Rat Small-Intestinal β -Galactosidases

STUDIES ON THE FRACTIONATION OF 'ACID' β -GALACTOSIDASE WITH ISOELECTRIC FOCUSING, GEL FILTRATION AND ION-EXCHANGE CHROMATOGRAPHY

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1. Different forms of the rat small-intestinal 'acid' β -galactosidase were separated by using the isoelectric-focusing technique. The isoelectric points of the different forms were at pH 4.2, 4.6, 5.4, 6.1 and approx. 8. 2. The two forms of 'acid' β -galactosidase isoelectric at pH 4.2 and 4.6 were completely excluded from the Sephadex G-200 gel, whereas the form isoelectric at pH 8 had K_{av} 0.4. The concentration and pH of the elution buffer influenced the distribution of enzyme activity between different forms. Thus, under certain conditions of ionic strength and pH, the enzyme seems to form high-molecular-weight aggregates with low isoelectric points. These may be homopolymeric aggregates or the result of binding of enzyme to, for example, membrane fragments. The forms isoelectric at pH 5.4 and 6.1 are probably aggregates of intermediate size. 3. During ion-exchange chromatography at pH 6.0 one fraction of 'acid' β -galactosidase was not retained on the column and was isoelectric at pH 8 and another fraction was eluted when the buffer concentration in the eluate had increased to about 50 mM. The main part of enzyme eluted in this second fraction was also isoelectric at pH 8, indicating that the elution of this fraction is not a simple ion-exchange procedure but probably also involves a splitting of high-molecular-weight aggregates, originally retained because of their low isoelectric points. The enzyme subunits have a higher isoelectric point, and are therefore no longer bound to the ion-exchange resin.

In a previous paper (Asp & Dahlqvist, 1968a) the fractionation of the 'acid' β -galactosidase in the rat small-intestinal mucosa into two components was reported. The following characteristics of the two components were compared: pH optimum, K_m for five different substrates, K_i for lactose as an inhibitor of the hydrolysis of hetero β -galactosides, K_i for phenyl β -galactoside as an inhibitor of the hydrolysis of lactose and relative V_{max} for the hydrolysis of different substrates (Asp & Dahlqvist, 1968b). No differences could be detected in any of the enzymic properties studied and it was therefore concluded that the two fractions probably represented different forms of the same enzyme rather than two different enzymes.

The fractionation of the rat small-intestinal 'acid' β -galactosidase has now been further studied by using isoelectric focusing, gel filtration and ion-exchange chromatography. Isoelectric focusing was performed both with crude preparations obtained by ultracentrifugation of intestinal homogenates and with fractions obtained by gel filtration and ion-exchange chromatography.

MATERIALS AND METHODS

Enzyme preparations. Albino rats of the Wistar strain 8-12 days old were decapitated. The distal third of the small intestine was removed, rinsed with about 5 ml of cooled 0.9% NaCl and homogenized in 4 vol. (v/w) of 0.9% NaCl with an Ultra-Turrax homogenizer. Previous studies have shown that such homogenates contain the highest specific activity of 'acid' β -galactosidase (e.g. see Koldovský & Chytil, 1965; Asp, Koldovský & Hošková, 1968). The homogenate was centrifuged at 100 000 *g* for 60 min at 4°C in a Spinco preparative ultracentrifuge (rotor no. 50). The supernatant, which contained about 60% of the 'acid' β -galactosidase of the homogenate, was then dialysed for 16-48 h against a buffer at 4°C (see figure legends and the Results section for specification). The preparation thus obtained is referred to in the text as crude 'acid' β -galactosidase preparation.

Isoelectric focusing. The separations were performed as described by Vesterberg & Svensson (1966). LKB 8100 Ampholine electrofocusing equipment was purchased from LKB-Produkter AB, S-161 25 Bromma 1, Sweden. The column with 110 ml capacity was used and the instruction manual was followed except that the density gradient was made up of aqueous solutions of glycerol

rather than sucrose. The denser solution contained 50% (v/v) of glycerol, which did not inhibit the assay of 'acid' β -galactosidase with the method used. Carrier ampholytes, pH 3-6, were used at an average concentration of 1%. A focusing time of 20-24 h was found to be satisfactory, although some of the experiments were run for 40-48 h. The voltage was kept at 500 V, giving a final load of 0.5 W. Cooling of the column was arranged by circulating water from a thermostatically controlled bath at 1.5°C. Fractions (3 ml) were removed from the column. The pH of the fractions was measured with a Radiometer pH-meter 27 with a Radiometer combination electrode

type GK 2021C at the same temperature as during the isoelectric-focusing process.

Gel-filtration chromatography. Sephadex G-200 was left to swell for 3 days at room temperature in excess of buffer. The gel was then packed into columns (1.0 cm diam. $\times$ approx. 95 cm). In each experiment 1.0-1.5 ml of enzyme preparation, dialysed against the same buffer as used for elution of the column, was put on the column. The flow rate was kept at about 3 ml/h. Fractions were collected every 40 min.

Blue Dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden) and glucose were run as reference substances

Fig. 1. (a) Isoelectric focusing of a crude 'acid' β -galactosidase preparation dialysed against 2 mM-sodium phosphate buffer, pH 6.0. A portion (1 ml) containing 0.95 unit of enzyme was put on the column. The recovery was 95%. (b) and (c). Refocusing of the peaks in (a) with isoelectric points at pH 4.2 (b) and 5.4 (c). The fractions of the peaks were pooled as indicated by broken lines in (a), dialysed against 1 mM-sodium phosphate buffer, pH 6.0, and then concentrated by evaporation in dialysis tubes. The recovery of 'acid' β -galactosidase after the concentration of the two peaks was 71 and 64% respectively. A portion (1 ml) of concentrated material containing 0.083 unit (b) or 0.100 unit (c) of 'acid' β -galactosidase was put on the isoelectric-focusing column. The recovery of activity after the isoelectric focusing was 70% (b) and 60% (c). ●, 'Acid' β -galactosidase activity; ○, protein concentration; ▲, pH.

to indicate the void volume and the total volume of the columns. Blue Dextran 2000 was measured spectrophotometrically at 630nm. Glucose was measured with tris-glucose oxidase reagent (Dahlqvist, 1968).

Ion-exchange chromatography. DEAE-cellulose was equilibrated with 10mM-sodium phosphate buffer, pH 6.0, and packed into a column (1cm diam. $\times$ 10–12cm). The column was then rinsed with about 500ml of buffer. Enzyme preparation (3ml) was dialysed against the same buffer and applied to the column. Elution was performed by increasing the buffer concentration gradually to 150mM. The flow rate was 4ml/h and fractions were collected every 30min. The buffer concentration in the eluate was determined by conductivity measurement.

Enzymic activity assay. 'Acid' β -galactosidase activity was assayed as described by Koldovský, Asp & Dahlqvist (1969), with *p*-nitrophenyl β -galactoside as substrate (final concn. 12mM). Incubations were performed at 37°C for 30 or 60 min.

Assay of protein concentration. The extinction at 280 nm was measured. Human serum albumin (Kabi AB, Stockholm 30, Sweden) was used as standard. The measurements on fractions obtained from isoelectric-focusing experiments met with certain difficulties, since the carrier ampholyte-glycerol mixture caused a relatively high

background, so that only protein concentrations higher than 0.1 mg/ml could be measured with accuracy. Usually, however, the protein concentration in these separations was less than 0.1 mg/ml. Therefore protein concentration is given only in some of the isoelectric-focusing experiments.

Calculations. Enzymic activity has been calculated as units/ml; one unit is defined as the amount of enzyme hydrolysing 1 μ mol of substrate/min at 37°C. The position of peaks in gel-filtration chromatograms was calculated as the K_{av} value defined from the formula:

$$K_{av} = (V_e - V_0)/(V_t - V_0)$$

where V_e is the elution volume for the substance under investigation. V_0 is the void volume and V_t is the total volume of the column.

RESULTS

Dialysis of 'acid' β -galactosidase preparations. All enzyme preparations were dialysed before separation by any of the three methods used. When crude 'acid' β -galactosidase preparations, i.e. the supernatant after ultracentrifugation of distal

Fig. 2. (a) Gel-filtration chromatography on a Sephadex G-200 column eluted with 50mM-sodium phosphate buffer, pH 7.0. A portion (1.5 ml) of crude 'acid' β -galactosidase preparation dialysed against the same buffer was put on the column. The recovery was essentially quantitative. (b) Isoelectric focusing of the small pre-peak at the void volume from a Sephadex G-200 chromatogram as described in (a). A portion (4 ml) containing 0.057 unit of activity was put on the column. The recovery was 97%. (c) Isoelectric focusing of the material in the main peak of a Sephadex G-200 chromatogram as described in (a). A portion (4 ml) containing 0.181 unit of activity was put on the isoelectric-focusing column without any treatment. The recovery was 90%. ●, 'Acid' β -galactosidase activity; ○, protein concentration; ▲, pH.

small-intestinal homogenates, were dialysed against water up to 90% of the 'acid' β -galactosidase activity was precipitated. Dialysis against 2–50mM-sodium phosphate buffer, pH 6.0, or 50mM-sodium phosphate buffer, pH 7.0, however, did not cause any precipitation and the recovery of 'acid' β -galactosidase activity after dialysis was at least 90%.

Isoelectric focusing of crude enzyme preparations. Fig. 1(a) shows a typical isoelectric-focusing experiment of a crude 'acid' β -galactosidase preparation that had been dialysed against 2mM-sodium phosphate buffer, pH 6.0, for 18h. Two major peaks with isoelectric points at pH 4.2 and 5.4 and three smaller peaks with isoelectric points at pH 4.6, 6.1 and approx. 8 were separated. The fractions in the two main peaks, referred to as peak 4.2 and peak 5.4, were pooled, dialysed against 1mM-sodium phosphate buffer, pH 6.0, concentrated by evaporation in dialysis tubes and applied again to the isoelectric-focusing column. The refocusing of peak 4.2 gave a single peak of activity with isoelectric point at pH 4.2 (Fig. 1b). The refocusing of peak 5.4, however, gave two peaks of activity with isoelectric points at pH 4.6 and 5.4 respectively (Fig. 1c). The result was the same whether the isoelectric focusing was run for 20 or 44h. The results of the refocusing experiments show that part of the 'acid' β -galactosidase activity originally occurring in peak 5.4 had been converted into a form with lower isoelectric point during the dialysis. The activity originally occurring in peak 4.2, however, did not change its isoelectric point during the refocusing procedure.

Gel-filtration chromatography. Fig. 2(a) shows a typical separation of crude 'acid' β -galactosidase on a Sephadex G-200 column eluted with 50mM-sodium phosphate buffer, pH 7.0. About 95% of the activity was eluted in a symmetrical peak with K_{av} 0.4. A small pre-peak at the void volume was also a constant finding. These conditions have been used in other separation studies (Asp & Dahlqvist, 1968a,b; Kraml *et al.* 1969), but the pre-peak was not subjected to further studies since it contains only a small part of the total 'acid' activity.

Isoelectric focusing of the pre-peak, without any treatment before the application to the isoelectric-focusing column, gave a single peak of activity with isoelectric point at pH 4.2 (Fig. 2b). Isoelectric focusing of the main Sephadex peak gave one major peak of activity with isoelectric point about pH 8 and small plateaux at pH 4.6, 5.4 and 6.1 (Fig. 2c). The result was the same whether a fraction at the ascending or at the descending part of the main Sephadex peak was taken.

In Sephadex G-200 chromatograms eluted with 10mM-sodium phosphate buffer, pH 6.0, all 'acid' β -galactosidase activity was eluted with the void

volume (Fig. 3a). Isoelectric focusing of this material gave a single peak of activity with an isoelectric point at pH 4.6 (Fig. 3b). Fig. 4(a) shows a Sephadex G-200 chromatogram run with 50mM-sodium phosphate buffer, pH 6.0. A major peak of 'acid' β -galactosidase was eluted with the void volume, followed by a smaller heterogeneous peak with K_{av} 0.20–0.25. Isoelectric focusing of the material in the latter peak gave three peaks with isoelectric points at pH 4.2, 5.3 and 8. The peak isoelectric at pH 8 was predominant (Fig. 4b).

Ion-exchange chromatography. Fig. 5(a) shows an ion-exchange chromatogram of a crude 'acid' β -galactosidase preparation dialysed against 10mM-sodium phosphate buffer, pH 6.0. As in previous experiments (Asp & Dahlqvist, 1968a,b) one fraction of the 'acid' β -galactosidase activity was not retained on the column (fraction I) whereas

Fig. 3. (a) Gel-filtration chromatography on a Sephadex G-200 column eluted with 10mM-sodium phosphate buffer, pH 6.0. A portion (1.5ml) of crude 'acid' β -galactosidase preparation dialysed against the same buffer and containing 1.200 unit of activity was put on the column. The recovery was 93%. (b) Isoelectric focusing of the peak of β -galactosidase activity in (a). A portion (1.7ml) containing 0.647 unit of activity was put on the isoelectric-focusing column. The recovery was 51%. ●, 'Acid' β -galactosidase activity; ○, protein concentration; ▲, pH.

Fig. 4. (a) Gel-filtration chromatography on a Sephadex G-200 column eluted with 50 mM-sodium phosphate buffer, pH 6.0. A portion (1.5 ml) of crude 'acid' β -galactosidase preparation dialysed against the same buffer and containing 1.157 units of activity was put on the column. The recovery was 78%. (b) Isoelectric focusing of the second small peak of 'acid' β -galactosidase in (a). Two fractions, indicated by broken lines, were pooled. A portion (1.7 ml) containing 0.033 unit of activity was put on the isoelectric-focusing column. The recovery was 50%. ●, 'Acid' β -galactosidase activity; ○, protein concentration; ▲, pH.

another fraction was eluted when the buffer concentration in the eluate had increased to about 50 mM (fraction II).

Isoelectric focusing of fraction I, without any treatment before the application to the column, showed that all the activity in this fraction occurred in the form isoelectric at pH 8 (Fig. 5b). It could not therefore be expected to be retained on the DEAE-cellulose column. Isoelectric focusing of fraction II also gave a peak isoelectric at pH 8 together with a smaller one at pH 6 (Fig. 5c). Since the 'acid' β -galactosidase activity of fraction II was originally retained on the DEAE-cellulose column the enzyme in this fraction would be expected to have isoelectric point below pH 6. Thus a shift in the isoelectric point of the enzyme in fraction II seems to occur during or after the elution from the DEAE-cellulose column.

DISCUSSION

These experiments show that the rat small-intestinal 'acid' β -galactosidase can appear in

several forms differing in isoelectric point. The isoelectric focusing of enzyme peaks eluted from Sephadex G-200 chromatograms indicates that the various forms differ also in molecular size.

The elution patterns in Sephadex G-200 chromatograms were influenced by ionic strength and by the pH of the elution buffer. Thus in Sephadex G-200 chromatograms with 50 mM-sodium phosphate buffer, pH 7, as eluent practically all 'acid' β -galactosidase activity appeared in a peak with K_{av} 0.4 and isoelectric point at pH 8. Kraml *et al.* (1969) estimated the minimal molecular weight of 'acid' β -galactosidase separated under these conditions as 83 000–105 000. In Sephadex G-200 chromatograms run with 10 mM-sodium phosphate buffer, pH 6.0, however, all 'acid' β -galactosidase activity was eluted with the void volume, indicating a much higher molecular weight. The isoelectric point of this form of the enzyme was at pH 4.6. The small pre-peak in the gel-filtration experiments run with 50 mM buffer, pH 7.0, also had a low isoelectric point at pH 4.2. These findings indicate that the two forms of enzyme with lowest isoelectric points are in fact high-molecular-weight aggregates of enzyme molecules. The aggregation may be either a simple polymerization of enzyme molecules with differences in the charge distribution of the polymers giving different isoelectric points, or a binding of the enzyme molecules to other macromolecules, e.g. mucopolysaccharides or membranes. The forms with isoelectric points at pH 5.4 and 6.1 have probably intermediate molecular sizes.

The earlier reported separation on DEAE-cellulose of two fractions of 'acid' β -galactosidase with identical properties (Asp & Dahlqvist, 1968a,b) seems to be fully explained by the present findings. Forms of the enzyme with isoelectric points higher than pH 6 are positively charged at pH 6 and would therefore not be retained on the DEAE-cellulose. The forms with lower isoelectric points, however, are retained on the column and eluted as fraction II. Since the main part of the activity of fraction II had an isoelectric point at pH 8 after elution from the column, the conclusion must be drawn that the elution of fraction II from the DEAE-cellulose column is not only due to a simple ion-exchange procedure but also to the fact that the originally retained enzyme with low isoelectric points is converted into the form isoelectric at pH 8 as the ionic strength in the elution buffer increases. Such an interpretation is in good agreement with the finding that the higher ionic strength of the elution buffer in gel-filtration chromatograms favoured the form isoelectric at pH 8.

A number of authors have separated apparently different forms of several enzymes and have interpreted their results in a variety of ways. Thus Hultberg & Öckerman (1969) separated three peaks

Fig. 5. (a) Ion-exchange chromatography of a crude 'acid' β -galactosidase preparation, dialysed against 10 mM-sodium phosphate buffer pH 6.0. A portion (3 ml) containing 2.29 units of 'acid' β -galactosidase activity was put on the column and the recovery was 83%. ●, 'Acid' β -galactosidase activity; ○, protein concentration; ▲, concentration of buffer. (b) and (c) Isoelectric focusing of fraction I (b) and fraction II (c) in the experiment shown in (a). Either 4 ml of fraction I containing 0.186 unit of activity (b) or 3 ml of fraction II containing 0.174 unit of activity (c) was put on the isoelectric-focusing column. The recovery was 29% and 45% respectively. ●, 'Acid' β -galactosidase activity; ▲, pH.

of 4-methylumbelliferyl β -galactosidase from human liver on Sephadex G-150. The first peak, which was eluted with the void volume, contained both the enzymes separated in the other two peaks. This was supposed to be due to an attachment of the enzymes to membranes or to the formation of other kinds of high-molecular-weight aggregates.

Furth & Robinson (1965) and Robinson, Price & Dance (1967) separated multiple forms of lysosomal β -galactosidase from rat liver, small intestine, and

other organs on DEAE-cellulose columns. They suggested that the β -galactosidase consists of a series of isoenzymes with elution patterns characteristic of the tissue of origin. It was also claimed, however, that the elution patterns obtained should be interpreted with caution in the absence of further evidence for the separate identity of multiple forms of the enzyme.

Jermyn (1962) fractionated an otherwise homogeneous preparation of β -glucosidase from *Strachy-*

botrys atra into several components on DEAE-cellulose columns. The different components were interpreted as a series of physically stable complexes with polysaccharides. A similar mechanism might be responsible for the heterogeneity of the intestinal 'acid' β -galactosidase reported in this paper, although the interconversion of activity of isolated peaks with other forms favours some other mechanism. Lamotta, McComb & Wetstone (1965) have reported evidence for the existence of some of the serum cholinesterase as aggregates differing in their rate of electrophoretic migration. Wallenfels *et al.* (1959) reported that crystalline β -galactosidase from *Escherichia coli* aggregated to form at least three different polymers under certain conditions of pH, ionic strength and concentration of enzyme.

Eriksson & Pettersson (1968) separated two cellulases from *Stereum sanguinolentum* differing in the content of diffusible carbohydrate. Since our preparations were dialysed extensively before chromatography such an explanation for the heterogeneity of the 'acid' β -galactosidase is unlikely.

Finally, some general conclusions might be drawn from the experiments presented. They clearly show that fractionation with any of the separation methods used must be interpreted with caution if the fractions obtained have similar properties. The fractionations as such obviously do not prove the existence of genetically different molecular species of the enzyme.

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