Characteristics of β -Galactosidase in the Mucosa of the Small Intestine of Infant Rats

PHYSICOCHEMICAL PROPERTIES

BY JIŘÍ KRAML, OTAKAR KOLDOVSKÝ, ALEŠA HERINGOVÁ, VĚRA JIRSOVÁ,
KAREL KÁCL, MILOŠ LEDVINA AND HANA PELICHOVÁ

*First Institute of Medical Chemistry, Charles University, Prague, Laboratory of Developmental Nutrition,
Institute of Physiology, Czechoslovak Academy of Sciences, Prague, and Institute for Mother and Child Care,
Prague, Czechoslovakia*

(Received 16 January 1969)

1. The characteristics of acid and neutral β -galactosidases isolated chromatographically from homogenates of the mucosa of the jejunum and ileum of suckling rats were studied. 2. The minimal molecular weight of the acid β -galactosidase, as estimated by gel filtration on Sephadex G-200, was in the range 83 000–105 000, whereas for the neutral β -galactosidase the estimated molecular weight was in the range 360 000–510 000. 3. The acid and neutral β -galactosidases were inhibited competitively by galactono-(1 $\rightarrow$ 4)-lactone, with respective K_i values of 0.15 mM and 1.1 mM. Only the acid β -galactosidase was inhibited competitively by sodium galactonate (K_i 0.17 mM). 4. Heat inactivation of both β -galactosidases occurred according to first-order kinetics. The neutral enzyme was more labile, but bovine serum albumin protected acid enzyme only. 5. Urea treatment inactivated both β -galactosidases, the neutral β -galactosidase being more sensitive than the acid β -galactosidase. 6. No differences were found between preparations from the jejunum and ileum.

Chromatography and gel filtration have revealed at least two β -galactosidases (EC 3.2.1.23) in the mucosa of the small intestine of the rat (Furth & Robinson, 1965; Asp & Dahlqvist, 1968*a,b*) and man (Hsia, Makler, Semenza & Prader, 1966).

These differ in their pH optimum and their affinity to some substrates (Asp & Dahlqvist, 1968*a,b*). We shall term the enzyme having a pH optimum at 5.5 the 'neutral' β -galactosidase and that with a pH optimum of 3.5 the 'acid' β -galactosidase. In the present work some further characteristics of both β -galactosidases and possible differences between jejunal and ileal preparations were examined, since during postnatal development the ratio of the enzymes in the ileum and jejunum changes (Koldovský & Chytil, 1965; Koldovský, Heringová & Jirsová, 1966*a*; Asp, Koldovský & Hošková, 1968).

MATERIALS AND METHODS

Determination of β -galactosidase activity. The method was based on that of Dahlqvist & Asp (1967). ONPG* was obtained from Dr Th. Schuchard, G.m.b.H., Munich,

Germany. For incubation 0.1 ml. of enzyme was added to 0.3 ml. of 50 mM-sodium citrate containing 12 mM-ONPG, 10 mM-mercaptoethanol and 1 mM-MgSO₄ (final concentrations). Sodium citrate buffer, pH 3.5 or 5.5, was used, depending on the pH optimum of the respective enzyme. Incubation was at 37°. The duration of incubation and the dilution of the enzyme were chosen so that reaction was proportional to time and amount of enzyme. The amount of substrate hydrolysed was less than 5–10% (usually incubation lasted for 10–30 min.). The reaction was stopped with 0.4 ml. of M-tris-HCl buffer, pH 8.5 (for incubations at pH 5.5) or pH 9.0 (for incubations at pH 3.5). The released *o*-nitrophenol was determined spectrophotometrically at 420 nm. against a blank containing 0.1 ml. of buffer instead of the enzyme. Activity was expressed in milliunits (nmoles of nitrophenol released/min.).

Protein determination. The method of Lowry, Rosebrough, Farr & Randall (1951) was used after precipitation of protein with 5% (w/v) trichloroacetic acid. The precipitate was dissolved in 0.1 M-NaOH. Bovine serum albumin (Mann Research Laboratories, New York, N.Y., U.S.A.) was used as standard. In the experiments designed for determination of molecular weight the extinction at 280 nm. was measured.

Preparation and treatment of homogenates from the intestinal mucosa. Rats of both sexes aged 10–13 days of the Wistar-Konáro-vice strain were decapitated and the small intestine of each was removed and rinsed with cold 0.15 M-NaCl. The intestine was then divided into thirds along its length. The

* Abbreviation: ONPG, *o*-nitrophenyl β -D-galactopyranoside.

proximal (jejunum) and distal (ileum) thirds were examined separately. The intestine was placed into a Petri dish on ice, cut open along its length and the mucosa was scraped off into an ice-cold dish. It was then treated by the procedure of Asp & Dahlqvist (1968a). Four volumes of cold 25 mM-sodium phosphate buffer (pH 7.0)–50 mM-KCl were added to the mucosa. This was then homogenized for 1–2 min. in a glass homogenizer with a Teflon pestle. To each 1 ml. of homogenate were added 0.125 mg. of papain (Mann Research Laboratories) and 0.075 mg. of cysteine hydrochloride and the homogenate was incubated for 60 min. at 37°. After centrifugation at 105 000g for 60 min. the supernatant was dialysed for 24 hr. at 4° against excess of 50 mM-phosphate buffer, pH 7.0. Mercaptoethanol and MgSO₄ were added to all buffers to give final concentrations of 10 mM and 1 mM respectively. Preparations from the proximal part of the jejunum were centrifuged at 105 000g before papain treatment (supernatant P₁), and the pellet was treated with papain. The second supernatant (P₂), obtained by centrifuging at 105 000g for 60 min., was dialysed as described above.

Separation on Sephadex G-200. Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was treated overnight with excess of 50 mM-phosphate buffer, containing mercaptoethanol and MgSO₄ as above, and packed into columns (100 cm. × 1 cm. or 100 cm. × 2.5 cm.). The same buffer was used to wash the columns (in a cold-room) and, after supernatant (2.5 ml. or 15 ml. for the respective column sizes) had been applied, fractions were collected automatically, about 1–2 ml./30 min. (narrower column) or 12–15 ml./30 min. (wider column). The following proteins of known molecular weights (Andrews, 1965) were used as standards: horse heart cytochrome c, type III (Sigma Chemical Co., St. Louis, Mo., U.S.A.), human haemoglobin, bovine serum albumin (Mann Research Laboratories), human serum γ -globulin (Institute of Sera and Vaccines, Prague, Czechoslovakia), ox liver catalase, thrice recrystallized (Reanal, Budapest, Hungary), β -galactosidase from *Escherichia coli* (preparation of Dr Janeček and Dr Spížek, Institute of Microbiology, Czechoslovak Academy of Sciences) and pig thyroglobulin (prepared from pig thyroid gland by the method of Derrien, Michel & Roche, 1948). Blue dextran

(Pharmacia Fine Chemicals) was used for determination of the void volume, V_0 . The protein content of fractions was estimated by the extinction at 280 nm. β -Galactosidases were assayed with ONPG as substrate.

Determination of K_m and K_i . The concentration of ONPG was changed within a range of 1–16 mM. Galactono-(1→4)-lactone (Lachema, Brno, Czechoslovakia) or sodium galactonate (prepared from barium galactonate; Lachema) was used as inhibitor, the former at a final concentration of 0.195–25 mM, the latter at 0.012–3.2 mM. K_m and K_i were determined by the methods of Lineweaver & Burk (1934) and Dixon (1953).

Heat inactivation. The enzyme obtained from the columns (peak fractions) was incubated at 40–53° and samples (0.1 ml.) were collected at regular intervals from 0 to 60 min. (0 to 10 min. for the higher temperatures) into thin-walled test tubes cooled in ice; the tubes contained substrate in 0.3 ml. of citrate buffer, pH 3.5 and 5.5. Activities were determined at 37°. Some of the heat-inactivation experiments was performed with bovine albumin added to give a final concentration of 2 mg./ml.

Urea inactivation. A 0.1 ml. portion of enzyme preparation was added to 0.3 ml. of citrate buffer, pH 3.5 or 5.5, containing urea of final concentration 0.25–8 M. The final concentration of citrate was 50 mM. Mercaptoethanol and MgSO₄ were present in a final concentration tenfold that in the other assays and buffers described in this paper, i.e. 0.1 M and 10 mM respectively (Shifrin & Steers, 1967). After 10–15 min. at room temperature 0.05 ml. of substrate in water was added to give a final concentration of 13.3 mM. The incubation was continued for 30 min. at 37°. The reaction was stopped with 0.4 ml. of 1 M-tris-HCl buffer, pH 8.5 or 9.0, as in the standard assay and *o*-nitrophenol was again determined against a blank containing the appropriate urea concentration.

RESULTS

Yields of neutral and acid β -galactosidases from jejunal and ileal homogenates. Tables 1 and 2 show that the 'acid' activity was much greater (five- to

Table 1. Preparation of β -galactosidases from the jejunum of 10–13-day-old rats

For details see the text. Numbers in parentheses refer to the numbers of determinations on different samples.

	Total vol. (ml.)	Protein (mg./ml.)	Activity (milliunits/ml.)		Sp. activity (milliunits/mg. of protein)	
			pH 3.5	pH 5.5	pH 3.5	pH 5.5
Homogenate	16.4	16.7	940	1200	56	71
105 000g supernatant (S ₁)	9.7	9.0	110	70	12	8
105 000g pellet (P ₁)	14.5	7.2	820	1080	113	150
Papain treatment						
Supernatant (S ₂)	11.5	3.8	540	730	144	194
Pellet (P ₂)	13.0	0.7	368	430	550	641
Dialysed S ₂	11.5	4.2	570	765	135	182
Sephadex G-200 (peak fractions)					703 (6)*	3280 (11)†

* Acid enzyme.

† Neutral enzyme.

Table 2. Preparation of β -galactosidases from the ileum of 10–13-day-old rats

Numbers in parentheses refer to the numbers of determinations on different samples.

	Total vol. (ml.)	Protein (mg./ml.)	Activity (milliunits/ml.)		Sp. activity (milliunits/mg. of protein)	
			pH 3.5	pH 5.5	pH 3.5	pH 5.5
Homogenate	10.3	11.0	8470	5320	770	483
Papain treatment						
105 000g supernatant	9.0	3.7	4460	2600	1210	706
Pellet	9.5	2.7	2780	1840	1037	686
Dialysed supernatant	9.0	4.2	3680	1980	876	471
Sephadex G-200 (peak fractions)					3000 (9)*	2930 (5)†

* Acid enzyme.
† Neutral enzyme.

Fig. 1. β -Galactosidase activities in a Sephadex G-200 chromatogram of a solubilized mucosal homogenate from the proximal part of the small intestine (jejunum) of 10-day-old rats. (a) Standard papain treatment of the homogenate; (b) supernatant of the homogenate after centrifugation at 105 000g before papain treatment; (c) pellet corresponding to (b) solubilized with papain. $\circ$, Activities measured at pH 5.5; $\bullet$, activities measured at pH 3.5.

seven-fold) in the ileum than in the jejunum, as shown previously (Koldovský & Chytil, 1965; Koldovský *et al.* 1966a; Asp *et al.* 1968). At pH 5.5 activity due to the 'acid' enzyme represents about 40% of the activity found at pH 3.5 (Asp *et al.*

1968). After subtraction of this 40% from the activity found at pH 5.5 the resultant activities at this pH were about the same in both ileum and jejunum. The 'neutral' enzyme is inactive at pH 3.5 (see below and also Asp & Dahlqvist, 1968b).

Fig. 2. β -Galactosidase activities in a Sephadex G-200 chromatogram of a solubilized mucosal homogenate from the distal part of the small intestine (ileum) of 10–11-day-old rats. (a)–(c) are analogous to (a)–(c) of Fig. 1. $\circ$, Activities measured at pH 5.5; $\bullet$, activities measured at pH 3.5.

For further purification the jejunum was taken as a source of neutral enzyme, to decrease the contamination with the acid one. The neutral enzyme is released only by papain treatment (Fig. 1). Papain treatment considerably decreased the total trichloroacetic acid-precipitable protein content, sometimes decreased total activity of neutral and acid enzymes, but solubilized the neutral enzyme and some of the acid one also (Figs. 1 and 2). The specific activity in the dialysed ultracentrifuge supernatant liquid was increased (two- to three-fold) in comparison with the homogenate only in the jejunal preparations of both enzymes. In the ileal preparations the acid enzyme specific activity remained unchanged whereas neutral activity was decreased. Further purification occurred as a result of gel filtration.

Separation of neutral and acid β -galactosidases from the jejunum and ileum on Sephadex G-200. Fig. 1 shows results for jejunal enzymes. The peak of the neutral enzyme is at 42–43 ml. elution volume, that of the acid one at 53–56 ml. Supernatant P₁ contained only part of the soluble acid enzyme. Sometimes a small amount of β -galactosidase activity was also eluted at 30–35 ml.

Fig. 2 shows the same for the ileal preparation with another column. The peak of the neutral

enzyme was at 37.8–40.7 ml. and that of the acid one at 53–54 ml.

Molecular weights. The minimal molecular weights as estimated by gel filtration were in the range 83 000–105 000 for the acid enzyme and in the range 360 000–510 000 for the neutral one (Fig. 3).

K_m and K_i of neutral and acid β -galactosidases in the jejunum and ileum. Table 3 compares K_m for both enzymes from both parts of the intestine. For the neutral jejunal enzyme substitution of acetate for citrate buffer at pH 5.5 had no effect. The K_m for the ileal acid enzyme was found to be the same at pH 3.5 and pH 5.5. Apparently there is no difference in the K_m of both enzymes along the length of the small intestine. The K_m for the acid enzyme is much lower than for the neutral one (see also Asp & Dahlqvist, 1968b).

Table 3 also shows the K_i values for galactonate and galactono-(1 $\rightarrow$ 4)-lactone with various enzyme fractions. Inhibition by both inhibitors was of the competitive type. Galactonate, however, did not inhibit the neutral enzyme, although the acid one was equally inhibited by both inhibitors. No differences between the ileum and jejunum were observed.

Heat inactivation. This showed first-order kinetics, except that at 47° the inactivation of the neutral enzyme was biphasic. The neutral enzyme was

Fig. 3. Plot of the V_e/V_0 ratio (where V_e is the elution volume and V_0 is the void volume) against $\log$ (mol.wt.) for proteins used as standards and β -galactosidases of the small intestine of the rat. Each value of V_e represents the mean of at least three determinations.

the more labile, and bovine serum albumin protected the acid enzyme only. A certain variability was found between different preparations, but no differences between the jejunum and ileum were detected. The first-order constants for inactivation and Arrhenius plots for the inactivation are shown in Fig. 4. The activation energy for inactivation of the neutral enzyme was 45.6 kcal./mole and 81 kcal./mole for the acid enzyme. For the acid enzyme addition of albumin had no effect on this energy. No acid activity appeared after heat inactivation of neutral activity.

Inactivation with urea. For this the enzymes were diluted in such a way that initial activity of both enzymes was the same. There were no differences between ileal and jejunal activities. The neutral enzyme was more easily inactivated than the acid one (Fig. 5). No acid activity was discovered after inactivation of the neutral enzyme with urea.

DISCUSSION

Our results confirm previous reports (Asp & Dahlqvist, 1968a) that the mucosa of the small intestine of rats contains two β -galactosidases.

Fig. 4. Arrhenius plot for the thermal inactivation of (a) neutral (pH 5.5) and (b) acid (pH 3.5) β -galactosidase from the small intestine of the rat. $\circ$, Without albumin; $\bullet$, albumin added (2 mg./ml.). K is the first-order rate constant (min.^{-1}) of inactivation.

These authors also found another high-molecular-weight fraction after separation on Sephadex (personal communication).

The heterogeneity of intestinal β -galactosidases has been shown chromatographically in man by Semenza, Auricchio & Rubino (1965), and has also been postulated in other species: rabbits (Doell & Kretchmer, 1962, 1963; Koldovský, Heringová, Jirsová, Chytil & Hošková, 1966b), mice and guinea pigs (Koldovský *et al.* 1966b). Neutral β -galactosidase is localized in the microvilli whereas acid β -galactosidase is found in other parts of the enterocytes (Koldovský *et al.* 1965b; Koldovský *et al.* 1966a). The former is closely concerned with lactose absorption (Koldovský, Muzyčénková, Heringová, Hahn & Jirsová, 1965a), whereas the latter has no such relationship.

The acid enzyme was found to be more sensitive than the neutral one to the inhibitors tested here. With this enzyme K_i for both galactonate and galactono-(1 $\rightarrow$ 4)-lactone was about the same. In this respect this β -galactosidase differs, for instance, from that of the rat epididymis (Levy, McAllan & Hay, 1962). Neutral β -galactosidase was affected by galactono-(1 $\rightarrow$ 4)-lactone only, and to a smaller extent than the acid enzyme. Apparently the neutral enzyme is more specific towards substrates

Table 3. K_m values for ONPG with neutral and acid enzymes from the solubilized mucosal homogenate from the small intestine of 10–13-day-old rats, and the corresponding K_i values for galactono-(1→4)-lactone and sodium galactonate (competitive inhibition)

Enzyme activities were obtained from the peak fractions after separation on Sephadex G-200.

		K_m (mM) ONPG	K_i (mM)	
			Galactono-(1→4)-lactone	Sodium galactonate
Neutral enzyme (pH 5.5)	Jejunum	14.4	1.09	No inhibition
	Ileum	11.75		
Acid enzyme (pH 3.5)	Jejunum	0.5	0.15	0.17
	Ileum	0.4

Fig. 5. Changes of enzyme activities of neutral and acid β -galactosidases of the small intestine of rats as a function of urea concentration. O, Neutral enzyme (pH 5.5); ●, acid enzyme (pH 3.5); ▲, acid enzyme (activity at pH 5.5). A_0 , Activity without urea; A , activity in the presence of urea. Preincubation with area was for 10–15 min. at room temperature (see the text).

and inhibitors (see also Asp & Dahlqvist, 1968a,b), as well as being the more sensitive to heat and urea inactivation.

No qualitative differences between β -galactosidases of the jejunum and ileum were found, although the relative activities of the enzymes differed in the two regions of the small intestine.

We thank Dr N. G. Asp, Dr Hořková and Dr D. Hiršová for their help, and Dr A. Dahlqvist for exchange of information. The technical assistance of Mrs A. Velechovská and Mrs J. Křivánková is gratefully acknowledged.

REFERENCES

- Andrews, P. (1965). *Biochem. J.* **96**, 595.
 Asp, N. G. & Dahlqvist, A. (1968a). *Biochem. J.* **106**, 841.
 Asp, N. G. & Dahlqvist, A. (1968b). *Biochem. J.* **110**, 143.
 Asp, N. G., Koldovský, O. & Hořková, J. (1968). *Physiol. bohemoslov.* **17**, 229.
 Dahlqvist, A. & Asp, N. G. (1967). *Biochem. J.* **103**, 86.
 Derrien, Y., Michel, R. & Roche, J. (1948). *Biochim. biophys. Acta*, **2**, 454.
 Dixon, M. (1953). *Biochem. J.* **55**, 1953.
 Doell, R. G. & Kretchmer, N. (1962). *Biochim. biophys. Acta*, **62**, 353.
 Doell, R. G. & Kretchmer, N. (1963). *Biochim. biophys. Acta*, **67**, 516.
 Furth, A. J. & Robinson, D. (1965). *Biochem. J.* **97**, 59.
 Hsia, D. Y. Y., Makler, M., Semenza, G. & Prader, A. (1966). *Biochim. biophys. Acta*, **113**, 390.
 Koldovský, O. & Chytil, F. (1965). *Biochem. J.* **94**, 266.
 Koldovský, O., Heringová, A., & Jirsová, V. (1966a). *Biol. neonat.* **10**, 241.
 Koldovský, O., Heringová, A., Jirsová, V., Chytil, F. & Hořková, J. (1966b). *Canad. J. Biochem.* **44**, 523.

- Koldovský, O., Muzyčenkova, H., Heringová, A., Hahn, P. & Jirsová, V. (1965a). *Canad. J. Physiol. Pharmacol.* **43**, 469.
- Koldovský, O., Noack, R., Schenk, G., Jirsová, A., Heringová, V., Braná, H., Chytil, F. & Friedrich, M. (1965b). *Biochem. J.* **96**, 492.
- Levy, G. A., McAllan, A. & Hay, A. J. (1962). *Biochem. J.* **82**, 225.
- Lineweaver, H. & Burk, D. J. J. (1934). *J. Amer. chem. Soc.* **56**, 568.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Semenza, G., Auricchio, S. & Rubino, A. (1965). *Biochim. biophys. Acta*, **96**, 487.
- Shifrin, S. & Steers, E. (1967). *Biochim. biophys. Acta*, **133**, 463.