Isoelectric Focusing of Human Liver Alkaline Phosphatase

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1. Isoelectric focusing of human liver alkaline phosphatase in a sucrose density gradient with LKB Ampholine as carrier ampholytes is described. 2. Problems due to the chelating properties of the ampholytes and the pH gradient were examined. 3. A reactivation procedure to counter these effects was devised that can probably be used for other alkaline phosphatases. 4. The isoelectric point of human liver alkaline phosphatase was found to be pH 3.9.

Isoelectric focusing in sucrose density gradients has been used to investigate a wide range of proteins including lactoperoxidase (Carlström & Vesterberg, 1967), α -crystallin (Bloemendahl & Schoenmakers, 1968), γ -globulin (Valmet, 1968) and lactate dehydrogenase (Weller, Heaney & Sjorgen, 1968). As this technique has been found capable of separating proteins that have isoelectric points differing by only 0.01 pH unit, it was proposed to apply it to human alkaline phosphatases (EC 3.1.3.1). The results obtained with the liver enzyme are described in the present paper.

MATERIALS AND METHODS

Enzyme preparation. Human liver (50 g) was obtained within 24 h of death and homogenized at 4°C with 100 ml of 0.05 M-veronal-HCl buffer, pH 8.0. The homogenate was placed in a water bath at 40°C, 125 ml of butan-1-ol added, and the mixture stirred for 20–30 min and then centrifuged at 2500g for 45 min at 4°C. The aqueous layer was removed, filtered through Whatman no. 1 filter paper and dialysed for 24 h at 4°C against 2 l of 0.9% NaCl and then against 2 l of water. The dialysis residue was concentrated to 20 ml by ultrafiltration, centrifuged at 20000g for 30 min at 4°C and the clear supernatant applied to a Sephadex G-75 column (20 cm $\times$ 1.5 cm). The phosphatase was eluted with 0.05 M-tris-HCl buffer, pH 7.4. Those fractions with alkaline phosphatase activity were pooled and concentrated by ultrafiltration. This concentrate, referred to as the liver phosphatase preparation, was stored at –20°C. Its total protein content was determined by comparing the E_{280} of the solution with that of a bovine albumin standard (100 μ g/ml; $E_{1\text{cm}}^{1\%}$ 6.8).

Isoelectric focusing. The apparatus used was the LKB 8101 electrofocusing column with the LKB 8121 gradient mixer (LKB Producter AB, Bromma, Sweden) and the technique essentially that of Haglund (1967). The anode solution (lower electrode) contained 0.2 ml of H_3PO_4 and 12 g of sucrose in 14 ml of water and the cathode solution (upper electrode) 0.2 ml of ethanolamine in 10 ml of water. For preparation of the gradient, the dense

solution contained 1.9 ml of 40% Ampholine type 8141 (LKB Producter AB) and 25 g of sucrose made up to 55 ml with water. The light solution contained 0.6 ml of 40% Ampholine and the liver phosphatase preparation (20 mg of total protein) made up to 5 ml with water. The gradient and sample were applied to the column with the aid of a peristaltic pump and a voltage of 300 V was applied for 40 h. The column was water-jacketed at 4°C. By means of the peristaltic pump, 3 ml fractions were then run off from the column and their pH and E_{280} measured.

Assay of alkaline phosphatase. The method was based on that of Bessey, Lowry & Brock (1946). The substrate was 5.5 mm-*p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate; Sigma Chemical Co., St Louis, Mo., U.S.A.) in 0.05 M-glycine–0.5 mm-MgCl₂ adjusted to pH 10.5 with 1 M-NaOH. Enzyme activity was assayed at 37°C by determining the increase in E_{400} after 30 min incubation and was expressed as μ mol of *p*-nitrophenol released/min per l of solution. Although 1% Ampholine did not show any apparent alkaline phosphatase activity with *p*-nitrophenyl phosphate as substrate, with phenyl phosphate and the Folin-Ciocalteu reagent for the colour reaction (King, 1951) there was significant apparent activity and this prevented this technique being used.

Chemicals. Unless otherwise specified, all chemicals were of the highest purity available from British Drug Houses Ltd., Poole, Dorset, U.K. Sephadex G-75 was obtained from Pharmacia, Uppsala, Sweden. Ampholine was obtained from LKB Producter AB, Bromma, Sweden.

RESULTS

Effect of Ampholine on alkaline phosphatase activity. Portions of the alkaline phosphatase preparation were diluted fivefold and tenfold with water or with 1% Ampholine and the phosphatase activities of the dilutions were assayed. In each case the enzyme activity was inhibited by some 75% in the presence of 1% Ampholine. Addition of Zn^{2+} to the phosphatase preparation diluted with 1% Ampholine was effective in reversing the inhibition of phosphatase activity (Table 1). The reversal

Table 1. *Effect of Zn^{2+} on alkaline phosphatase activity in the presence of 1% Ampholine*

The activity is expressed as a percentage of that obtained with liver phosphatase diluted to the same volume with water.

Concn. of Zn^{2+} (mm)	Activity (%)		
	Expt. 1	Expt. 2	Expt. 3
0	25	16	23
0.001	22	16	23
0.01	29	22	27
0.1	87	76	77
0.5	100	—	98
1.0	92	89	85
5.0	80	—	81
10.0	30	38	36

Table 2. *Effect of pH on alkaline phosphatase activity in the presence of 1% Ampholine*

Liver phosphatase preparation (0.1ml) was added to 3ml fractions from a control column and the activity of each fraction assayed after being kept at 4°C for 48 h. The activity is expressed as a percentage of that obtained with 0.1ml of liver phosphatase diluted to the same volume with water. The results from alternate fractions from two columns are given.

pH of fraction	Activity (%)	
	Expt. 1	Expt. 2
3.2	0	2
3.75	20	12
4.1	8	11
4.6	9	10
5.2	12.5	11
5.8	16	12
6.1	22	13
6.7	41	35
7.3	62	48
7.8	62	74
8.25	77	83
8.55	86	—
9.05	83	—

was virtually complete at a final Zn^{2+} concentration of 0.5mm. Mg^{2+} (0.1–5.0mm) did not produce this effect.

Effect of pH gradient on alkaline phosphatase activity. As the normal electrofocusing procedure takes up to 48h, it was necessary to examine the effect on alkaline phosphatase activity of 1% Ampholine in the pH range 3–10. Fractions (3ml) from a control column run without a sample were collected and to each was added 0.1ml of the liver phosphatase preparation. After being kept at 4°C for 48h, the residual phosphatase activity of a

Fig. 1. Isoelectric focusing of human liver preparation. Enzyme activity (○) is expressed as μ mol units/3ml fraction. The pH gradient (▲) is also indicated.

Fig. 2. Isoelectric focusing of human liver preparation: comparison of activity-pH plots obtained with fresh preparation (○) and that with the peak fractions from the first column used as sample for the second column (●).

portion of each fraction was assayed with a number of buffer substrates containing various amounts of Zn^{2+} (0.001–10mm). Maximum activity was found when the substrate was 1mm with respect to Zn^{2+} (Table 2). Zn^{2+} was added (final concentration 1mm) to 2.0ml of the remaining portions of a representative number of fractions. After standing for 24h at 4°C, the phosphatase activity was again assayed. Below pH5.0 reactivation was poor, 63% of the original activity being found at pH4.6 and 17% at pH3.95. An alternative procedure based on the findings of Scutt & Moss (1967, 1968) and Butterworth (1968) was investigated. A 0.1ml portion of the liver phosphatase preparation was added to fractions from another control column, and after 48h at 4°C all fractions below pH7.0 were titrated to this pH with 0.1M-sodium hydroxide and their activities measured, taking into account the increase in volume of the neutralized

fractions. Zn^{2+} was then added to give a final concentration of 1 mM in each fraction and after 24 h at 4°C alkaline phosphatase activities were measured with buffered substrate containing 1 mM- Zn^{2+} (Table 3). This reactivation procedure gave satisfactory results and led to its adoption after the isoelectric focusing experiments with alkaline phosphatase, since the alkaline phosphatase activity appeared only in fractions of pH less than 7.0.

Isoelectric focusing of human liver alkaline phosphatase. A 1 ml portion (20 mg) of the alkaline phosphatase preparation was applied to the

Fig. 3. Isoelectric focusing of human liver preparation: comparison of activity-pH plots obtained with fresh preparation (○) and preparation stored at -20°C for 6 months (□).

electrofocusing column. Fractions (3 ml) were collected, their E_{280} and pH measured and all with a pH below 7.0 were reactivated by using the procedure as described above. Their phosphatase activities were then assayed (Fig. 1). A peak containing 64% of the phosphatase activity of the sample occurred at pH 3.9 and those fractions with highest activity were used as a sample for a second electrofocusing. The peak activity was once again at pH 3.9 (Fig. 2).

After 6 months, the same liver preparation that had been stored at -20°C was applied to an identical electrofocusing procedure. The isoelectric point was again pH 3.9 but the peak was not as symmetrical as with the unstored preparation and there was indication of an additional small peak at pH 4 (Fig. 3).

DISCUSSION

Isoelectric focusing of human liver alkaline phosphatase in a sucrose density gradient would seem to have value in relation to characterization of the enzyme and the separation of high-specific-activity preparations.

Various difficulties have been encountered as a result of the nature of the carrier ampholytes and the pH gradient. Alkaline phosphatase activity could not be measured satisfactorily in the presence of 1% Ampholine by the King-Armstrong method. This was due to the high apparent activity given by 1% Ampholine alone. With *p*-nitrophenyl phosphate as substrate, the 75% inhibition of alkaline

Table 3. Reactivation of liver alkaline phosphatase in the presence of 1% Ampholine

Liver phosphatase preparation was added to fractions from a control column and the activity measured after 48 h at 4°C. Acid fractions were titrated to pH 7.0 and their activities again assayed. Zn^{2+} was added to all fractions to a final concentration of 1 mM and after a further 24 h at 4°C their activities were again assayed.

pH of fraction	Activity after 48 h at 4°C (%)	Activity after titration to pH 7.0 (%)	Activity after addition of Zn^{2+} to 1 mM (%)
3.1	2	1	2
3.5	9	25	33
3.8	12	24	77
4.1	11	14	74
4.35	10	14	83
4.6	10	10	82
4.9	9	12	90
5.2	11	12	84
5.45	12	15	81
5.9	12	14	83
6.2	13	14	84
6.5	32	14	100
6.85	35	21	68
7.2	48	—	88
7.8	74	—	75
8.25	83	—	72

phosphatase activity found with 1% Ampholine was nearly completely reversed by adding Zn^{2+} to a concentration of 0.5 or 1mM. The dependence of alkaline phosphatase activity on Zn^{2+} has been well established (Mathies, 1958; Engström, 1961a,b; Plocke, Levinthal & Vallee, 1962). The Zn^{2+} content is considered to be about 0.2% of the enzyme and firmly bound to it.

One of the most serious drawbacks to the use of isoelectric focusing is that it exposes enzymes to regions of pH at which they may be unstable. The instability of alkaline phosphatase at pH 6.0 and below is well recognized (Martland & Robinson, 1927; Bauer, 1936). With Ampholine fractions below pH 6.0 obtained from a focused column, there was 80% inhibition of added liver phosphatase activity. By titrating all acidic fractions to pH 7.0 followed by the addition of Zn^{2+} to a concentration of 1mM, satisfactory reactivation was achieved (Table 3). The ability of Zn^{2+} partially to reactivate acid fractions agrees with the results of Kunitz (1960), who showed that chick intestinal alkaline phosphatase at pH 4.5 could be reactivated by adding Zn^{2+} at the same pH. Scutt & Moss (1967, 1968) have also described results with human liver and intestinal alkaline phosphatases in which the enzymes inactivated at pH 2.1 and reactivated by incubation at pH 7.2 showed an increase in activity at pH 9.9. If taken directly from pH 2.1 to pH 9.9 there was no reactivation. Added metal ions were not necessary for reactivation. This latter fact was confirmed by Butterworth (1968), who reactivated the pig kidney enzyme but found that activity was regained as readily at pH 10.0 as at pH 7.6.

The reactivation procedure established for human liver alkaline phosphatase activity after isoelectric focusing in Ampholine has also been successfully applied to the calf intestinal and human kidney enzymes (A. L. Latner, M. E. Parsons & A. W. Skillen, unpublished work). With the former

enzyme 93% recovery of alkaline phosphatase activity was obtained from the column.

The isoelectric point of the human liver enzyme is at pH 3.9. The peak obtained from a fresh preparation was symmetrical. After storage at -20°C for 6 months, a small additional peak appeared. Whether or not this reflects some change in the enzyme on storage requires further study. It is, however, noteworthy that the mobilities of certain human alkaline phosphatase isoenzymes have been found to be altered after butanol extracts were frozen and thawed (Moss & King, 1962) or after storage at -20°C for 4 months (Moss, 1963).

REFERENCES

- Bauer, E. (1936). *Hoppe-Seyler's Z. physiol. Chem.* **242**, 35.
Bessey, O. A., Lowry, O. H. & Brock, M. (1946). *J. biol. Chem.* **164**, 321.
Bloemendahl, H. & Schoenmakers, J. G. G. (1968). *Sci. Tools*, **15**, 6.
Butterworth, P. J. (1968). *Biochem. J.* **108**, 243.
Carlström, A. & Vesterberg, O. (1967). *Acta chem. scand.* **21**, 271.
Engström, L. (1961a). *Biochim. biophys. Acta*, **52**, 36.
Engström, L. (1961b). *Biochim. biophys. Acta*, **52**, 49.
Haglund, H. (1967). *Sci. Tools*, **14**, 17.
King, E. J. (1951). In *Micro-Analysis in Medical Biochemistry*, 2nd ed. p. 70. London: J. and A. Churchill Ltd.
Kunitz, M. (1960). *J. gen. Physiol.* **43**, 1149.
Martland, M. & Robinson, R. (1927). *Biochem. J.* **21**, 665.
Mathies, J. C. (1958). *J. biol. Chem.* **233**, 1121.
Moss, D. W. (1963). *Nature, Lond.*, **200**, 1206.
Moss, D. W. & King, E. J. (1962). *Biochem. J.* **84**, 192.
Plocke, D. J., Levinthal, C. & Vallee, B. L. (1962). *Biochemistry, Easton*, **1**, 373.
Scutt, P. B. & Moss, D. W. (1967). *Biochem. J.* **105**, 43P.
Scutt, P. B. & Moss, D. W. (1968). *Enzymologia*, **35**, 157.
Valmet, E. (1968). *Sci. Tools*, **15**, 8.
Weller, D. L., Heaney, A. & Sjorgen, R. E. (1968). *Biochim. biophys. Acta*, **168**, 576.