Isoelectric Fractionation of the *Escherichia coli* Alkaline Phosphatase and Resolution of the Purified Enzyme into Isoenzymes

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Alkaline phosphatase from *E. coli* has been investigated with respect to heterogeneity and isoelectric point, using the method of isoelectric focusing in stable pH gradients.

The enzyme was found to be resolvable into five isoenzymes with isoelectric points between pH 5.06 and 5.39. Some facts which may underlie alkaline phosphatase isoenzyme formation are discussed.

The isoelectric separation and focusing of proteins was introduced by Svensson *et al.*[1–4] In this type of electrophoresis, a stable pH gradient is created; amphoteric molecules migrate into a pH region where they are isoelectric and at this pH they concentrate as a sharp zone. The method is characterized by extraordinary resolving power. A great advantage is also that it allows a convenient and direct way for accurate determinations of isoelectric points. Hence we chose this method to investigate the heterogeneity and isoelectric point of *E. coli* alkaline phosphatase. This enzyme has been the object of a number of investigations and several preparation and purification methods have been described.[5–15] In all cases the sedimentation velocity studies showed only one boundary, apparently symmetrical, suggesting that the preparations consisted of a single molecular species. On the other hand, some DEAE-cellulose chromatography and electrophoresis results indicated the presence of multiple forms of the enzyme. In fact, existence of two or three isoenzymes was reported.[9–11,15–17]

The finding that *E. coli* alkaline phosphatase may contain a number of isoenzymes prompted the further investigations which are reported in this paper.

EXPERIMENTAL

Protein. Alkaline phosphatase (orthophosphoric monoester phosphohydrolyase EC 3.1.3.1) of *Escherichia coli* was prepared and purified as described in an accompanying paper I.[15]

Chemicals. Tris "Sigma 121" was used for the Tris buffers; *p*-nitrophenyl phosphate disodium tetrahydrate was also from Sigma. Analytical grade salt was used without further purification as source of the metal ion.

To create the pH gradients, Ampholite carrier ampholytes, 40 % water solutions, were purchased from LKB-produkter, Box 76, Stockholm-Bromma 1, Sweden. Sucrose, analytical reagent grade, from Mallinckrodt Chemical Works, St. Louis, Mo., USA, was used for the density gradient.

Electrolysis apparatus. An electrolysis column of 110 ml volume (LKB 8101) from LKB-Produkter was used.

Preparation of the column and isoelectric focusing by electrolysis. This was essentially done as described earlier.[18] The concentration of carrier ampholytes was about 2 %. The enzyme sample was added to the less dense solution. Then the enzyme will be found everywhere in the column from the start. The column was thermostated to 4°C during operation. In order to get pI values at 25°, the electrolysis was continued for about 4 h after the steady state was reached, with thermostated water of 25°C circulating through the cooling mantle. The time required for the focusing was about 60 h with a power of about 1 W.

pH and spectroscopic measurements. The pH (25°C) and the UV absorption (280 nm) were measured continuously on the column effluent by means of flow-through cells.[19] The instrument used for the pH measurements was a Beckman Expandomatic pH meter, equipped with a Beckman 97633 capillary pH electrode assembly and a Beckman ten-inch, linear, potentiometric recorder. For the UV absorption measurements a Vitatron universal photometer UFD, equipped with a flow-through cell and a Vitatron linear/logarithmic integrating recorder was used. For subsequent enzyme activity measurements, fractions were collected manually. The size of the fractions were varied according to the distribution of the enzyme in the column. This could be done simply by following the UV absorption course on the recorder.

Enzyme activity measurements. The enzyme activity was determined in 1.0 M Tris-HCl buffer, pH 8.0, at 27°C with 1.0×10^{-3} M *p*-nitrophenyl phosphate. The unit of enzyme activity and the specific enzyme activity were defined according to Malamy and Horecker.[10]

RESULTS

A pre-run with the broadest available pH range (pH 3 − 10) was first made to get an estimation of the isoelectric point of the enzyme. Ten ml of purified enzyme solution containing 7 mg of protein was applied to the column. By means of focusing, fractionation and analysis, the isoelectric spectrum shown in Fig. 1 was recorded. One main protein peak with alkaline phosphatase activity separated from a couple of small, inactive peaks was obtained. The enzyme peak showed some slight asymmetry and was found to be isoelectric around pH 5.4.

By applying a more shallow gradient, the resolution capacity can be markedly increased. Therefore, experiments were performed with carrier ampholytes isoelectric between pH 5 and 7. Fig. 2 shows the pH, UV absorption and the enzyme activity curves of a representative run. Five fully resolved components, isoelectric between pH 5.06 and pH 5.39, can be seen. 5 mg of protein was used in this experiment.

DISCUSSION

Alkaline phosphatase from *E. coli* prepared by the method described in the accompanying paper [15] appears homogeneous by rechromatography, zone electrophoresis, and in the ultracentrifuge. However, disc electrophoresis
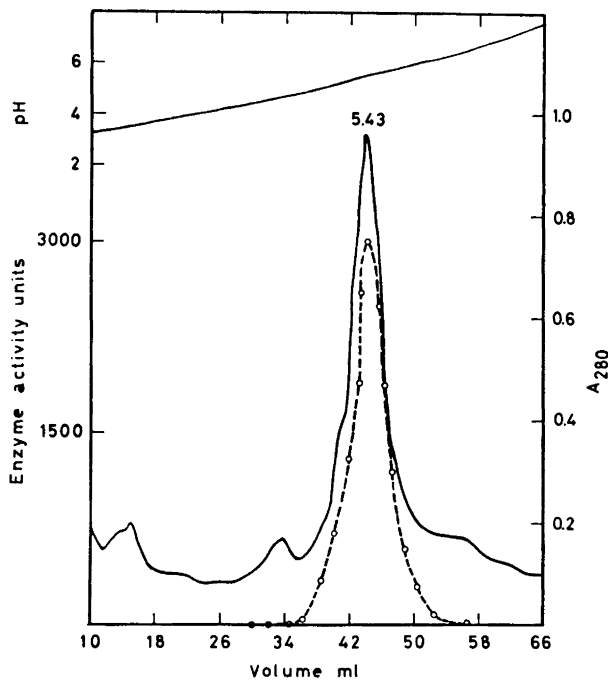
Fig. 1. pH (+25°C) course (upper solid surve), UV absorption (280 nm) (lower solid curve), and enzyme activity (O) of the column solution after isoelectric separation of *E. coli* alkaline phosphatase. The figures above the enzyme activity peaks give the pI values of the enzyme components.

indicated that the enzyme exists in multiple forms,[15] in agreement with results obtained by some other investigators.[9,11,15,17]

The isoelectric focusing experiments demonstrate that by application of a favourable pH gradient purified preparations of *E. coli* alkaline phosphatase separate into five isoenzymes. In the run described in Fig. 2 only four of the five UV absorption peaks showed enzyme activity. However, addition of zinc to these fractions resulted in an activation of the fifth peak and an increased specific activity for the other four peaks. The specific activity became almost identical with the specific activity of the original material, *i.e.* there was a loss of alkaline phosphatase activity during the electrolysis procedure that could be restored by addition of zinc. According to the manufacturer, the carrier ampholytes form complexes with metals, consequently enzyme activity may disappear or be reduced by complex formation with the metal of the enzyme, but can be restored by adding metal to those fractions containing inactivated enzyme. In one experiment, performed with an enzyme preparation of highest available specific activity, the loss of activity during the electrolysis was not so large as in runs with enzyme of lower specific activity.

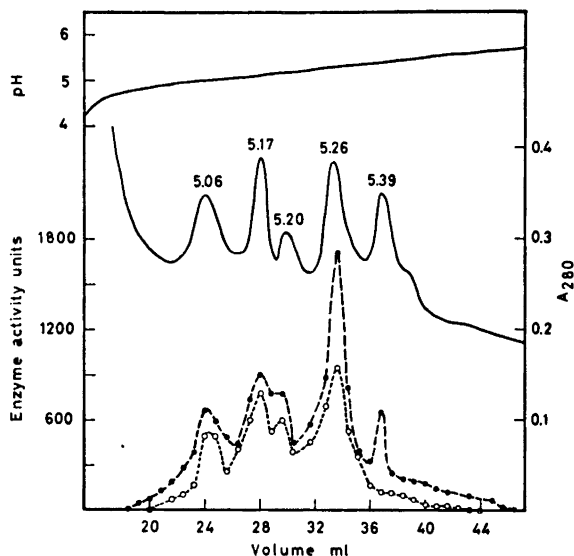So far only two or three isoenzymes had been reported, on the basis of

Fig. 2. pH ($+25°C$) course (upper solid curve), UV absorption (280 nm) (lower solid curve), and the enzyme activity in the absence ($\bigcirc$) and the precence ($\bullet$) of zinc, of the column solution after isoelectric separation of *E. coli* alkaline phosphatase. The figures above the enzyme activity peaks give the pI values of the enzyme components.

chromatography and gel electrophoresis experiments.[9,11,15,17] Lazdunski and Lazdunski [11] separated alkaline phosphatase on a DEAE-cellulose column and obtained three isophosphatases. Several kinetic and structural properties of these isoenzymes were similar. The method of isoelectric focusing used in our study is known to be the most sensitive one available for separation of protein molecules with small differences in net charge. All our experiments afforded similar isoelectric spectra. The number of components were always five, while the distribution of the enzyme material between them varied somewhat. The question arises if these components exist *in vivo* and because of their similarity are difficult to separate by standard methods; another possibility is that they are produced by the isoelectric focusing method. However, as one can see, some of the peaks of the isoelectric spectra differ in isoelectric points by minute values and consequently they are not easily detectable by standard methods.

On the basis of this study one cannot explain why *E. coli* alkaline phosphatase consists of so many isoenzymes. However, certain facts which might contribute to isoenzyme formation should be mentioned. Since alkaline phosphatase is a zinc metalloprotein,[20] metal-protein interactions can account for the presence of multiple forms of this enzyme. Proteolytic splitting of part of the protein chain without essentially changing the activity and chemical reactions occurring in a homogeneous protein and changing its charge are other factors. Furthermore, it should be noted that for most oligomeric pro-

teins, isoenzymes related by subunit association have been observed. Valuable knowledge about the existence and the nature of subunits in alkaline phosphatase isolated from *E. coli* has been obtained.[6] According to Rothman and Byrne this enzyme is a dimer containing two subunits.[6] The recent discovery of the formation of a tetrameric form of *E. coli* alkaline phosphatase by Reynolds and Schlesinger [22] is of great interest. Their data show that alkaline phosphatase under certain conditions behaves as a tetramer.[22]

The purpose of this study was, however, not to determine the reasons underlying isoenzyme formation but to test an observed heterogeneity with a very sensitive method, in order to establish the number of isoenzymes in *E. coli* alkaline phosphatase and to determine their isoelectric points.

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