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β-GLUCOSIDASE (PHLORIZIN HYDROLASE) ACTIVITY OF THE LACTASE FRACTION ISOLATED FROM THE SMALL INTESTINAL MUCOSA OF INFANT RATS, AND THE RELATIONSHIP BETWEEN β-GLUCOSIDASES AND β-GALACTOSIDASES

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SUMMARY

Sephadex G-200 chromatography reveals the presence of two β-glucosidase (phlorizin hydrolase) peaks in the supernatant obtained after papain treatment of mucosal homogenate from the jejunum of infant rats, the major one being identical with the neutral β-galactosidase (lactase) peak in position, shape, and pH optimum (pH 5–6). The other one, also with a nearly neutral pH optimum, emerges shortly after the acid (lysosomal) β-galactosidase, which has pH optimum 3.5.

In the 105 000 × *g* supernatant of the mucosal homogenate, not previously treated with papain, the soluble (lysosomal) acid β-galactosidase, which has no phlorizin hydrolase (β-glucosidase) activity, predominates.

Kinetic data (mutual inhibition between β-glucosides and β-galactosides, inhibition by Tris and aldonolactones) show common properties of the β-glucosidase and β-galactosidase active site(s) in the chromatographic fraction of the neutral β-galactosidase (lactase). However, with the artifical substrates (β-glucoside and β-galactoside) the mixed type of mutual inhibition was obtained, which cannot unequivocally exclude, at present, the existence of more than a single binding site for the two substrates in the lactase fraction. The acid β-galactosidase is more specific to the glycone part of the substrate molecule (C-4 configuration) than the neutral β-galactosidase, as the former enzyme does not effectively split cellobiose and hetero-β-glucosides (phlorizin and *p*-nitrophenyl-β-glucoside) and is not inhibited by phlorizin and gluconolactone. On the other hand, the neutral β-galactosidase (lactase) fraction, which is released from the mucosal membrane by the proteolytic action of papain similarly as the other phlorizin hydrolase (hetero-β-glucosidase), splits β-glucosides as well as β-galactosides and the lactase activity is competitively inhibited by phlorizin. Phloretin β-galactoside is hydrolyzed by the two β-galactosidases and exhibits a competitive inhibitory effect on both enzymes.

Unlike the lactase fraction, the second phlorizin hydrolase (hetero-β-glucosidase)

Abbreviations: PNP-, *p*-nitrophenyl-; ONP-, *o*-nitrophenyl-.

does not apparently split cellobiose and is inhibited by 0.1 mM *p*-chloromercuribenzoate.

INTRODUCTION

The existence of a β-glucosidase catalyzing phlorizin hydrolysis to phloretin and glucose has been demonstrated by MALATHI AND CRANE[1] in the microvillus membrane of hamster intestinal brush border. This enzyme was found to be distinct from the other hitherto known brush border glycosidases, including lactase. A number of α-glycosidases and β-glycosidases from the small intestine of various species was separated by Sephadex G-200 chromatography of the papain-treated mucosa[2–7]. It could be shown that cellobiase activity of intestinal tissue is inseparable from the lactase activity[3]. However, on the basis of their experimental results, MALATHI AND CRANE[1] excluded the possibility that also such hetero-β-glucosides as phlorizin (phloretin-β-glucoside) or *p*-nitrophenyl-β-glucoside (PNP-β-glucoside) could be split by lactase (brush border β-galactosidase).

As phlorizin hydrolase is suspected of being involved in the mechanism of phlorizin inhibition of sugar transport and cellular metabolism[1,8,9], we thought it useful to contribute to the knowledge of the specificity of this enzyme(s) and to elucidate the relationship of the phlorizin hydrolase activity to β-galactosidases. This relationship was followed on the Sephadex G-200 chromatographic fractions[7] of the papain-solubilized mucosa of infant rats[10], where the activities of β-galactosidases (both of the neutral β-galactosidase–lactase, which is bound in the brush border[11,12,28], and of the acid, lysosomal[12,28] β-galactosidase) are much higher than in adult animals. Another advantage of this material is that there are only minute amounts of α-glucosidases present in the intestine of infant rats[13].

MATERIALS AND METHODS

Chemicals

p-Nitrophenyl-β-glucoside (PNP-β-glucoside), *p*-nitrophenyl-β-galactoside (PNP-β-galactoside) and *o*-nitrophenyl-β-galactoside (ONP-β-galactoside) were purchased from Lachema, Brno (Czechoslovakia); Lactose and cellobiose were obtained from Lachema, Brno (Czechoslovakia) or from Koch-Light Laboratories (England) and were recrystallized from ethanol–water before use. Phlorizin was obtained from Fluka A.G. (Switzerland) and recrystallized from ethanol–water. Phloretin β-galactoside and one batch of phlorizin were synthesized by Dr. Vereš from the Isotope Laboratory, Czechoslovak Academy of Sciences. 1,4-Gluconolactone and 1,4-galactonolactone were gifts from Dr. O. Koldovský, Institute of Physiology, Czechoslovak Academy of Sciences and 1,5-gluconolactone was obtained from Nutritional Biochemicals, Cleveland, Ohio. Tris (tris-hydroxymethylaminomethane) was purchased from Lobachema, Wien (Austria). Glucose oxidase reagent (Blood Sugar Test) was obtained from C. F. Böhringer and Söhne GmbH, Mannheim (Germany), 2-fold recrystallized papain (suspension 15 U/mg) from Mann Research Laboratories, N.Y. (U.S.A.) and Sephadex G-200 from Pharmacia, Uppsala (Sweden). Lyophilized human albumin was purchased from the Institute for Production of Vaccines and Sera, Prague (Czechoslovakia). All chemicals were reagent grade.

Methods

All experiments were carried out with the enzymes obtained from the small-intestinal mucosa of rats of both sexes aged 10–13 days. The preparation and treatment of the homogenates from the intestinal mucosa is described elsewhere[10]. In the majority of experiments the proximal third of the small intestine (jejunum) was used because of a relatively lower content of the acid (lysosomal) β-galactosidase in this material, as compared with the ileum, where the acid enzyme prevails at this developmental stage[10]. The mucosa was homogenized and the first supernatant (1 h, 105 000 × *g*) designated as supernatant P_1. The pellet was then incubated with papain and the second 105 000 × *g* supernatant designated as supernatant P_2 (the corresponding supernatants from the ileum were designated as supernatant D_1 and supernatant D_2). Sephadex G-200 chromatography was carried out on two types of columns, 100 cm × 2.5 cm or 100 cm × 3.7 cm in size with 50 mM sodium phosphate buffer, pH 7.0.

Enzyme activities with hetero-β-glycosides (PNP-β-glucoside, PNP-β-galactoside, ONP-β-galactoside) as substrates were determined with 12 mM substrate in 50 mM sodium citrate buffer, pH 3.5 and/or 5.5, as described previously[7,10]. For K_m and K_i determinations the substrate concentration was changed between 1 and 16 mM. The released *o*-nitrophenol or *p*-nitrophenol was determined spectrophotometrically at 425 nm.

Lactase and cellobiase activities were determined with 29 mM lactose and/or cellobiose, as suggested by DAHLQVIST[14] and ASP AND DAHLQVIST[7] and the released glucose was estimated by Tris–glucose oxidase method[14]. For K_m and K_i determinations the lactose and cellobiose concentrations were varied within a range of 6–64 mM and 1–12 mM, respectively.

Phlorizin hydrolase activity was estimated according to MALATHI AND CRANE[1] employing the reducing sugar method. 2.5 mM recrystallized phlorizin was used as substrate. The liberated sugar was estimated by the method of SOMOGYI[16] and NELSON[17]. For K_m determination, the concentrations of phlorizin and/or phloretin β-galactoside were changed within a range of 0.25–2.5 mM.

The amount of protein in the homogenate fractions was determined by the method of LOWRY *et al.*[18]. Lyophilized human albumin was used as standard.

Calculations

The enzyme activities were expressed in units or m units (U or mU), *i.e.* μmoles or nmoles of nitrophenol or glucose released/min at 37°, and specific activities were related to 1 mg protein. K_m and K_i were determined graphically by the methods of LINEWEAVER AND BURK[19] and DIXON[20]. v_{max} values were calculated from the mean K_m of the respective substrate and from the velocities at the respective substrate concentration in the standard assays. The relative v_{max} was expressed in percent of lactase activity (v_{max} for lactose = 100%).

RESULTS AND DISCUSSION

Chromatographic separation of β-glucosidases and β-galactosidases on Sephadex G-200

The distribution of the β-glucosidase and β-galactosidase activities in the chromatographic fractions was followed both in the supernatants before (P_1 and D_1)

and after (P_2 and D_2) papain treatment. As substrates both hologlycosides (cellobiose, lactose) and heteroglycosides (PNP-β-glucoside, phlorizin, ONP-β-galactoside and PNP-β-galactoside) were employed. In the supernatants before papain treatment (P_1 and D_1) practically only the soluble acid β-galactosidase (pH optimum 3.5) was found. Furthermore, the high-molecular-weight fraction ("prepeak") was present, which is eluted with the void volume (V_0), the major part of it being related to the acid β-galactosidase[21,22]. Only traces of the PNP-β-glucosidase activity at pH 5.5 were eluted from the column between the "prepeak" and the acid β-galactosidase peaks. Practically no β-glucosidase activities were found in the peak of the acid

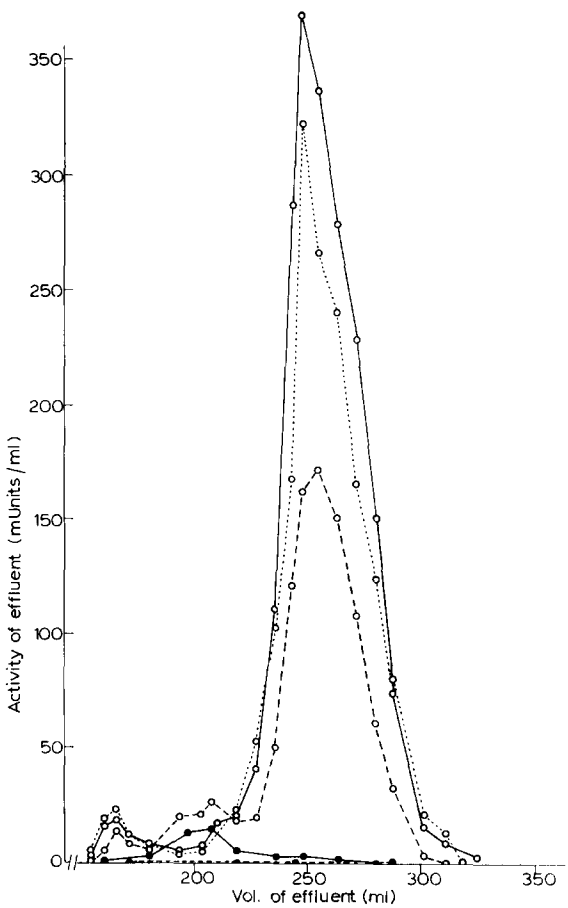

Fig. 1. β-galactosidase and β-glucosidase activities in a Sephadex G-200 chromatogram of a 105 000 × *g* supernatant (supernatant D_1) obtained from the homogenate of mucosa from the distal part of the small intestine (ileum) of infant rats. Activities were measured at pH 5.5 or 3.5. Substrates (12 mM): ○—○, *o*-nitrophenyl-β-galactoside (pH 3.5); ○---○, *o*-nitrophenyl-β-galactoside (pH 5.5); ○...○, *p*-nitrophenyl-β-galactoside (pH 3.5); ●---●, *p*-nitrophenyl-β-glucoside (pH 3.5) and ●—●, *p*-nitrophenyl-β-glucoside (pH 5.5). The first small peak is the high-molecular-weight fraction of the acid enzyme (elution volume between 160–170 ml = V_0), the second minor peak (with its maximum between 200–210 ml) are traces of the neutral β-galactosidase, accompanied also by some *p*-nitrophenyl-β-glucosidase, and the major peak (with its maximum between 240–250 ml) corresponds to the acid β-galactosidase.

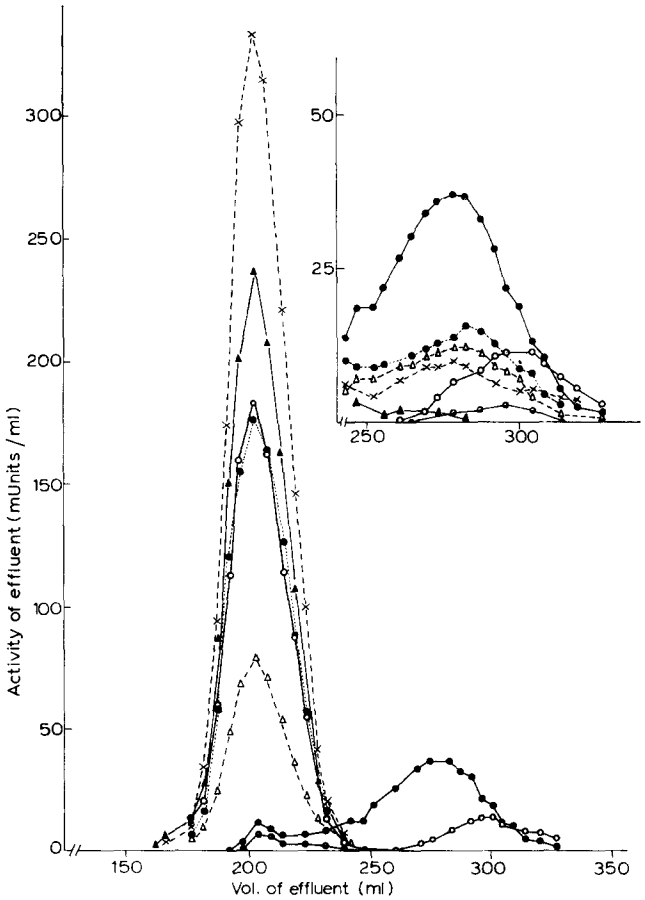

Fig. 2. β-galactosidase and β-glucosidase activities in a Sephadex G-200 chromatogram of a 105 000 × *g* supernatant (supernatant P_2) obtained from the papain-treated homogenate of mucosa from the proximal part (jejunum) of infant rats. The soluble portion of the homogenate (supernatant P_1) was removed by centrifugation before the pellet was treated with papain. Substrates: ×---×, 29 mM lactose (pH 5.5); ▲—▲, 29 mM cellobiose (pH 5.5); ○—○, 12 mM *p*-nitrophenyl-β-glucoside (pH 5.5); ◒—◒, 12 mM *p*-nitrophenyl-β-glucoside (pH 3.5); ●···●, 12 mM *o*-nitrophenyl-β-galactoside (pH 5.5); ●—●, 12 mM *o*-nitrophenyl-β-galactoside (pH 3.5); and △---△, 12 mM *p*-nitrophenyl-β-galactoside (pH 5.5). The major peak (elution volume 200–210 ml) belongs to the neutral β-galactosidase (lactase), the peak of the acid β-galactosidase has its maximum between 270–285 ml, and the second hetero-β-glucosidase peak (substrate: *p*-nitrophenyl-β-glucoside) has its maximum at 300 ml. Activities obtained with the elution volume 240–320 ml are given in the larger scale in the insertion. The values of lactase activity are reduced 2-fold.

β-galactosidase, which is apparent in Fig. 1, representing the chromatogram obtained from the supernatant D_1 from the ileum. The traces of β-glucosidase activities are located in the position of the peak of the neutral β-galactosidase (lactase), when compared with the chromatogram in Fig. 2.

In the supernatants after papain treatment (supernatants P_2 and D_2) only the supernatants of P_2 revealed two distinct PNP-β-glucosidase peaks (Fig. 2). In the supernatant D_2 the second peak was practically absent or extremely reduced. The first, major peak, was identical in shape and position with that of the neutral β-

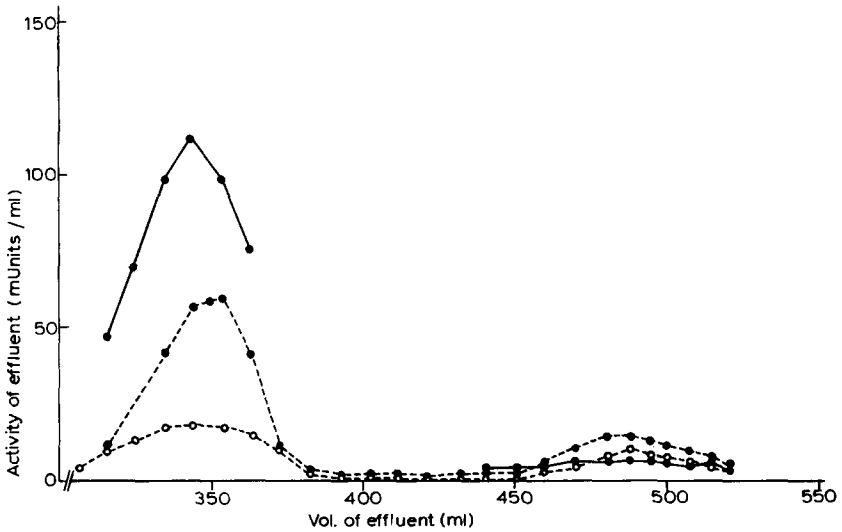

Fig. 3. Hetero-β-glucosidase activities in a Sephadex G-200 chromatogram of the material analogous to that presented in Fig. 2. A wider type of column was used than in the experiment in Fig. 2. Thus the corresponding elution volumes are larger. Substrates: ●---●, 2.5 mM phlorizin (pH 5.5); ○---○, 2.5 mM phlorizin (pH 3.5); and ●—●, 12 mM *p*-nitrophenyl-β-glucoside (pH 5.5). The two peaks of phlorizin hydrolase correspond to those of *p*-nitrophenyl-β-glucosidase. The major peak is identical with that of the neutral β-galactosidase.

galactosidase (lactase), the second one was hidden in the peak of the acid β-galactosidase; however, it was shifted towards higher elution volumes. Both hetero-β-glucosidase peaks had a higher activity at pH 5.5 than at pH 3.5. It should be pointed out that the presence of PNP-β-glucosidase activity in the analogous human lactase fraction (enzyme I) was observed also by GRAY AND SANTIAGO[23] and GRAY *et al.*[24].

In order to correlate the PNP-β-glucosidase with phlorizin hydrolase, in another experiment the supernatant P_2 was chromatographed on Sephadex G-200 column (wider type, Fig. 3). Two phlorizin hydrolase peaks could be identified, with a more

TABLE I

K_m AND v_{max} VALUES OF THE INTESTINAL NEUTRAL β-GALACTOSIDASE (LACTASE) FRACTION FOR VARIOUS β-GALACTOSIDES AND β-GLUCOSIDES AS SUBSTRATES

K_m values were estimated by the graphical method of LINEWEAVER AND BURK[19]. Ranges of values are given in parentheses. The relative v_{max} was expressed in percents of the lactase activity, where v_{max} for lactose was taken as 100% (0.64–1.58 units/ml enzyme). All activities were assayed at pH 5.5.

Substrate	*K_m (mM)*		*v_{max} (%)*	
Lactose	40.0	(28.0 –50.0)	100.0	
Cellobiose	1.1	(0.96– 1.3)	15.5	
ONP-β-galactoside	13.1	(11.8 –14.4)	30.95	(19.9–42.0)
PNP-β-galactoside	22.3	(15.5 –30.0)	16.5	(14.3–18.8)
PNP-β-glucoside	4.1	(3.1 – 5.0)	18.3	(14.4–22.2)
Phlorizin	0.4	(0.34– 0.5)	3.2	
Phloretin-β-galactoside	0.4		0.7	

neutral pH optimum (values at pH 5.5 higher than at 3.5). Both phlorizin hydrolase peaks were found in the same position as PNP-β-glucosidase peaks.

β-Glucosidase activity of the neutral β-galactosidase (lactase) fraction

The striking similarity of the distribution and chromatographic behavior of the neutral β-galactosidase (lactase) and of the major chromatographic fraction of cellobiase and hetero-β-glucosidase (PNP-β-glucosidase and phlorizin hydrolase) led us to the assumption that these activities may belong to a single enzyme molecule in infant rats, which, of course, would be a result different from the findings of MALATHI AND CRANE[1] obtained with intestinal brush borders of adult hamsters. Our hypothesis was tested on the neutral β-galactosidase (lactase) fraction in a number of ways, both kinetic and immunochemical[21,25]. In this paper we are dealing only with kinetic data. Table I represents the K_m and v_{max} values for different β-glucosides and β-galactosides as substrates at pH 5.5. Whereas β-glucosides show generally a higher affinity for the enzyme (low K_m), they are split with a much lower maximum velocity than lactose. The K_m and v_{max} values for β-galactosides seem to be in relatively good agreement with those published by ASP AND DAHLQVIST[26].

Criteria for a common active site for β-glucosides and β-galactosides in the molecule of isolated rat lactase

Although some additional criteria revealed that the isolated chromatographic fraction of lactase was relatively pure (it showed one band in the acrylamide gel electrophoresis but in the Ouchterlony double-diffusion test in agar gel there was one stronger and another much weaker precipitation line, when the appropriate rabbit antiserum was employed[21]), there was still some doubt, whether the β-galactosidase and β-glucosidase activities belonged to the same protein molecule and possibly to a single active site. However, SEMENZA and co-workers also could demonstrate some phlorizin hydrolase activity in their purified preparation.

In a competitive inhibition experiment PNP-β-glucoside was used as substrate and lactose as inhibitor. Released *p*-nitrophenol was measured. K_i for lactose, calculated by the graphical method of DIXON[20], was 43.7 mM.

Table II shows the relative rates of hydrolysis of PNP-β-glucoside and PNP-β-galactoside in mixture. At equal relative concentrations ($\Theta = S/K_m$, ref. 27) of PNP-β-glucoside (K_m = 5 mM) and PNP-β-galactoside (K_m = 30 mM) the mixed substrate velocity was measured in terms of nmoles *p*-nitrophenol or glucose released/min at 37° per 0.1 ml enzyme. In the range of Θ = 0.166–1.0 the resulting velocity was not in any case the sum of the individual velocities for each substrate separately, thus showing possible competition of the two substrates for the same active site(s). However, the resulting rates were lower than could be expected if the fully competitive inhibition[27] were assumed. Even though the individual velocities for each of the two substrates separately were comparable at the lower relative concentrations, PNP-β-galactoside was hydrolyzed in the mixture at a higher rate than PNP-β-glucoside, and with increasing relative concentrations of both substrates (kept in an invariable proportion) the amount of glucose released from PNP-β-glucoside increased only to Θ = 0.5. At higher relative mixed substrate concentrations the amount of liberated glucose even decreased.

The competition of these analogous substrates was analyzed in more detail,

TABLE II

MIXED SUBSTRATE INCUBATION (*p*-NITROPHENYL-β-GLUCOSIDE AND *p*-NITROPHENYL-β-GALACTOSIDE) WITH THE NEUTRAL β-GALACTOSIDASE FRACTION

$[S]/K_m$ (Θ)	*nmoles p-nitrophenol released/min per 0.1 ml enzyme*			*nmoles glucose released/min per 0.1 ml enzyme*
	PNP-β-glucoside	*PNP-β-galactoside*	*PNP-β-glucoside + PNP-β-galactoside*	*PNP-β-glucoside + PNP-β-galactoside*
1.0	4.4	4.93	5.15	1.48
0.5	2.9	3.43	3.96	1.64
0.33	2.38	2.75	3.43	1.47
0.21	2.02	2.07	2.77	1.26
0.166	1.72	1.76	2.48	1.09

applying the Lineweaver–Burk plot for PNP-β-glucoside as substrate and PNP-β-galactoside as inhibitor. Released glucose was measured as the product for the velocity estimations. The apparent K_m for PNP-β-glucoside increased with the concentration of PNP-β-galactoside in the medium but the v_{max} did not remain constant, as one should expect with the fully competitive inhibition, but rather decreased with the concentration of PNP-galactoside as inhibitor. Thus the kinetics with the two artificial substrates are of the mixed type, and the existence of more than a single binding site for the two substrates in the lactase fraction cannot be excluded at present.

These results were supplemented by an immunochemical test[21] in which both the β-galactosidase and β-glucosidase activities could be detected in the same immunoprecipitation line in the Ouchterlony test in agar gel, when indigogenic substrates 4-chloro-5-bromo-3-indolyl-β-glucoside or galactoside were employed for enzyme detection in the method as recommended by LOJDA[28,29]. This precipitation line was formed by the isolated rat neutral β-galactosidase fraction and its appropriate rabbit antiserum. It should be mentioned, that this line was active also against the analogous fucoside[25]. Furthermore, our results are in agreement with the histochemical findings of LOJDA[28,29].

Inhibitors of the β-glucosidase and β-galactosidase activities of the isolated rat lactase (neutral β-galactosidase)

Another test for the identity or non-identity of β-glucosidase and β-galactosidase molecules or active sites is the inhibitory effect of Tris or aldonolactones. Tris is a competitive inhibitor of several carbohydrases, including lactase, in which imidazole groups are assumed to be involved in their catalytic sites[30,31]. Aldonolactones inhibit competitively the enzymatic hydrolysis of their configurationally analogous substrates[32]. Whereas Tris, galactonolactone and gluconolactone inhibited the brush border lactase in the hamster[1], Tris and galactonolactone did not inhibit the phlorizin hydrolase activity in the same species[1]. However, Tris, galactonolactone and gluconolactone inhibit the β-glucosidase activity of the rat lactase fraction as well as the β-galactosidase activity (Table III). There was no substantial difference in the extent and the type of inhibition by 1,4- or 1,5-gluconolactone, the latter being a slightly more efficient inhibitor. Comparing various substrates, for the β-galactoside-lactose, gluconolactone is even a more efficient inhibitor (lower K_i) than for the analogous β-glucoside-cellobiose. It is also a more efficient inhibitor than galactonolactone when

TABLE III

EFFECTS OF TRIS AND ALDONOLACTONES ON THE HYDROLYSIS OF β-GALACTOSIDES AND β-GLUCOSIDES BY THE INTESTINAL NEUTRAL β-GALACTOSIDASE FRACTION

K_i values were estimated by DIXON's[20] graphical method. As substrate lactose was employed at 1–64 mM, *o*-nitrophenyl-β-galactoside, *p*-nitrophenyl-β-galactoside and *p*-nitrophenyl-β-glucoside at 1–12 mM and phlorizin at 0.5–1.25 mM. Tris as inhibitor was tested at 1–50 mM. 1,4-gluconolactone as inhibitor was used at 0.025–0.25 mM, 1,4-galactonolactone at 0.25–2.5 mM, except phlorizin as substrate, where both inhibitors were tested at 25–100 mM. All activities were measured at pH 5.5. The inhibition was of the competitive type except of +, where the kinetics were atypical.

Substrate	*Inhibitor K_i (mM)*		
	Tris	*Galactonolactone*	*Gluconolactone*
Lactose	4.4—4.5	0.35	0.027
Cellobiose	1.8	0.36	0.94
ONP-β-galactoside	25.0	1.1	0.01
PNP-β-galactoside	6.0—6.8	0.48–0.5	+
PNP-β-glucoside	7.5—9.8	0.45	0.5
Phlorizin	53.0	82.0	8.0

using the same β-galactosides as substrates (lactose, ONP-β-galactoside, PNP-β-galactoside). A similar result was reported also by SWAMINATHAN AND RADHAKRISHNAN[33] for the two lactase fractions from monkey small intestine, *i.e.* gluconolactone was a more efficient inhibitor than galactonolactone when lactose was used as substrate. These results would indicate a close relationship between the β-glucosidase and β-galactosidase active site(s) also in the fraction of rat lactase. With phlorizin as substrate the K_i values of all the three inhibitors were relatively high, as compared with the other substrates. With PNP-β-galactoside the inhibition by gluconolactone was atypical, however, this inhibitory effect was much higher (about one order of magnitude) than that of galactonolactone.

Substrate specificity of the acid β-galactosidase

From the chromatogram presented in Fig. 1 it is apparent that no β-glucosidase activity against PNP-β-glucoside could be detected in the peak of the acid (lysosomal) β-galactosidase. The same lack of hydrolysis was observed with cellobiose and phlorizin as substrates. The failure of the analogous human enzyme (enzyme II) to split cellobiose and PNP-β-glucoside was observed also by GRAY AND SANTIAGO[23] and GRAY[24]. On the other hand, phloretin β-galactoside was split by the acid β-galactosidase fraction isolated from the rat small intestine, the K_m value being 1.42 mM. Thus the acid β-galactosidase seems to be a more strictly specific enzyme as far as the glycone part of the substrate molecule (C-4 configuration) is concerned. This observation is supported also by the lack of inhibition of the isolated acid β-galactosidase by gluconolactone up to 50 mM concentration of the inhibitor when the enzyme activity was measured with 12 mM ONP-β-galactoside at pH 3.5, whereas for galactonolactone the K_i value was previously[10] estimated as 0.15 mM (competitive inhibition). Thus the acid enzyme of the rat behaves in this respect similarly to that in the brush border-free homogenate from the hamster intestine[1]. The lack of inhibition of the lysosomal β-galactosidase from various species and organs by gluconolactone and cellobiose was demonstrated also by the histochemical findings of LOJDA[29].

Phlorizin and phloretin β-galactoside as inhibitors of the isolated neutral and acid β-galactosidases of the rat

Whereas phlorizin did not inhibit the acid β-galactosidase up to 10 mM concentration (substrate: 1–12 mM ONP-β-galactoside at pH 3.5) it did inhibit the neutral β-galactosidase at 0.01–0.1 mM concentration with 6–64 mM lactose as substrate, or at 0.05–5 mM concentration with 1–16 mM ONP-β-galactoside as substrate at pH 5.5. The inhibition with phlorizin was of the competitive type, when lactose was used as substrate, K_i value according to Dixon's plot being 0.047 mM. It should be noted that with lactose as substrate the concentrations of phlorizin in the original incubation mixture higher than 1 mM have to be avoided, as they inhibit the glucose oxidase reaction. With ONP-β-galactoside the phlorizin inhibition was again atypical. On the other hand, both the neutral and the acid β-galactosidases were inhibited competitively by phloretin β-galactoside, when using ONP-β-galactoside as substrate, K_i values were found to be the same (1.46 mM) for the two enzymes (measured at pH 5.5 for the neutral and pH 3.5 for the acie enzyme). The lack of inhibition of the acid enzyme with phlorizin would agree with the concept that the acid β-galactosidase is more specific than the neutral enzyme as far as the glucone part of the substrate molecule (C-4 configuration) is concerned.

Comments on the number of β-glucosidases in the small intestine of the rat and of other species

There is some evidence now that the neutral β-galactosidase (lactase) in the rat may be a hetero-β-glucosidase (phlorizin hydrolase) as well. However, some of the presented kinetic data (the mixed type of mutual inhibition between PNP-glucoside and PNP-β-galactoside, the difference in K_i values of Tris and/or aldonolactones with phlorizin and/or the other β-glucosides and β-galactosides as substrates, and the difference in K_i and K_m values of phlorizin) represent still an important argument for the assumption of more than a single binding site for the artifical substrates in the lactase fraction. On the other hand, the PNP-β-glucosidase and PNP-β-galactosidase activities of the lactase fraction were inseparable, in our preliminary experiments, also on Sepharose 4B and on DEAE-cellulose (with concentration gradient), and even after solubilization by Triton X-100. The relation of the other hetero-β-glucosidase, demonstrated in our chromatograms (Figs. 2 and 3), and reported by other authors in the hamster[1] and in the rat[9], to the neutral β-galactosidase fraction, is still to be elucidated. This second β-glucosidase, observed also by Asp[34], seems to be an enzyme different from lactase. Unlike the latter enzyme, it does not apparently split cellobiose. Thus it would be a true hetero-β-glucosidase. Moreover, we found that 0.1 mM *p*-chlormercuribenzoate inhibits this hetero-β-glucosidase to 20%, which is similar to the properties of the acid β-galactosidase[35] and would indicate the involvement of sensitive sulfhydryl groups in the active center of this enzyme. However, the neutral β-galactosidase (lactase) is not inhibited by the sulfhydryl reagent at all[35].

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