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THE DIVERSITY OF ALKALINE PHOSPHATASE FROM RAT INTESTINE

ISOLATION AND PURIFICATION OF THE ENZYME(S)

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SUMMARY

The molecular constitution of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) from intestines of rats was examined. The enzyme, purified 1200-fold, catalysed the release of 21.3 mmole phenol/h per mg protein from phenylphosphate and 4.10 mmole *p*-nitrophenol/h per mg protein from *p*-nitrophenylphosphate. Measurements of phosphate released from β-glycerophosphate gave K_m = 2.27 mM and inhibition of this reaction by L-phenylalanine was uncompetitive, K_i = 0.73. The purified material contained three main isozymes which differed with respect to substrate specificity and each of them consisted of a microheterogeneous set of glycoproteins.

INTRODUCTION

Mammalian intestine is one of the richest sources of alkaline phosphatase activity and highly purified enzyme has been prepared from intestinal tissues of several species[1–3]. Recently[4], it was shown that the high post-prandial phosphatase activity of rat sera was partially sensitive to antibodies obtained from enzyme from intestinal mucosa. Other findings also supported a suggestion that the post-prandial elevation of rat serum alkaline phosphatase activity was due to the entry of intestinal phosphatase into the circulation. Results were also obtained[4] which indicated heterogeneity of the intestinal alkaline phosphatase, and in this paper we report the results of an examination of highly purified preparations of the enzyme. Extensive molecular diversity of the purified material was observed and in a preliminary communication[5] we have reported the detection of the microheterogeneous glycoprotein isozymes which constitute this enzyme.

METHODS

Rats of the Wistar strain, 150–300 g body weight, were obtained from the

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Animal House, University of Sydney. Protein was estimated by the copper–phenol reagent method[6]. The procedures associated with electrophoresis in gels of starch and polyacrylamide and the assay methods using phenyl phosphate and *p*-nitrophenyl phosphate have been described earlier[4,5].

Kinetic parameters were determined with respect to β-glycerophosphate hydrolysis and the method of CHEN *et al.*[7] was used for measurement of the inorganic phosphate produced. 0.35 ml of buffered substrate (9 mM β-glycerophosphate, 0.05 M carbonate–bicarbonate, pH 8.88) was added to 0.05 ml enzyme solution. The purified enzyme solutions used did not require deproteinisation and hydrolysis was terminated by addition of 0.8 ml of phosphomolybdate reagent. Buffer solution A, used extensively during enzyme purification, was composed of $MgSO_4$ 2.5 mM–Tris·HCl 5.0 mM, pH 7.4.

The preparation of anion-exchange cellulose columns

DE11 and DE32 celluloses (Whatman) were used following the precycling treatment recommended by the supplier. The material was finally washed extensively with buffer soln. A and then it was resuspended in that buffer (9%, w/v). The suspension was allowed to sediment under gravity in the column, which was made in sections, each approx. 5 cm in height. The top of the growing column was loosened immediately before each addition of fresh material. Columns were kept at 4° for 24 h before they were used.

Gel filtration

Sephadex G-200 (Pharmacia) was soaked for five days in buffer soln. A. Twice daily, buffer was removed by decantation and replaced with fresh solution. Sufficient material to make a column was de-aerated in suspension in the buffer and poured into a separating funnel. The funnel was attached to a column which was completely filled with buffer soln. A. The tap at the bottom of the column was slowly opened and buffer leaving the column was replaced by Sephadex suspension from the funnel. Column packing was then achieved by control of the rate of flow of buffer.

RESULTS

Enzyme purification

Five preparations were made, each from groups of 200 rats. Table I gives details of the purification evidence for one preparation.

Homogenisation of intestinal tissues

The animals were killed by decapitation. The whole of the small intestine, from the pylorus to the ileo-caecal junction, was taken out and put directly into crushed ice. After a group of fifty rats had been killed, deionised water was forced through each intestine and attached mesentery was removed. The material was weighed and stored at —20°. Frozen intestines were cut into small pieces with scissors and then homogenised at room temperature for 1 min in 2.5 vol. (w/v) of buffer soln. A. The homogenate was kept at room temperature for 2 h.

TABLE I

RESULTS OF THE ENZYME PURIFICATION STEPS

Purification step	*Volume (ml)*	*Total activity* $\times 10^{-3}$	*Total protein (g)*	*Specific activity (μmole/h per mg protein)*	*Yield*	*Purification*
Crude extract	5850	211	64.4	3.28	100	1
Butanol treatment	4700	677	29.4	23.0	321	7
Dialysis	5700	940	16.5	56.9	445	17
Ethanol precipitation (65%, v/v)	360	554	5.5	99.3	263	30
DE-11 cellulose chromatography	100	520	2.00	260	246	79
Sephadex G-200 gel filtration	220	508	0.356	1430	240	426
DE-32 cellulose chromatography (NaCl gradient 0–0.2 M)	200	432	0.120	3600	205	1100
DE-32 cellulose chromatography (NaCl gradient 0–0.05 M)	110	365	0.090	4060	173	1240

Butanol treatment

1 vol. of redistilled *n*-butanol was added to the homogenate and the mixture was frequently shaken vigorously during 1.25 h. The emulsion formed was centrifuged at 23 000 × *g* for 10 min. The combined aqueous and solvent layers were filtered through 4 layers of muslin and kept overnight in 2-l measuring cylinders. The aqueous layer was removed and dialysed for 24 h against buffer soln. A. The buffer was changed twice. The dialysed solution was clarified by filtration through Whatman No. 1 paper.

Ethanol precipitation

Ethanol, which had been cooled to —20°, was added slowly to the dialysed solution (at 4°) to give a final concentration of 65% (w/v). The mixture was allowed to stand for 1 h. The material precipitated by the ethanol treatment was collected by centrifugation at 23 000 × *g* for 10 min. Buffer soln. A was added and the mixture was shaken gently. The small amount of enzymically inactive material which did not dissolve was removed by centrifugation and the clear, brownish-yellow supernatant solution was again dialysed for 16 h against buffer soln. A.

The first anion-exchange cellulose column

The enzyme solution was allowed to pass slowly into a column (70 cm × 3.5 cm) of DE 11 cellulose (Whatman), followed by 2-, 2- and 30-ml amounts of buffer soln. A. The column was then connected to an assembly from which a linear gradient of 0–0.4 M NaCl in buffer soln. A was obtained, from 500-ml amounts of the appropriate solutions.

Gel filtration

Enzymically active fractions from the DE11 column were pooled and treated

with ethanol as described above. The precipitate was dissolved in buffer soln. A and dialysed for 6 h at 4° against buffer soln. A. A column (100 cm × 3.75 cm) of Sephadex G-200 was prepared and buffer soln. A was passed through it for 24 h at 4°. The enzyme solution was then allowed to run into the column in small portions. After the addition of several small amounts, buffer soln. A was passed through the column continuously and the effluent was collected in 10-ml fractions.

Further anion-exchange cellulose columns

Manipulative procedures very similar to those described above were applied in two further purification steps. DE 32 (Whatman) cellulose was used in 40 cm × 2.7 cm columns. Linear gradients of NaCl concentration were used. There was an upper limiting solution of 200 ml of 0.2 M NaCl for the penultimate column and an upper limiting solution of 500 ml of 0.05 M NaCl for the final column.

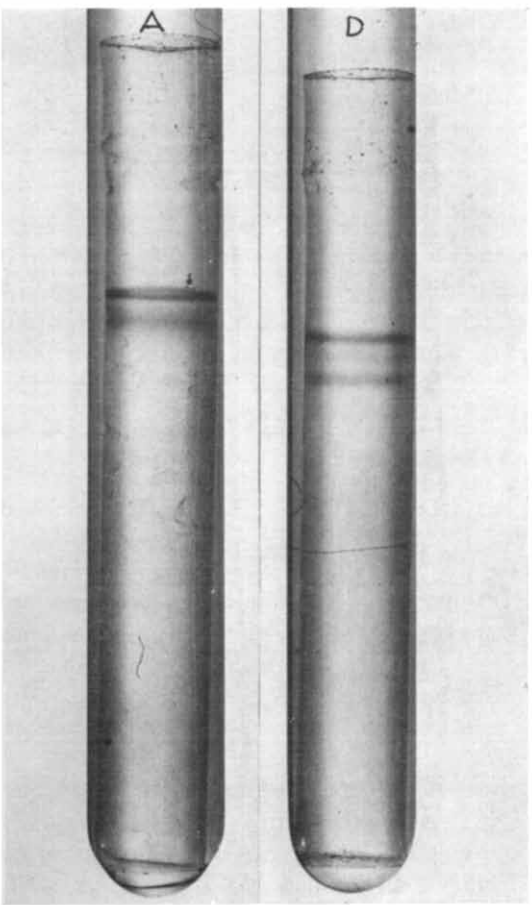

Fig. 1. Polyacrylamide gels stained after disc electrophoresis by periodate–Schiff reagent using methods already described[5]. Samples A and D were taken from fractions which represented points close to the middle of the ascending and descending parts, respectively, of the single protein peak obtained after the final chromatography step of the enzyme purification procedure.

Diversity of the molecule forms of phosphatase

We have reported[5] that successive fractions from the final column produced three protein bands. However, when samples from successive column fractions were run in the same gel, small but definite increases in mobilities of the bands were found. Additional evidence of complex microheterogeneity is given in Fig. 1. Two of the fractions from the final column were examined by disc electrophoresis. It can be seen (Fig. 1) that the 'isozymes' in the latter fraction had higher mobilities and also that they were more widely separated.

Catalytic activities of the purified material

Specific activities were determined by the assay procedures given in METHODS. The results obtained with the respective substrates (mmoles/h per mg protein) are given in the following list: phenylphosphate, 21.3; *p*-nitrophenylphosphate, 4.10; β-glycerophosphate 4.80. Other workers have shown a preferential hydrolysis of phenylphosphate by intestinal alkaline phosphatases from other species[8,9]. We extended estimation of a K_m for β-glycerophosphate hydrolysis to include an examination of inhibition by L-phenylalanine. The concentrations of the β-glycerophosphate solutions used were in the range 0.5–1.5 mM. It was then also possible to measure reaction rates when solutions in this range were made 2.0, 3.5 and 5.0 mM, respectively, with respect to L-phenylalanine.

TABLE II

CATALYTIC PROPERTIES OF THE PURIFIED ENZYME

Initial velocities for the release of inorganic phosphate from solutions of β-glycerophosphate in the range 0.5–1.5 mM were measured using the method of CHEN *et al.*[7]. K_m, K_p and v_{max} were computed by the method of CLELAND[10] and since K/v_{max} was constant, uncompetitive inhibition conditions were assumed for the calculation of K_i, so that $K_i = I/(K_m/K_p - 1)$

Phe concentration (mM)	K^* *(mM)*	K/v_{max}	K_i
0	2.27	2.01	—
2.0	0.634	1.98	0.77
3.5	0.420	1.90	0.79
5.0	0.258	2.06	0.64

* $K = K_m$ or K_p (K_m apparent).

The results are given in Table II. The values of K_m, K_p and v_{max} were computed by use of the programme of CLELAND[10]. It is clear from Table II (Column 3) that L-phenylalanine does not influence the K/v_{max} values so that the enzyme shows the rather unusual 'uncompetitive' type of inhibition[11]. Inhibition constants were therefore calculated from the expression $K_i = I/(K_m/K_p - 1)$ where K_i = inhibition constant, K_m = Michaelis constant, K_p = an apparent Michaelis constant and I = molarity of L-phenylalanine.

DISCUSSION

The well known high alkaline phosphatase activity of mammalian intestine

suggested that the tissue would be a rich source of the enzyme. However, as the purification procedure was developed, high specific activities were obtained and it became clear that the starting material was only a moderately rich source of the enzyme. While the specific activity of the most highly purified material was comparable with that of the first crystalline mammalian alkaline phosphatase[12], there was clear evidence that some of the constituents were relatively more active than others. After electrophoresis the slowest band was the most prominent after treatment with protein or lipid stains but enzyme activity was relatively low[5]. Thus the specific activity of the material of the most mobile of the bands (Fig. 1) is likely to be exceptionally high. It could be said, on the basis of data from Table I, that the enzyme constitutes 0.1–1.0 % of the protein of the original tissue homogenate. This estimate obviously involves much approximation. The purity of the final product was not rigorously established and the specific activity values relate to mixtures of enzymes. Another feature of the purification data which complicates this estimation is the increased activity observed after butanol treatment and again after the extract had been dialysed. This could have been due to the variable specificity of the method used for protein analysis and to the loss of relatively small molecules during the dialysis. It was consistently observed that both the butanol treatment and the dialysis of the extract resulted in increased activity. These effects may have been associated with the detachment of the enzyme from cellular structures and they may be comparable with the enhanced activities which have been observed during the preparation of alkaline phosphatase from *Escherichia coli*[13,14].

The values for reaction rates and the kinetic parameters derived from them are obviously of operational significance only, in view of the varying catalytic activities of the constituents of the purified material. Inhibition by L-phenylalanine was shown to correspond closely to the uncompetitive type of kinetic system, in which it is proposed that the inhibitor forms an inactive complex with the enzyme–substrate complex[15]. The affinity of this inhibitor was relatively high (Table II) and this may be associated with the high specific activities observed when phenylphosphate was used as substrate.

When we examined samples from successive fractions during chromatography with Sephadex and anion-exchange cellulose by various electrophoresis techniques, very small differences were detected consistently in the mobilities of the enzyme molecules. In some cases such resolution was quite clear during elution with NaCl concentration of a linear gradient between 0 and 0.005 M (ref. 5). Resolution of the different molecular species of enzyme would be dependent, according to the particular procedure, upon their size, not charge and also upon affinity between the carbohydrate moiety and the stationary phase. Thus it is possible that our failure to detect any resolution of the catalytically active components of crude extracts by electrophoresis was due to the presence in them of a large number of closely related enzyme molecules. MANWELL AND BAKER[16] were led to believe that a number of active phosphatases do not resolve well in electrophoresis, so that the microheterogeneity of alkaline phosphatase may not be uncommon. Isozyme bands were seen only after one or more purification steps and discrete bands were formed only when polyacrylamide gels were used. This may have been due to the low affinity of carbohydrate moities of the enzyme molecules for the polyacrylamide. It could be that the rates of migration in this medium were mainly a function of the net charges of the polypeptide moieties

and that the number of types of polypeptide was much less than the number of types of carbohydrate.

Finally we wish to comment on some biochemical aspects of the presence of bound carbohydrate components in the structures of these enzymes. It has been proposed that the attachment of carbohydrate to proteins promotes their transport into intracellular environments[17], but alkaline phosphatase from intestinal mucosa of calves is included in the list of intracellual proteins which are not glycoproteins. However, PORTMANN *et al.*[3] found substantial amounts of hexose and hexosamine in their highly purified preparation of the calf enzyme and both enzyme activity and the periodate–Schiff's reagent stain were coincident with the protein after paper electrophoresis. Establishment of the presence of bound carbohydrate in our purified enzyme from rats rests on similar criteria. The entry of intestinal alkaline phosphatase into the circulation of rats could therefore be promoted by bound carbohydrate.

SPIRO[18] has pointed out that recent advances in the understanding of the enzyme mechanism operative in the assembly of carbohydrate units of glycoproteins gives some insight into the causes of microheterogeneity observed in most carbohydrate units of glycoproteins. The great diversity of alkaline phosphatase from rat intestine which we have observed can therefore be associated with the very extensive changes in enzyme activity which WEBSTER AND HARRISON[19] and MOOG AND GREY[20] have shown to occur during the differentiation of intestinal epithelial cells. The material from which we prepared the enzyme would contain cells at many different stages of development.

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REFERENCES

1 R. K. MORTON, *Biochem. J.*, 57 (1954) 595.
2 L. ERGSTROM, *Biochim. Biophys. Acta*, 52 (1961) 36.
3 P. Z. PORTMANN, R. ROSSIER AND H. CHARDONNERS, *Helv. Physiol. Pharmacol. Acta*, 18 (1960) 414.
4 P. K. SAINI AND S. POSEN, *Biochim. Biophys. Acta*, 177 (1969) 42.
5 P. K. SAINI AND J. DONE, *FEBS Lett.*, 7 (1970) 86.
6 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
7 P. S. CHEN, T. Y. TORIBA AND H. WARNER, *Anal. Chem.*, 28 (1956) 1756.
8 N. R. KEIDING, *Scand. J. Clin. Lab. Invest.*, 18 (1966) 134.
9 P. R. V. NAYUDU AND F. MOOG, *Biochem. Genetics*, 1 (1967) 155.
10 W. W. CLELAND, *Adv. Enzymol.*, 29 (1967) 1.
11 N. K. GHOSH, AND W. H. FISHMAN, *J. Biol. Chem.*, 241 (1966) 2516.
12 N. K. GHOSH AND W. H. FISHMAN, *Biochem. J.*, 108 (1968) 779.
13 M. MALAMY AND B. L. HORECKER, *Biochem. Biophys. Res. Commun.*, 5 (1961) 104.
14 M. MALAMY AND B. L. HORECKER, *Biochemistry*, 3 (1964) 1893.
15 H. GUTFREUND, *An Introduction to the Study of Enzymes*, Blackwell, Oxford, 1965.
16 C. MANWELL AND C. M. A. BAKER, *Molecular Biology and the Origin of Species*, Sidgwick and Jackson, London, 1970, p. 130.
17 E. H. EYLAR, *J. Theoret. Biol.*, 10 (1965) 89.
18 R. G. SPIRO, *Ann. Rev. Biochem.*, 39 (1970) 599.
19 H. L. WEBSTER AND P. D. HARRISON, *Exp. Cell Res.*, 56 (1969) 245.
20 F. MOOG AND R. D. GREY, *Develop. Biol.*, 18 (1968) 481.