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Rat Small-Intestinal β-Galactosidases

SEPARATION BY ION-EXCHANGE CHROMATOGRAPHY AND GEL FILTRATION

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1. The chromatography of rat small-intestinal β-galactosidase activities on gel-filtration and ion-exchange columns has been studied. Five different substrates were used to measure β-galactosidase activity (lactose, phenyl β-galactoside, *o*-nitrophenyl β-galactoside, *p*-nitrophenyl β-galactoside and 6-bromo-2-naphthyl β-galactoside) and the activity was measured at one acid and one more neutral pH value. 2. By gel filtration one acid β-galactosidase, hydrolysing lactose and the hetero-β-galactosides at about the same rate, and one more neutral β-galactosidase, hydrolysing lactose much more rapidly than the hetero-β-galactosides, were separated. 3. By ion-exchange chromatography the acid enzyme was fractionated into two components. These may be individual enzymes or different forms of the same enzyme.

Studies on the influence of pH on β-galactosidase activity (Dahlqvist & Asp, 1967) have indicated that there exist at least two β-galactosidases in mucosal homogenates from the small intestine of adult rats. One of the enzymes appeared to be mainly a hetero-β-galactosidase and had a pH optimum at 3–4. The other one appeared to be chiefly a disaccharidase and had a pH optimum at 5–6.

We have now attempted to separate these enzymes by ion-exchange and gel-filtration chromatography. Five substrates were used for measuring β-galactosidase activity, namely lactose, phenyl β-galactoside, *o*-nitrophenyl β-galactoside, *p*-nitrophenyl β-galactoside and 6-bromo-2-naphthyl β-galactoside.

MATERIALS AND METHODS

Mucosal homogenates. Small-intestinal mucosal homogenates were prepared from albino rats weighing 100–200g., as described by Dahlqvist & Asp (1967).

Solubilization of mucosal homogenates. To each millilitre of mucosal homogenate were added potassium phosphate buffer (0·025M or 0·050M), pH 7, and either 0·125mg. of crystalline papain (Mann Research Laboratories, New York, N.Y., U.S.A.) plus 0·075mg. of cysteine hydrochloride as an activator for the papain, or 1mg. of crystalline trypsin (Trypure Novo, Ferrosan AB, Malmö, Sweden). The tube was incubated at 37° for 60min. and then chilled with crushed ice.

Ultracentrifugation. The homogenate, either before solubilization or after solubilization with papain or trypsin, was centrifuged at 100000*g* for 1hr. in a Spinco preparative ultracentrifuge. The supernatant liquid was used for chromatography after dialysis overnight at 4° against the same buffer as was used for the preparation of the column.

Ion-exchange chromatography. DEAE-cellulose (medium grade; Sigma Chemical Co., St Louis, Mo., U.S.A.) (1g.) was equilibrated with 0·01M-sodium phosphate buffer, pH 6·0, and packed into a column (1cm. diam. × approx. 8cm. long). About 4ml. of the dialysed ultracentrifuge supernatant was applied to the column. Elution was performed by increasing the buffer concentration gradually from 0·01M to 0·15M at constant pH. The chromatography was performed at 4° with a flow rate of 3–7ml./hr. About 50–60 fractions were collected, each containing 2–3ml. of effluent.

In one experiment TEAE-cellulose* (Porath, 1957) was used instead of DEAE-cellulose.

Gel filtration. About 3g. of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was mixed with 300ml. of 0·05M-sodium phosphate buffer, pH 7·0, left to swell without stirring overnight, and then packed to form a column (1cm. diam. × 85–95cm. long). Of the dialysed ultracentrifuge supernatant liquid, 2·0–2·5ml. was applied to the column. Chromatography was performed at 4° with a flow rate of 2–3ml./hr., and 60–80 fractions were collected, each containing about 1ml. of effluent.

Assay of protein concentration. After suitable dilution of the fractions the extinction at 280mμ was measured. Human serum albumin (KABI, Stockholm, Sweden) was used as a standard.

Enzyme activity assay. The enzyme activity of each fraction was tested with the five β-galactoside substrates by methods based on those described previously (Dahlqvist & Asp, 1967). The following modifications were made. To

* Abbreviation: TEAE-cellulose, triethylaminoethylcellulose.

develop the colour of free *o*-nitrophenol and *p*-nitrophenol, tris buffer was added to give a final pH of 8·3. For this purpose 0·1 ml. of M-tris buffer was added to 0·2 ml. of reaction mixture containing 0·05 M-sodium citrate. When the citrate buffer had a pH of 3·0, tris buffer, pH 9·0, was used; when the citrate buffer had a pH of 5·0 or 5·5 we used tris buffer, pH 8·5. In the previous study an excess of sodium carbonate was used instead, but this was found to give some hydrolysis of the substrate due to too high a pH value, which caused unnecessarily high blanks.

For lactase assay we used the method of Dahlqvist (1961, 1964), but with a smaller volume of reagent (0·4 ml. of tris–glucose oxidase reagent for each 0·2 ml. of incubated enzyme–substrate mixture) to increase the sensitivity.

The following pH values were used for incubation with the different substrates: lactose, pH 3·5 and 5·5; phenyl β-galactoside, pH 4·0 and 5·5; *o*-nitrophenyl β-galactoside, pH 3·0 and 5·5; *p*-nitrophenyl β-galactoside, pH 3·0 and 5·0; 6-bromo-2-naphthyl β-galactoside, pH 3·5 and 5·0. A 0·05 M-sodium citrate buffer was used to bring the reaction mixture to the desired pH. When 6-bromo-2-naphthyl β-galactosidase activity was to be assayed at pH 3·5, 0·05 M-glycine–HCl buffer was used instead The substrates were used in the following concentrations: lactose, 29 mM; phenyl β-galactoside, 20 mM; *o*-nitrophenyl β-galactoside and *p*-nitrophenyl β-galactoside, 12 mM; 6-bromo-2-naphthyl β-galactoside, 0·39 mM. The enzyme activity was calculated as μmoles of substrate hydrolysed/min. at 37°.

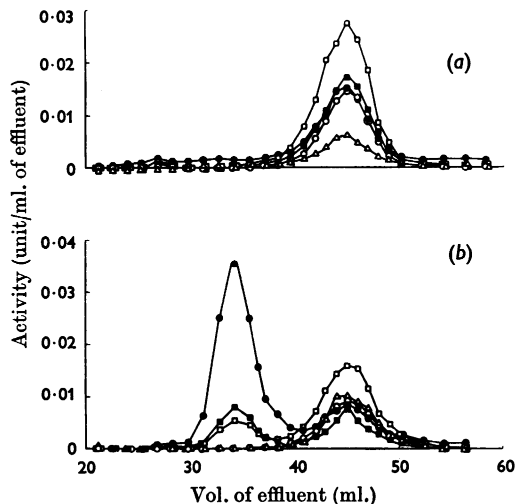

Fig. 1. β-Galactosidase activities in a Sephadex G-200 chromatogram of a solubilized rat small-intestinal mucosal homogenate. (*a*) Activities measured at pH 3·0–4·0; (*b*) activities measured at pH 5·0–5·5. ●, Lactase; ○, phenyl β-galactosidase; ■, *o*-nitrophenyl β-galactosidase; □, *p*-nitrophenyl β-galactosidase; △, 6-bromo-2-naphthyl β-galactosidase.

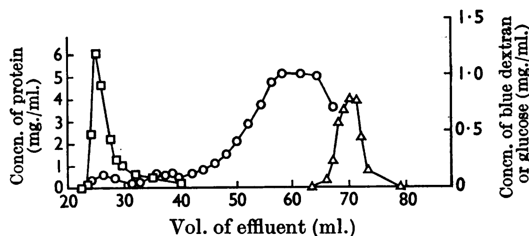

Fig. 2. Protein contents and reference substances in the chromatogram shown in Fig. 1. ○, Protein; □, blue dextran; △, glucose.

RESULTS

Solubility of the β-galactosidase activities. When the β-galactosidase activities were measured at pH 5·0–5·5 with the different substrates in a freshly prepared mucosal homogenate that had not been solubilized, and then the same activities were measured in the supernatant of the same homogenate obtained after ultracentrifugation at 100 000 g for 60 min., it was found that a considerable proportion of the different activities had been removed during centrifugation. The proportion that had been removed could be recovered in the resuspended sediment, and therefore represented enzymes that were associated with insoluble material, but nevertheless active. Only 10% of the lactase activity, but 60% of the 6-bromo-2-naphthyl β-galactosidase, was soluble in the fresh homogenate. The other activities had a solubility that was between these two figures.

When the β-galactosidase activities were measured at pH 3·0–4·0, about 60% of the total activity was soluble with all the substrates.

Solubilization of the particle-bound enzymes. Incubation of the homogenate with trypsin or papain resulted in a partial loss of the β-galactosidase activity (up to 40% of the total activity). The lactase, *o*-nitrophenyl β-galactosidase and *p*-nitrophenyl β-galactosidase activities at pH 5·0–5·5 were solubilized by this treatment, so that 70–90% of the activities remaining after solubilization was now present in soluble form. There was no apparent solubilization of the initially insoluble fraction of the phenyl β-galactosidase or 6-bromo-2-naphthyl β-galactosidase activities when measured at pH 5·0–5·5, or of any of the activities when measured at pH 3·0–4·0.

Storage at −20° for about 5 months also resulted in a partial solubilization of the lactase, *o*-nitrophenyl β-galactosidase and *p*-nitrophenyl β-galactosidase activities.

Gel-filtration chromatography. Chromatography of solubilized homogenates on Sephadex G-200 columns gave two β-galactosidase activity peaks (Figs. 1 and 2). The same results were obtained whether the solubilization had been performed with

Table 1. *Specificity of the first β-galactosidase peak obtained on Sephadex G-200 chromatograms*

The activity towards lactose has been arbitrarily set at 100. Only the activities at pH 5·0–5·5 are given. At pH 3·0–4·0 no activity could be measured with any of the substrates.

Substrate	Activity at pH 5·0–5·5
Lactose	100
Phenyl β-galactoside	Not measurable
o-Nitrophenyl β-galactoside	24
p-Nitrophenyl β-galactoside	13
6-Bromo-2-naphthyl β-galactoside	Not measurable

Table 2. *Specificity of the second β-galactosidase peak obtained on Sephadex G-200 chromatograms*

The activity towards lactose at pH 3·5 has been arbitrarily set at 100.

Substrate	Activity	
	pH 3·0–4·0	pH 5·0–5·5
Lactose	100	44
Phenyl β-galactoside	70	39
o-Nitrophenyl β-galactoside	93	37
p-Nitrophenyl β-galactoside	133	85
6-Bromo-2-naphthyl β-galactoside	45	50

papain, with trypsin or by storage at −20°. The first peak hydrolysed lactose, o-nitrophenyl β-galactoside and p-nitrophenyl β-galactoside at pH 5·0–5·5 in the incubation medium. At pH 3·0–4·0 no hydrolysis of these substrates by the first peak could be measured. Hydrolysis of phenyl β-galactoside or 6-bromo-2-naphthyl β-galactoside could not be demonstrated at any pH. The material of the second peak hydrolysed all five substrates studied, and all the substrates except 6-bromo-2-naphthyl β-galactoside were hydrolysed more rapidly at pH 3·0–4·0 than at pH 5·0–5·5. 6-Bromo-2-naphthyl β-galactoside, however, was hydrolysed somewhat more rapidly at pH 5·0 than at the lower pH.

When the supernatant of a fresh homogenate, not treated with proteolytic enzymes, was chromatographed the first peak was very small but the second peak was large. In fresh homogenates therefore the enzyme eluted in the first peak was soluble only to a small extent, but the enzyme eluted in the second peak seemed to be more soluble.

The specificity of the two peaks separated on Sephadex G-200 is shown in Tables 1 and 2. The first peak was clearly characterized as a disaccharidase, as it hydrolysed the hetero-β-galactosides much more slowly than the disaccharide. The second peak hydrolysed the disaccharide and the hetero-β-galactosides at comparable rates.

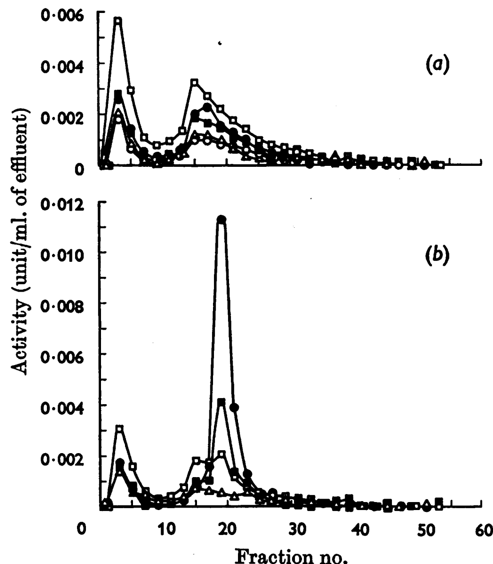

Fig. 3. β-Galactosidase activities in a DEAE-cellulose chromatogram of a solubilized rat small-intestinal mucosal homogenate. (*a*) Activities measured at pH 3·0–4·0; (*b*) activities measured at pH 5·0–5·5. ●, Lactase; ○, phenyl β-galactosidase; ■, o-nitrophenyl β-galactosidase; □, p-nitrophenyl β-galactosidase; △, 6-bromo-2-naphthyl β-galactosidase.

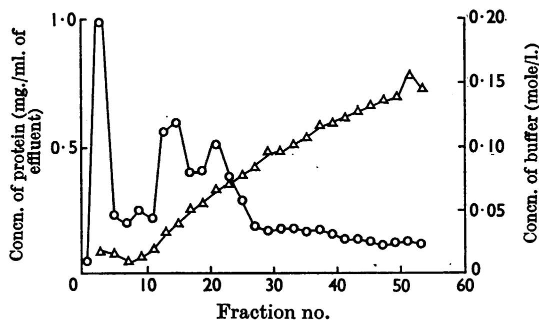

Fig. 4. Protein contents and buffer concentration in the chromatogram shown in Fig. 3. ○, Concn. of protein; △, concn. of sodium phosphate buffer, pH 6·0.

Ion-exchange chromatography. Two β-galactosidase peaks were also obtained on chromatography on DEAE-cellulose columns (Figs. 3 and 4). The first peak hydrolysed all five substrates, and with all the substrates the activity in this peak was higher at pH 3·0–4·0 than at pH 5·0–5·5. The specificity of this enzyme peak was very similar to that of the

second peak of the gel-filtration chromatograms (see Table 2).

The second β-galactosidase peak obtained from the DEAE-cellulose columns also hydrolysed all five substrates, but this peak was not homogeneous. In the beginning of the peak the hydrolysis of all the substrates was more rapid at pH 3·0–4·0 than at pH 5·0–5·5, but in the later part of the peak lactose, *o*-nitrophenyl β-galactoside and *p*-nitrophenyl β-galactoside were hydrolysed much more rapidly at the higher pH than at the lower.

In one experiment TEAE-cellulose was used instead of DEAE-cellulose with the same result.

DISCUSSION

The results indicate that there are at least two β-galactosidases in the rat small-intestinal mucosa, and thus support our previous investigation of the influence of pH on the hydrolysis of β-galactosides (Dahlqvist & Asp, 1967). One enzyme has an acid pH optimum and is extracted in crude homogenates, in accord with previous reports (Doell & Kretchmer, 1962; Koldovský *et al.* 1965). This enzyme seems to hydrolyse all five substrates used in the present investigation (lactose, phenyl β-galactoside, *o*-nitrophenyl β-galactoside, *p*-nitrophenyl β-galactoside and 6-bromo-2-naphthyl β-galactoside), and all the substrates are hydrolysed at comparable rates. The other enzyme has a higher pH optimum. This enzyme is chiefly insoluble in fresh mucosal homogenates, but can be solubilized by incubation with trypsin or with papain, or by storage in the frozen state for a long time. This enzyme hydrolyses lactose rapidly. *o*-Nitrophenyl β-galactoside and *p*-nitrophenyl β-galactoside are also hydrolysed, but much more slowly, and no hydrolysis at all can be measured with phenyl β-galactoside or 6-bromo-2-naphthyl β-galactoside as substrate.

The enzyme with a higher pH optimum appears to be specifically designed to hydrolyse lactose, and the localization of this enzyme also supports the concept that it has a digestive function in the intestine. The enzyme with a more acid pH optimum seems to be less specific, and can hydrolyse β-galactosides independently of the structure of the aglycone. Since this enzyme is not located in the brush borders (Koldovský *et al.* 1965) but in the cytoplasm (or possibly in lysosomes), it is not likely to participate in intestinal digestion but may have a function in the metabolism of the mucosal cells.

The two enzymes are separated from each other during Sephadex chromatography. On DEAE-cellulose chromatography, however, the enzyme with the more acid pH optimum is fractionated into two peaks, one of which is eluted close to the enzyme with a higher pH optimum. It is difficult to say at present whether the activities at pH 3·0–4·0 are caused by two β-galactosidases that appear to have very similar properties, or are exerted by a single enzyme that can appear in different forms that can be separated by ion-exchange chromatography but not by gel-filtration chromatography. We have the impression that the distribution of the enzyme with an acid pH optimum between the peaks in an ion-exchange chromatogram alters with storage of the preparation at −20°. This would support the concept that the peaks represent two different forms of the same enzyme.

Dahlqvist, Bull & Thomson (1965) separated the lactase and 6-bromo-2-naphthyl β-galactosidase activities completely from each other on a TEAE-cellulose column. Although the same separation was obtained in the present investigation on a Sephadex column, the complete separation on a TEAE-cellulose column could not be reproduced. In the previous study (Dahlqvist *et al.* 1965) the lactase activity was measured at pH 6·0 and the 6-bromo-2-naphthyl β-galactosidase at pH 4·6. Therefore the low lactase activity in the first peak, which has its pH optimum at 3·0–4·0, would not have been detected. The 6-bromo-2-naphthyl β-galactosidase activity of the second peak in the ion-exchange chromatogram in our present experiments is low when measured at pH 5·0, which explains why it was not observed in the previous study.

The two peaks isolated by us on Sephadex columns seem to correspond to those separated by Hsia, Makler, Semenza & Prader (1966) from extracts of human small-intestinal mucosa, with lactose and 6-bromo-2-naphthyl β-galactoside as substrates. These authors also concluded that one of the enzymes is a disaccharidase and the other a more unspecific β-galactosidase. The pH optima were not reported.

Furth & Robinson (1965) and Robinson, Price & Dance (1967) studied β-galactosidase activity in rat tissues with hetero-β-galactosides as substrates. They found β-galactosidase activity in all the tissues studied. The activity had an acid pH optimum and all the substrates used were hydrolysed at about the same rate. Lactose was not used, however. A considerable fraction of the activity appeared to be located in lysosomes; liver, spleen and kidney were used for the localization studies. On DEAE-cellulose chromatography two large peaks of β-galactosidase were found, but the one that was eluted last appeared heterogeneous. The results are similar to those we obtained with the acid enzyme, and this may thus be rather widespread in the body; this agrees with the concept that it may not be a factor in the intestinal digestion but may have a more metabolic function in the cells.

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