RAT INTESTINAL ALKALINE PHOSPHATASE. A MICROHETEROGENEOUS SERIES OF GLYCOPROTEINS

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1. Introduction

Alkaline phosphates (orthophosphoric monoester phosphohydrolase EC 3.1.3.1) from several sources contain carbohydrate. Tests with neuraminidase (*N*-acetyl-neuraminate glycohydrolase EC 3.2.1.18) showed that human liver [1], kidney [2] and placental [3] alkaline phosphatases are sialoproteins. Highly purified preparations obtained from calf intestine [4] and human placenta [5] both contained substantial amounts of carbohydrate.

We wish to record a direct demonstration of the glycoprotein nature of rat intestinal alkaline phosphatase isoenzymes and also some observations of the extent of their microheterogeneity.

2. Experimental

The starting material was 1.5 kg of small intestine tissue from 200 rats. The purification procedure consisted essentially of the following steps:- butanol extraction [6], fractional precipitation with ethanol, chromatography on DE 11 cellulose (Whatman), gel filtration on Sephadex G-200 (Pharmacia) and finally chromatography on DE 32 cellulose (Whatman).

The assay procedure was similar to that of Kind and King [7]. 2 ml 18 mM disodium phenylphosphate in 50 mM carbonate-bicarbonate buffer pH 9.8, at 25° was added to 0.1 ml enzyme solution. Phenol, which was liberated at a linear rate for at least 5 min, was usually determined after incubation for 1 min.

Total carbohydrate content was estimated by the Anthrone method of Chung et al. [8]. A mixture of galactose and mannose (1:1) was used for standard solutions.

Protein was estimated by the method of Lowry et al. [9] using bovine serum albumin as standard.

Disc electrophoresis [10] gave satisfactory results when the conditions outlined below were adopted. The main gels were made by mixing 6.0 ml of stock buffer (0.12 M tris, 0.15 M $MgSO_4$, pH adjusted to 8.2 with boric acid), 6.0 ml water and 6.0 ml 22.5% acrylamide, 0.75% bisacrylamide. No deaeration was required and 0.02 ml *N,N,N',N'*-tetramethylenediamine and 0.15 ml freshly prepared 10% ammonium persulphate were added. In the spacer gels, the acrylamide concentration was 4.5%. The stock buffer was eluted 1:3 for the electrode reservoirs and was discarded after each run. The apparatus was kept at room temperature during electrophoresis with indicated current 40 mA and potential 100 V.

Electrophoresis in gradient polyacrylamide gel [11] (Gradiopore, 4–25; Townson and Mercer Australia) was conducted for 15 hr at 4°C, indicated current 25 mA and potential 150 V.

The starch gel electrophoresis and the localisation of enzyme activity were performed essentially by the procedure described previously [12], but for this work, more satisfactory results were obtained when the gels were made in 0.076 M tris-HCl, pH 7.4.

Enzyme activity in polyacrylamide gels after electrophoresis was detected by calcium phosphate precipitates formed when they were immersed for about 10 min in a solution containing 0.5% $CaCl_2$, 0.5% $MgSO_4$, 0.5% β-glycerophosphate and 2.0% barbitone [13].

Proteins were detected by treatment of the gels with 1% Amido Black in 7% acetic acid. Subsequently they were destained in 7% acetic acid.

Glycoprotein in the bands was detected by periodate

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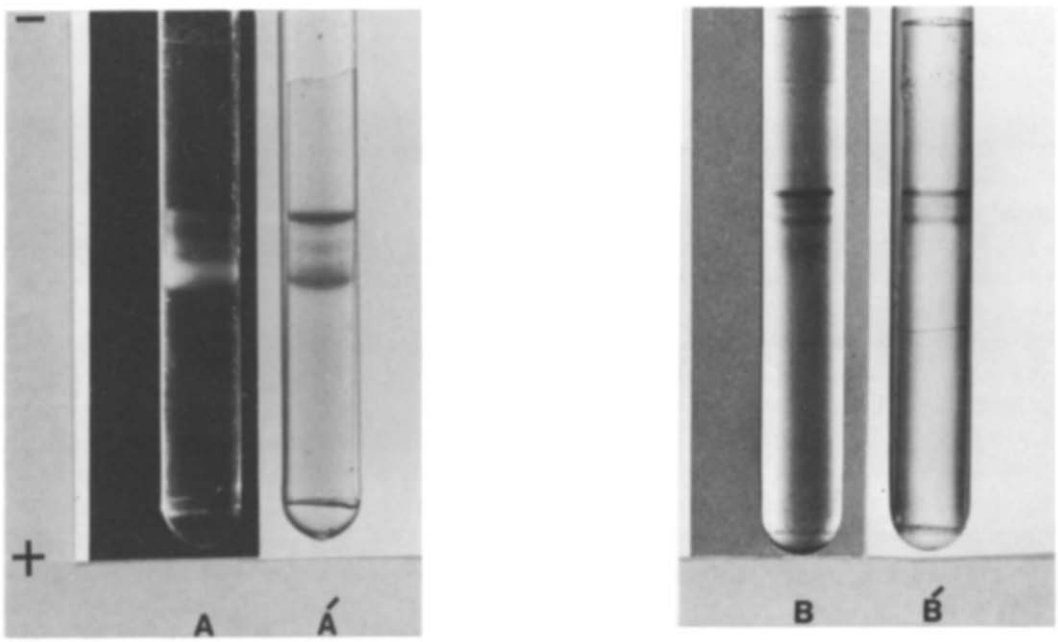

Fig. 1. Polyacrylamide gels after electrophoresis (for details see the text) of samples of purified rat intestinal alkaline phosphatase from one of the latter fractions of the final DE 32 cellulose column.

A Enzyme activity detected by $CaPO_4$ deposits formed in a gel, after 6 hr electrophoresis.

A′ Bands of stained proteins formed by treatment of the same gel (A) with 1% Amido Black in 7% acetic acid and subsequently destained with 7% acetic acid.

B Glycoprotein detected by a periodate-Schiff method [14] after electrophoresis for 4 hr of the second sample from the same fraction.

B′ Bands formed by treatment as described for A.

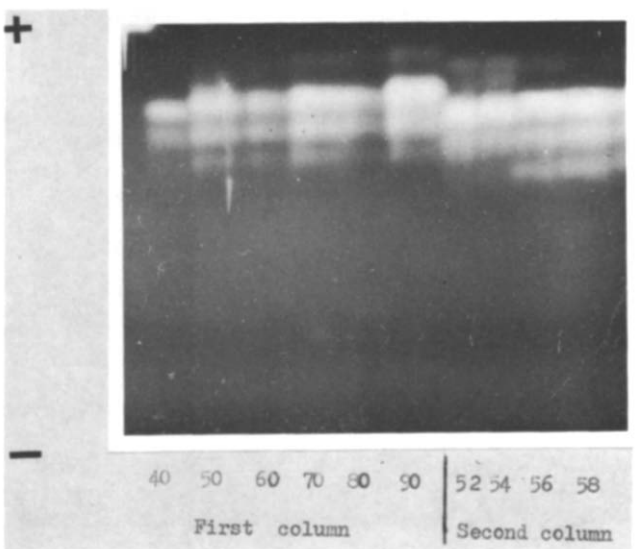

Fig. 2. A polyacrylamide gel (Gradiopore, 4–25; Townson and Mercer, Australia) after electrophoresis of samples from two successive columns of DE 32 cellulose. The numbers on the photograph represent the successive fractions from the two columns.

First column: Elution by a linear gradient of NaCl, 0 to 0.2 M, in 0.005 M tris-HCl, 0.0025 M $MgSO_4$, pH 7.4.

Second column: Elution by a linear gradient of NaCl, 0 to 0.005 M, in 0.005 M tris-HCl, 0.0025 M tris-HCl, 0.0025 M $MgSO_4$, pH 7.4.

Schiff method using the fuchsin sulphite stain [14].

3. Results and discussion

Recently Zacharius et al. [14] developed an effective method for the detection of glycoproteins after disc electrophoresis. We applied this to samples from our final DE 32 column. Results are illustrated in fig. 1. There is a clear coincidence of the bands of calcium phosphate formed as a result of enzymic activity with the regions stained by the treatments with Amido Black and Fuchsin respecively. Thus the preparation contained a highly purified group of isoenzymes which were glycoproteins.

The total carbohydrate content of a sample of the pooled fractions from the final column measured by the Anthrone method of Chung et al. [8] was 26.2% of the total protein content as measured by the Copper-phenol reagent method of Lowry et al. [9].

Microheterogeneity of the preparation became evident when the fractions from single "peaks" from chromatographic columns were examined by electrophoresis (figs. 2 and 3). There were small but definite increases in the mobilities of the isoenzymes in successive fractions. Treatment with neuraminidase (Sigma, from *Clostridium perfringens*) according to the method of Robinson et al. [15] did not alter the electrophoretic mobility.

We think now that the blurred bands observed in our earlier work, especially with unfractionated material (and regarded as due to "bad electrophoresis") were a manifestation of microheterogeneity.

The catalytic activity of the different proteins is being investigated. Preliminary results are consistent with those of Moog [16] in that the most intense cathodic protein band showed more activity with phenyl phosphate than with β-glycerophosphate. The association of the carbohydrate composition of the isoenzymes with such differences in catalytic characteristics and also with the stage of development of the intestinal epithelial cells may have to be considererd.

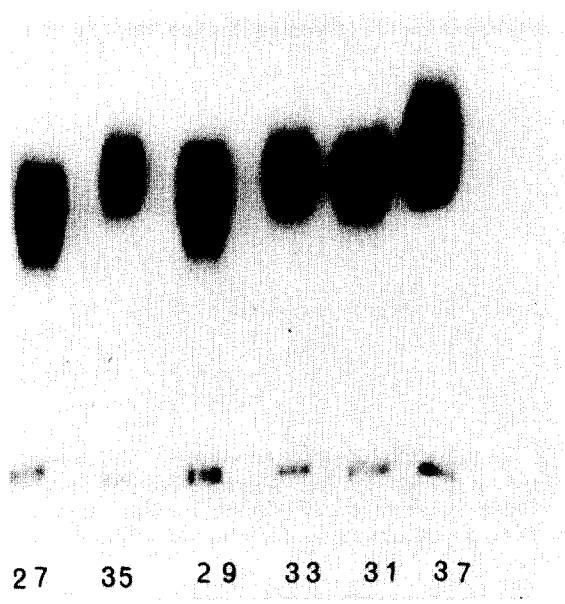

Fig. 3. A starch gel after electrophoresis of samples from fractions (as numbered on the photograph) from a Sephadex G-200 column. The buffer used was 0.005 M tris-HCl, 0.0025 M $MgSO_4$, pH 7.4.

Acknowledgements

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