Properties of Alkaline-Phosphatase Fractions in Extracts of Human Small Intestine

By D. W. MOSS

Department of Chemical Pathology, Postgraduate Medical School, London, W. 12

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1. The chromatographic and electrophoretic heterogeneity of human-intestinal alkaline-phosphatase activity is described. 2. The phosphatase activity has been divided into two main components, of which certain properties have been compared. The components resemble each other in their Michaelis constants for the hydrolysis of phenyl phosphate and in their behaviour towards certain inhibitors, but differ in their stability to heat. 3. The addition of Mg^{2+} or Ca^{2+} ions to the electrophoresis buffers sharpens the electrophoretic zones. 4. The results presented do not support the existence of more than one alkaline phosphatase in small intestine.

When alkaline-phosphatase-containing extracts of human small intestine are submitted to starch-gel electrophoresis the main part of the enzyme activity appears as a diffuse zone with a mobility about half that of serum albumin at pH 8.6, with one or two minor components visible near the origin (Boyer, 1961; Hodson, Latner & Raine, 1962; Moss & King, 1962; Chianidussi, Greene & Sherlock, 1962). Considerable heterogeneity within the main phosphatase activity of some intestinal preparations has been demonstrated, however, by chromatography on substituted-cellulose ion-exchange columns (Grossberg, Harris & Schlamowitz, 1961; Landau & Schlamowitz, 1961), and Moss (1963) showed that on starch-gel electrophoresis the major intestinal phosphatase zone could be fractionated into at least two components, which could be correlated with those obtained by column chromatography.

Moss & King (1962) compared certain properties of the various zones of human intestinal phosphatase separated by starch-gel electrophoresis and, because of their similarity, concluded that there was only one alkaline phosphatase in this tissue. In that work the main phosphatase activity was treated as a single zone; therefore, in view of the chromatographic and electrophoretic heterogeneity of this zone demonstrated in the investigations referred to above, it appeared desirable to re-examine the validity of their conclusion. The results of this re-examination are presented in this paper.

MATERIALS AND METHODS

Enzyme preparations. Specimens of human small intestine obtained within 24 hr. of death were washed with a jet of tap water, freed from fat, connective tissue etc. and cut into

small pieces. Phosphatase-containing extracts were prepared by homogenizing with water and butan-1-ol, according to the modification by Ahmed & King (1960) of Morton's (1950) original method. The aqueous extracts were concentrated to small volume by dialysis against 30% (w/v) Carbowax 20M solution, followed by dialysis for 18 hr. at 4° against a large volume of distilled water. The alkaline-phosphatase activity of the concentrates was several hundred King & Armstrong (1934) units/100 ml.

Column chromatography. The concentrates were eluted from columns (21 cm. $\times$ 1 cm. or 42 cm. $\times$ 2.5 cm.) of DEAE-Sephadex A-25 (Pharmacia A.B., Uppsala, Sweden) by an increasing concentration of NaCl in 0.05 M-tris-HCl buffer, pH 7.7 (Grossberg *et al.* 1961). Fractions (3 ml.) were collected, on which alkaline-phosphatase estimations were carried out by the method of Kind & King (1954); extinction at 280 m μ and chloride concentration (Cotlove, Trantham & Bowman, 1958) were also measured. Fractions eluted between chloride concentrations of 0.05 and 0.09 M ('B'), and above 1.0 M ('A') were separately pooled and concentrated by dialysis against Carbowax 20M followed by dialysis against a large volume of distilled water at 4° for 18 hr. The protein content of the concentrated fractions was estimated by a modified Lowry method (Bailey, 1962).

Starch-gel electrophoresis. This was carried out on horizontal gels as described by Smithies (1955), in a discontinuous tris-citrate-borate buffer system at pH 8.65 (Poulik, 1957). This was modified in certain experiments by the addition of other ions to both the electrode-vessel buffer and gel buffer in the following concentrations: NaCl and KCl, 0.05 M; $MgCl_2$, $CaCl_2$ and $ZnCl_2$, 0.017 M. The applied voltage was 10 v/cm. except when studying the effect of added ions, when it was lowered to 6 v/cm. to reduce heating. Some two-dimensional electrophoretic runs were carried out in which the extracts were separated first on Whatman no. 1 paper, followed by electrophoresis on starch gel. The buffer used for the paper electrophoresis was barbiturate buffer, pH 8.6 (King & Wootton, 1956), with tris-citrate-borate buffer in the second dimension. Concentrated intestinal extracts were dialysed for 18 hr. at 4° against 0.1 M-EDTA, adjusted

to pH 8.6, before some electrophoresis experiments. Alkaline phosphatase was located on the cut surfaces of the gels after electrophoresis by the method of Estborn (1959).

Enzyme studies. Phosphatase activity was determined by colorimetric estimation of phenol liberated at 37° from phenyl phosphate (Kind & King, 1954). Michaelis constants for hydrolysis of phenyl phosphate by fractions A and B were determined at the optimum pH for each substrate concentration (Motzok, 1959) in Na_2CO_3 - NaHCO_3 buffers (Delory & King, 1945). The pH values of the reaction mixtures were measured with the glass electrode. The Mg^{2+} ion concentration was 5 mM. Apparent K_m values were derived from plots of reciprocal of reaction velocity at optimum pH against reciprocal of substrate concentration. Lines were fitted to the experimental points by the method of least squares.

The effect on the double-reciprocal plots of inhibition of the two fractions by inorganic phosphate was studied by determining the optimum reaction velocity with and without addition of Na_2HPO_4 (2.5 mM) at various substrate concentrations. The rates of hydrolysis of phenyl phosphate by fractions A and B were also determined in the presence of 5 mM-DL-phenylalanine and 5 mM-sodium deoxycholate; in each case the substrate concentration was 5 mM, that of Mg^{2+} ions 5 mM and the pH was 9.9.

The stability of the enzyme activity of the chromatographic fractions to heat was tested by incubating portions for various times in 0.01 M-tris buffer, pH 9.2, then measuring the residual enzyme activity. Incubation was carried out both with and without the addition of Mg^{2+} ions (10 mM), at 45° and 55°.

RESULTS

Chromatograms of intestinal alkaline phosphatase on DEAE-cellulose are shown in Fig. 1, and the type of separation obtained on starch-gel electrophoresis is shown in Fig. 2(a). There was some variation in the appearance of the chromatograms with different tissue specimens, but the activity eluted by concentrations between 0.05 M- and 0.09 M-sodium chloride was always greater than that eluted at higher concentrations. Starch-gel electrophoresis of the concentrated fractions A and B showed them to be essentially free from each other. On two-dimensional electrophoresis it was apparent that each fraction was heterogeneous to some extent, since the zones of activity appeared as spots elongated along a line passing through the origin of the electrophoretic runs. The heterogeneity of the faster fraction, A, was more marked than that of fraction B, and with some extracts was clearly evident also on the chromatogram (Fig. 1b).

Recovery of the phosphatase activity from the chromatographic columns was 85–90%. The concentrated dialysed fractions contained 150–170 μg . of protein/ml. by the Lowry method, and the enzyme activity was 10–30 King-Armstrong units/100ml. Specific activities were thus between 4 and 12 King-Armstrong units/mg. of protein N, an increase of 5–10-fold over the starting material,

Fig. 1. DEAE-Sephadex chromatograms of two intestinal extracts: (a) on a 21 cm. $\times$ 1 cm. column; (b) on a 42 cm. $\times$ 2.5 cm. column. —, Alkaline-phosphatase activity; ---, concn. of chloride. A and B, Fractions of main phosphatase activity.

Fig. 2. Alkaline-phosphatase zones after starch-gel electrophoresis at pH 8.65 of intestinal extracts. (a) Unmodified tris-citrate-borate buffer; (b) with MgCl_2 (0.017 M) added to buffers; (c) with CaCl_2 (0.017 M) added to buffers. The chromatogram of this extract is shown in Fig. 1(b). A and B, Fractions of main phosphatase activity corresponding to chromatographic fractions; S, minor slow-moving components.

which is about the same degree of purification as that obtained by starch-gel electrophoresis of tissue phosphatases (Moss, Campbell, Anagnostou-Kakaras & King, 1961). There was no correlation

between the chromatographic zones of enzyme activity and any protein zone revealed by extinction measurements at 280 m μ .

The addition of Mg²⁺ ions to the starch-gel electrophoresis buffers produced a considerable sharpening of the phosphatase zones of intestinal extracts compared with gels in which potassium chloride or sodium chloride had been added to raise the ionic strength by an equivalent amount, the effect on the leading enzyme zone being particularly marked (Fig. 2). The addition of Ca²⁺ ions produced a similar effect. When Zn²⁺ ions were added inhibition was such that the enzyme zones could only just be detected after electrophoresis; it was therefore not possible to decide whether these ions affected the pattern in a similar way.

Dialysis of the intestinal extracts against EDTA before starch-gel electrophoresis markedly decreased the phosphatase activity, but, though faint, the zones appeared to have the same relative intensities and positions as those of an undialysed control.

Fractions A and B gave similar apparent K_m values, 0.2 mm, for the hydrolysis of phenyl phosphate (Fig. 3). Both fractions were competitively

inhibited by inorganic phosphate (Fig. 4), and the values of K_i for each were similar (0.6 mm).

Sodium deoxycholate produced some activation of both fractions A and B, the activity in each case being 120% of that of controls without deoxycholate. DL-Phenylalanine inhibited both fractions to approximately the same extent: 50% for fraction A, 60% for fraction B.

The effect on the activity of the two fractions of heating at 45° and 55°, with and without added Mg²⁺ ions, is shown in Fig. 5. Fraction A is less stable to heat than fraction B, and this difference is accentuated to some extent in the presence of Mg²⁺ ions. The plots of log(residual activity) against time of heating are not linear, particularly those for fraction A, indicating that the fractions are heterogeneous. The various discontinuities in these plots were not associated with changes in the pattern of phosphatase zones on starch-gel electrophoresis.

Fig. 3. Plots of $1/v$ (at optimum pH) against $1/[S]$ for phosphatase fractions B (○) and A (●) for hydrolysis of phenyl phosphate. $K_m^A = K_m^B = 0.2$ mm. Similar agreeing K_m values were obtained with fractions from two other intestinal preparations.

Fig. 4. Plots of $1/v$ (at optimum pH) against $1/[S]$ for phosphatase fractions A and B, with and without inorganic phosphate (2.5 mm). ○, Fraction A without phosphate; ●, fraction A with phosphate; □, fraction B without phosphate; ■, fraction B with phosphate. $K_i^A = K_i^B = 0.6$ mm.

Fig. 5. Effect on phosphatase activity of fractions A and B of heating at 45° (a) and 55° (b) in 0.01 M-tris buffer, pH 9.2, with and without added Mg^{2+} ions (10 mM). ○, Fraction A without added Mg^{2+} ions; ●, fraction A with added Mg^{2+} ions; □, fraction B without added Mg^{2+} ions; ■, fraction B with added Mg^{2+} ions. Points are mean values of two determinations on fractions from a single intestinal preparation; similar results were obtained with fractions from a second extract.

DISCUSSION

The properties of the two alkaline-phosphatase fractions indicate that they are both 'intestinal type': both are inhibited by DL-phenylalanine, which has been shown by Fishman, Green & Inglis (1962) to inhibit intestinal phosphatase but not phosphatases of other tissues, and neither is inhibited by sodium deoxycholate, which inhibits non-intestinal alkaline phosphatases (Bodansky, 1937). The Michaelis constants of the two fractions for the hydrolysis of phenyl phosphate are indistinguishable, as are their inhibitor constants for inhibition by inorganic phosphate. This close similarity of enzymic properties suggests that the fractions do not constitute distinct phosphatase isoenzymes, but that one is probably derived from the other in some way, e.g. by degradation. The two forms do not appear to differ greatly in molecular size, since they

are not resolved by gel-filtration on Sephadex G-200 columns (Moss, 1963). Intestinal alkaline phosphatase is a large molecule; a molecular weight of 100 000 has been reported for the calf-intestinal enzyme (Engström, 1961), so that considerable modification of the protein can presumably take place without affecting the properties of the active centre.

The effect of Ca^{2+} and Mg^{2+} ions on the electrophoretic separation of the fractions suggests that conversion of one form into the other may involve a loss of bivalent cations. Loss of ions to varying extents by different molecules could account for the electrophoretic and chromatographic heterogeneity of the fractions, which is particularly marked in the faster component. The greater sensitivity to heat of the faster fractions may then be accounted for by supposing the cations to have some stabilizing effect on the protein molecules, which is not, however, restored by adding ions after degradation has taken place. Alternatively, enzyme molecules may acquire varying amounts of negative charge in some other way, such as the unmasking of previously-protected ionizable groups by alterations in tertiary structure. Neutralization of these charges by bivalent cations during electrophoresis may then produce a homogeneous population of altered molecules. The sharpening effect of Mg^{2+} or Ca^{2+} ions is only observed if these ions are added to the buffers; pre-dialysis of the tissue extracts against magnesium chloride solution does not affect the electrophoretic pattern, indicating that the altered molecules in particular do not bind these ions strongly.

Engström (1961) also observed that highly purified calf-intestinal alkaline phosphatase is chromatographically and electrophoretically heterogeneous, and that the separation of the chromatographic zones was sharpened by the addition of Mg^{2+} ions to the buffers.

The relative amounts of the two components in intestinal extracts are not constant, but vary from one tissue specimen to another (Moss, 1963). This would be expected if the faster components are indeed degraded forms of the native enzyme, since the proportions of them would depend on the condition of the tissue from which the extract was made. Conversion of one form of the enzyme into the other has not, however, been reproduced in the laboratory, though on prolonged storage of intestinal extracts at -20° the faster-moving zones become more compact and further separated from the slower zones (Moss, 1963).

The heterogeneity of intestinal alkaline phosphatase can thus be explained by supposing that the various forms arise by modification of a single enzyme; present evidence does not appear to justify the postulation of separate isoenzymic forms of phosphatase in this tissue.

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