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Purification of *Escherichia coli* Alkaline Phosphatase on an Ion-Exchange High-Performance Liquid Chromatographic Column Using Carboxymethyl Dextrans

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Carboxymethyl dextrans (CM-Ds) were used on an HPLC ion-exchange column to obtain significantly enriched alkaline phosphatase (EC 3.1.3.1) from a sample of *Escherichia coli* periplasmic space proteins without significant loss of enzymatic activity. The ability of CM-Ds to separate alkaline phosphatase even when the column was 80–85% saturated with protein demonstrates the potential for high column capacity using CM-Ds. In addition, the fractions containing alkaline phosphatase and CM-Ds were reapplied to the same ion-exchange column under different buffer conditions and purified to homogeneity by salt gradient elution chromatography, thus demonstrating the compatibility of CM-Ds with the latter chromatographic method. The two-step chromatographic procedure yielded enzyme of purity comparable to that of electrophoretically purified *E. coli* alkaline phosphatase obtained commercially. These studies demonstrate that HPLC displacement chromatography is a mild procedure which allows rapid, quantitative purification of an enzyme. Scaling up with larger columns should allow purification of enzymes of a commercial basis.

Proteins with biological activity are often obtained from microorganisms or other complex biological mixtures along with a variety of contaminating materials including membrane fragments, lipopolysaccharides, and nucleic acids, as well as other proteins (1). Column chromatography has proven to be the method of choice for purifying preparative amounts of biologically active proteins. These chromatographic methods are based on ion-exchange, reverse-phase, hydrophobic interaction, size exclusion, and affinity principles (2).

We have employed an ion-exchange displacement method which utilizes increasingly substituted carboxymethyl dextrans (CM-Ds)[3] to space the protein components according to column affinities (3–5). After application of the sample and spacer CM-Ds, the train of contiguous, successively higher affinity bands is driven through the column by the addition of CM-D (final displacer) which has a column affinity higher than that of any of the other components (5,6). The displacement system has been shown to offer high capacity, high resolution, and the convenience of allowing analysis of fractions directly by gel electrophoresis (7).

A variety of proteins have been separated using CM-Ds as spacer displacers (5–8), and CM-Ds have been used to concentrate proteins, including a labile enzyme (DNA-dependent RNA polymerase), without signifi-

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[3] Abbreviations used: CM-Ds, carboxymethyl dextrans; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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cant loss of activity (9). However, no attempt has been made previously to purify an enzyme from a complex biological mixture using CM-Ds.

In this study, we demonstrate that CM-Ds can be used on a high-performance ion-exchange column to provide rapid purification of alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum); EC 3.1.3.1] from *Escherichia coli* without significant loss of activity. *E. coli* alkaline phosphatase was chosen because it has been well characterized (10,11), can be readily assayed (12), and is a periplasmic space protein with an N-terminal signal sequence (10). Thus, the preparation and purification of a periplasmic protein using CM-Ds may also serve as a model for the purification of other proteins which are produced by genetically engineered *E. coli* under conditions leading to their secretion into the periplasmic space (13,14).

MATERIALS AND METHODS

Bacterial growth and enzyme preparation. *E. coli* strain CW3747, an inducible overproducer of alkaline phosphatase (12), was obtained from Dr. J. E. Coleman. Two-liter batches of bacteria were cultured and crude alkaline phosphatase was obtained using methods described previously (12). Briefly, cell density (turbidity at 590 nm) (13) and alkaline phosphatase activity (12) were followed at 1- to 2-h intervals. Bacteria were harvested by centrifugation at 6000 rpm for 15 min at 4°C in a Sorvall RC-2 centrifuge, and crude alkaline phosphatase was recovered by the osmotic shock method of Neu and Heppell (14) or by conversion of cells into spheroplasts by treatment with EDTA and lysozyme (15).

Enzyme assay. Alkaline phosphatase activity was assayed spectrophotometrically by following the release of 4-nitrophenol from 4-nitrophenyl phosphate. To start the reaction, 10 μl of sample or an appropriate dilution was added to 1 ml of 1 mM 4-nitrophenyl phosphate in 0.1 M Tris-HCl, pH 8.0, and the absorbance at 410 nm was read after 5 min at room temperature. One unit of enzymatic activity was defined as the amount required to hydrolyze 1 μmol of 4-nitrophenyl phosphate per minute based on an extinction coefficient of $1.62 \times 10^4\ M^{-1}$ (12).

Delipidation. Samples were delipidated twice at room temperature with 2 vol of *n*-butanol/isopropyl ether (40/60, v/v) after sodium chloride was added to 0.35 M (16). After the final centrifugation at 2000 rpm for 5 min, the aqueous phase was dialyzed extensively against 20 mM Tris–acetate, pH 7.8, at 4°C to remove organic solvents.

Concentration of alkaline phosphatase. After addition of NaCl to 0.3 M, samples were concentrated by ultrafiltration using a 300,000-Da cutoff filter (Amicon). Although smaller than 300,000 alkaline phosphatase was quantitatively recovered in the retentate.

Removal of nucleic acid. After overnight dialysis against 20 mM Tris–acetate, pH 7.8, containing 10 mM $MgCl_2$ at 4°C, samples were incubated at 37°C for 45–60 min to promote digestion of nucleic acid by endogenous nucleases known to be present in periplasmic samples (14,17). Samples were then dialyzed exhaustively at 4°C against column buffer, filtered through a 0.45-μm filter, and injected onto the column.

Nucleic acid and protein content of samples. Nucleic acid and protein concentrations of periplasmic lysates were determined from the ratio of A_{280}:A_{260} using a standard nomograph (18). The relative amounts of RNA and DNA were estimated to be 3:1, respectively, after visual inspection of an ethidium bromide-stained agarose gel (19). Protein concentrations of alkaline phosphatase samples were determined by the Coomassie brilliant blue G-250 binding assay (20).

Column chromatography. A Beckman Model 421 controller equipped with two Model 112 pumps, a Model 165 detector, and a Kipp and Zonen BD 41 dual-pen recorder was used. A 5.0-ml stainless-steel sample loop was used with an Altex 210 sam-

ple injector and the column effluent was collected in an ISCO Cygnet fraction collector. The preparation of narrow-range CM-Ds and their characterization using the RPV (reciprocal of pellet volume) assay have been described previously (5). The higher the RPV value of a CM-D, the greater its column affinity under given conditions of pH and buffer concentration (6).

Stainless-steel columns (2.1 × 45 mm; 150 μl) were purchased from Rainin and packed with ion-exchange polymer beads (Altex, DEAE-5PW) in water with pressure rising to 2000 psi. Helium-degassed buffers were used in all experiments. CM-Ds were used as 1% solutions in 20 mM Tris–acetate buffer (pH 7.8). All samples, displacers, and buffers were filtered through 0.45-μm filters. In both displacement and elution experiments the flow rate was 0.1 ml/min, and 2-min fractions were collected in 1.8-ml polypropylene centrifuge tubes. After each chromatographic experiment, the column was washed with 1.0 ml of 0.5 M potassium chloride in order to remove displacer and residual protein. Before samples were injected, the column was thoroughly equilibrated to pH 7.8 with the degassed buffer.

Fractions from displacement experiments containing alkaline phosphatase activity greater than 25 units/ml were pooled and subjected to salt gradient elution chromatography on the same 150-μl DEAE-5PW column equilibrated with 20 mM imidazole acetate, pH 7.4. After addition of the sample and equilibration of the column, a 7-ml gradient from 150 to 250 mM NaCl, in the same imidazole buffer, was begun.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). Analysis of samples by SDS–PAGE was performed using the methods of Laemmli and Favre (21) on 10% acrylamide running gels with 6% stacking gels. Molecular weight standards (Dalton Mark VII-L) were obtained from Sigma Scientific Co. (St. Louis, MO). For comparative purposes, a commercial preparation of *E. coli* alkaline phosphatase, type III R, electrophoretically prepared, substantially free of ribonuclease and deoxyribonuclease, was obtained from Sigma. Gel protein staining was performed using a silver stain kit (Bio-Rad, Richmond, CA).

Two-dimensional gel electrophoresis. Two-dimensional polyacrylamide gel electrophoresis was performed using the methods of Anderson and Anderson (22). Bio-Rad Bio-Lytes 5/7 (4 parts) and 3/10 (1 part) were employed in the first dimension. Gradient SDS–PAGE slab gels (9–18% acrylamide) were used in the second dimension. Silver staining of the slab gels was performed as described by Tollaksen *et al.* (23).

RESULTS AND DISCUSSION

In each of five separate experiments, bacterial growth and alkaline phosphatase production were similar. Periplasmic space protein preparation obtained using either osmotic shock or EDTA–lysozyme treatment gave similar concentrations of protein and nucleic acid and similar specific activities of alkaline phosphatase (Table 1). The relatively easy isolation of large amounts of crude alkaline phosphatase using these methods is due to two factors: first, alkaline phosphatase biosynthesis is induced by *in situ* phosphate starvation (12), and, second, alkaline phosphatase is a periplasmic space enzyme which is readily released from the cell as a result of cold-water shock or spheroplast formation, with minimal contamination by cytoplasmic proteins (14–17).

Delipidation with organic solvents resulted in reduction of the protein concentration of the crude periplasmic space proteins by about 40%, based upon the Coomassie brilliant blue G-250 dye-binding assay (data not shown). Since organic solvents can give high dye-binding values, samples were exhaustively dialyzed prior to the measurement of protein concentrations. Although almost half of the protein was removed from the aqueous phase, alkaline phosphatase activity was quantitatively recovered in it. It is

TABLE 1

PURIFICATION OF *E. coli* ALKALINE PHOSPHATASE (APase)

Step	Protein concentration (mg/ml)	Volume (ml)	APase specific activity (units/ml)	Yield (%)
Osmotic shock[a]	0.53	150	5.8	—
Concentrated, delipidated nucleic acid-free sample	1.83	4[b]	5.7	100
Displacement chromatography	2.5	0.48	30.1	86
Gradient elution chromatography	0.50	0.96	48.0	55

[a] Similar results were obtained using EDTA–lysozyme preparations.

[b] A 4-ml sample volume was used in each displacement chromatography run. This volume does *not* represent the *total* volume of concentrated, delipidated nucleic acid-free sample recovered from the 150 ml of osmotic shock.

therefore likely that the delipidation step removed denatured proteins and fragments of cell membranes. Confirmation of the removal of a significant amount of protein by the organic solvents was also evidenced by a decrease in the A_{280}:A_{260} ratio and by gel electrophoresis. Concentration by ultrafiltration followed by nucleic acid digestion and dialysis resulted in an approximately threefold concentration of both total protein and alkaline phosphatase without significantly affecting enzyme specific activity (Table 1). Based on frontal analysis, the column capacity for periplasmic proteins was 9 mg, i.e., the 7.3-mg sample applied (4 ml × 1.83 mg/ml, Table 1) saturated approximately 80% of column capacity.

In displacement chromatography one generally attempts to bracket the target protein with displacers of lower and higher affinities (6). The intention is to separate proteins with a lower column affinity from the target protein and then displace the protein of interest from proteins with higher column affinities. Application of 5 mg of the lowest affinity CM-D (RPV = 5.0) displaced alkaline phosphatase in the first peak (Fig. 1). Separation was not improved when smaller amounts of sample or of CM-D were used. Although the separation resulted in a specific activity approximately fivefold greater (30 units/mg) than that of the concentrated sample (Table 1), several contaminants were also present by SDS–PAGE analysis (not shown).

Subsequent application of 11 mg of a high-affinity CM-D resulted in a second chromatographic peak which contained the vast majority of the periplasmic proteins with only trace amounts of alkaline phosphatase (Fig. 1). It is uncertain whether differences exist in the apparent alkaline phosphatase activity present in the two peaks in Fig. 1. The second peak may represent tightly bound alkaline phosphatase or another phosphatase enzyme, i.e., acid phosphatase or 5′-nucleotidase, known to be present from the periplasmic space preparations (14).

Fractions 4 and 5 (Fig. 1), which contained 86% of the initial enzyme activity, were pooled, diluted to 2 ml with 20 mM imidazole acetate at pH 7.4, and dialyzed overnight against three changes of the same buffer. Gradient elution chromatography revealed four peaks, two of which were associated with enzyme activity (Fig. 2). The two enzyme-rich peaks contained 55% of the activity applied; alkaline phosphatase from each peak had a specific activity of 48 units/mg protein.

Analysis of the individual highly enriched alkaline phosphatase fractions 13–17 (Fig. 2) by silver-stained SDS–PAGE revealed purity comparable to that of electrophoretically pu-

rified *E. coli* alkaline phosphatase obtained commercially (sp act 44 units/mg) (not shown). Analysis of the pooled fractions by two-dimensional gel electrophoresis revealed two major spots and one minor spot at 47 kDa (not shown), possibly representing different charge forms of alkaline phosphatase.

These experiments have demonstrated that CM-Ds can be used to separate an enzyme from a complex biological mixture by column chromatography without significant loss of activity. Chromatography using CM-Ds increases column efficiency compared to gradient elution conditions, since columns can be loaded with more protein without sacrificing resolution (6). In addition, the presence of CM-D in protein samples did not interfere with subsequent salt gradient elution chromatography or with analysis of samples by gel electrophoresis, as demonstrated previously (6,24).

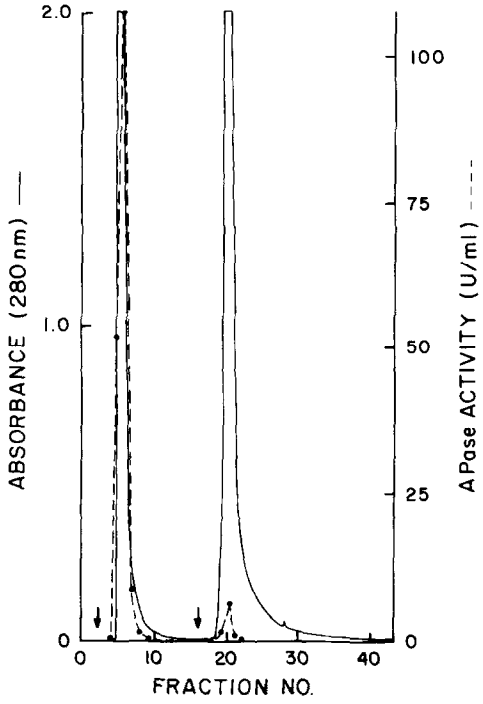

FIG. 1. Initial HPLC chromatography of 4 ml of delipidated, nucleic acid-free *E. coli* periplasmic space protein (7.3 mg protein) on a 150-μl DEAE-5PW column equilibrated with 20 mM Tris-acetate, pH 7.8. At the left arrow, 0.5 ml of 1% CM-D (RPV 5.0) was added, and, at the right arrow, 1.1 ml of 1% CM-D (RPV 26.6) was added. All the CM-D solutions contained 20 mM Tris-acetate, pH 7.8. The solid line indicates absorbance at 280 nm, while the dotted line represents alkaline phosphatase activity. Flow rate was 0.1 ml/min, chart speed was 0.1 cm/min, and fractions were collected at 2-min intervals.

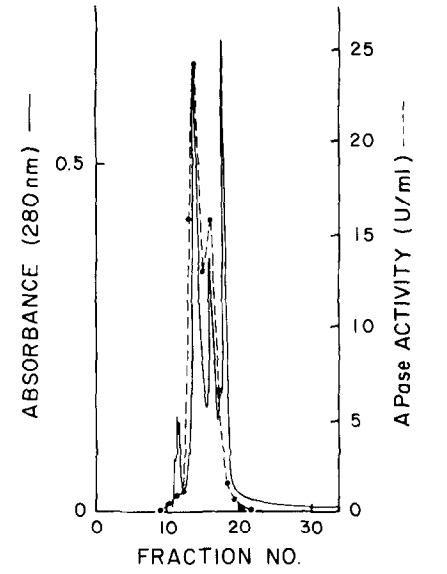

FIG. 2. Salt gradient elution chromatography of dialyzed, pooled fractions 4 and 5 from CM-D chromatography (Fig. 1) on the same 150-μl DEAE-5PW column, now equilibrated with 20 mM imidazole acetate, pH 7.4. A 70-min gradient from 150 to 250 mM NaCl in 20 mM imidazole acetate, pH 7.4, was started at fraction 0. The solid line indicates absorbance at 289 nm, while the dotted line represents alkaline phosphatase activity. Flow rate was 0.1 ml/min, chart speed was 0.1 cm/min, and fractions were collected at 2-min intervals.

The two-stage chromatographic technique developed, which involved separation using low-affinity CM-D followed by salt gradient elution chromatography on the same DEAE-5PW ion-exchange column, yielded highly enriched *E. coli* alkaline phosphatase with specific activity comparable to that achieved by others (25). Bacterial alkaline phosphatase and other periplasmic proteins, including those produced by genetic engineering, could be obtained in larger amounts using this approach by increasing the

amounts of absorbent and displacers in proportion to the protein load.

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