosphatase.

Amino acid	Amino acid residues per molecule of protein		
	Rothman *et al.*[7]	Simpson *et al.*[30]	Our results
Lysine	50	50.3	49.1
Histidine	17	16.4	16.3
Arginine	23	23.1	23.4
Aspartic acid	84	92.5	92.6
Threonine	70	42.0	69.1[c]
Serine	40	76.0	40.3[c]
Glutamic acid	79	88.4	86.8
Proline	36	39.4	38.0
Glycine	77	87.0	85.4
Alanine	110	124.4	116.0
Half-Cystine	7	8.0	7.8[b]
Valine	43	43.0	42.2[a]
Methionine	12	14.4	14.8[b]
Isoleucine	26	27.7	26.0[a]
Leucine	66	75.4	70.0[a]
Tyrosine	19	20.3	20.1
Phenylalanine	15	16.0	16.1
Tryptophan	7	7.9	8.0[d]

[a] After 72 h of hydrolysis. [b] Determinations by the method of Moore.[33]
[c] Values are obtained after extrapolation to zero time of hydrolysis.
[d] Determinations by the method of Spies and Chambers.[32]

Disc-gel electrophoresis. The enzyme homogeneity was also tested by disc-gel electrophoresis according to the method by Smith.[38] A 7 % gel solution was made. Tris-borate buffer, pH 9.5, was used for the preparation of the gel solution. The polyacrylamide was polymerized in small glass tubes. The enzyme (10 ng) in a 40 % sucrose solution (20 ng) was applied to the gel and 3–4 mA current was passed through for 50 min. Coomassie Brilliant Blue R-250, 0.25 % in 20 % trichloroacetic acid, was used to stain the protein bands. After washing with 7 % acetic acid three close distinct protein bands were obtained. The gel was developed with *p*-nitrophenyl phosphate for enzymatic activity estimation. All three protein bands showed alkaline phosphatase activity.

Zinc analysis. The purified enzyme was analysed for zinc as described above. The results are shown in Table 2.

Amino acid analysis. The results from the determination of the amino acid composition of alkaline phosphatase are given in Table 3. The values obtained for the various amino acids in the samples hydrolysed for 20 or 72 h were averaged. However, only the values obtained after 72 h of hydrolysis are reported for valine, leucine, and isoleucine. The values listed for serine and threonine were obtained after extrapolation to zero time of hydrolysis, assuming first order kinetics for the decomposition. The value for tryptophan is the average of five determinations on native enzyme samples.

DISCUSSION

The results delineated in this work have demonstrated that the *E. coli* growth conditions can be changed so that the yield of bacterial cells and the production of alkaline phosphatase increases markedly. The culturing procedure described has the advantage of offering a better starting material for the isolation of the enzyme compared to earlier methods.

Alkaline phosphatase can be reached by external substrate in the intact *E. coli* cells, and this finding indicates a location of the enzyme in the periplasmic space, a region between the protoplasmic membrane and cell wall layers. Procedures which affect the integrity of the cell wall – such as osmotic shock or conversion of *E. coli* cells into spheroplasts – cause the release of the enzyme. This is the reason why we employed treatment with lysozyme and EDTA to release the alkaline phosphatase from the cells.[26]

The data presented in Table 1 shows that when cells are converted to spheroplasts the total number of alkaline phosphatase units was higher than in intact cells, measured under similar conditions. This finding is in agreement with previous observations,[26] and with the report of Torriani and Levinthal [39] that the apparent Michaelis constant for hydrolysis of *p*-nitrophenyl phosphate is greater for the whole cells than for the cell extracts. As suggested,[39] the reason for this might be the existence of a cell wall barrier.

The procedure described here for the purification of alkaline phosphatase gives a considerably higher yield of enzyme than earlier methods. The enzyme shows apparent homogeneity, as judged on the basis of data derived from rechromatography, ultracentrifugation, zone electrophoresis, and starch gel electrophoresis. However, disc gel electrophoresis indicated the presence of multiple forms of the enzyme. Isoelectric focusing electrophoresis – a method with extremely high resolving power – has also revealed the presence of isoenzymes, as described in an accompanying paper.[40]

The purified protein exhibits properties similar to alkaline phosphatase prepared from *E. coli* with other procedures. Our ultracentrifugation data give a sedimentation constant with a value of 6.1×10^{-13} sec^{-1} which is in good agreement with data from other laboratories.

The amino acid composition of alkaline phosphatase prepared with the new procedure (see Table 3) is very similar to those obtained for alkaline phosphatases prepared by other methods. The fact that, for amino acids appearing in low amounts, the nearest integral is an even number, is consistent with earlier findings [7,8] that *E. coli* alkaline phosphatase is a polymeric enzyme consisting of identical subunits.

Several studies showing that alkaline phosphatases, from a number of sources, need divalent cations for activity have been published.[1,2] Mg^{2+} is is the most common activating ion but also Zn^{2+} is an essential part of several phosphatases examined.[1,2] Investigations carried out on *E. coli* alkaline phosphatase give direct evidence that at least 2 zinc ions per enzyme molecule are obligatory for catalytic activity. Additional zinc ions and the presence of Mg^{2+} may effect the stability of the protein. Zinc analyses of our enzyme preparations gave a value of 2.7 and 3.3 g atoms per mol of enzyme. This is very close to the value reported by Harris *et al.*[29], obtained on *E. coli* alkaline phosphatase prepared in a different manner. Actually, the zinc content of *E. coli* alkaline phosphatase preparations has been determined also by many other workers, and the values reported vary considerably. For example, Plocke *et al.*[6] mention 2 g atoms of zinc/mol; Reynolds and Schlesinger [41] first found 3 g atoms of zinc per mol of enzyme, and later the same authors [42] reported values between 2 and 6 g atoms of zinc per mol of enzyme. Mean values between 2.5 and 3.5 g atoms of zinc/mol have also been reported.[29] Simpson *et al.*[30] give the value 4 g atoms of zinc/mol as the metal content of their enzyme preparations.

However, the described preparative methods which resulted in alkaline phosphatase with varying zinc contents give enzymes with rather comparable specific activities. There may be several reasons for the variation found in the zinc content of the enzyme. For instance, they might be related to the use of different bacterial strains, or to the methods by which the enzyme has been released from the cells, purified and stored. It has recently been shown that the use of EDTA during preparation strongly influences determinations of the number of zinc ions considered necessary for activity.[43] Naturally, a multitude of other factors causing discrepancies could be envisaged.

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