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INTRACELLULAR DISTRIBUTION OF β-GALACTOSIDASES IN MUCOSAL CELLS FROM HOG SMALL INTESTINE

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Summary

1. Intracellular distribution of three types of β-galactosidases (β-D-galactoside galactohydrolase EC 3.2.1.23) i.e. hetero β-galactosidase, lactase and acid β-galactosidase, was studied by examining the properties of subcellular fractions isolated by a systematic fractionation of mucosal cells of the small intestine of the hog. Localization of hetero β-galactosidase in cytosol could be shown.

2. Localization of lactase in the brush borders was shown by analyzing the purified brush borders prepared separately.

3. To demonstrate the lysosomal localization of acid β-galactosidase, lysosomes were purified separately and their extract was chromatographed on a hydroxylapatite column. The activities of various enzymes in the purified lysosomes as well as in the intermediary fractions obtained during lysosome purification and the pattern of the hydroxylapatite chromatography led us to conclude that acid β-galactosidase is a lysosomal enzyme.

Introduction

It has generally been accepted that at least two types of β-galactosidases occur in mammalian small intestines [1]. One is lactase with a neutral optimum pH which is located in the brush borders. The other is hetero β-galactosidase acting only on synthetic β-galactosides such as *o*-nitrophenyl-β-galactoside. Controversial results have, however, been reported for its optimum pH and subcellular localization. Alpers [2] isolated a β-galactosidase with an acidic optimum pH from purified lysosomes prepared from human and rat small intestines and claimed that this could be identified as hetero β-galactosidase. Other investigators, however, have reported that hetero β-galactosidase from the small intes-

Abbreviation: Nph-Gal, *o*-nitrophenyl-β-galactoside.

tines of man, the monkey, and the rabbit, has a neutral optimum pH and is located in cytosol [3—5]. A β-galactosidase with an acidic optimum pH was then discovered by Asp [6] from the human small intestine which differed from hetero β-galactosidase in its ability to act on lactose. This acid β-galactosidase appears to be similar to one of the three types of β-galactosidases isolated by us [7,8] from the small intestine of the hog with respect to optimum pH and to substrate specificity, including a relatively high activity towards 2-naphthyl-β-galactoside. Our enzyme was best characterized by its activity towards galactosyl linkages occurring at the non-reducing termini of glycopeptides and glycoproteins while the other two types of β-galactosidases were lactase and hetero β-galactosidase, respectively.

The intracellular distribution of acid β-galactosidase has been studied with the results reported in this paper. They show that acid β-galactosidase is a lysosomal enzyme whereas lactase is localized in the brush borders and hetero β-galactosidase is located in cytosol.

Materials and Methods

Fractionation of mucosal cells from hog small intestine

Fresh hog small intestines (about 50 cm long from the pylorus) were cut and washed with chilled saline to wash away the intestinal fluid contents. The mucosal layer was collected by scraping the sample with a glass slide. Scrapings were homogenized in a homogenizer of the Potter-Elvehjem type, equipped with a Teflon pestle moderately tightly fitted, with 10 volumes of 0.3 M mannitol adjusted to pH 7.4 with potassium bicarbonate at 600 rev./min through 6 strokes. The homogenate was filtered through a sheet of gauze to remove contaminating mucus and blocks of smooth muscle. The filtrate, referred to as the homogenate, was fractionated by differential centrifugation according to Takesue and Sato [9]. Five subcellular fractions (I—V), were prepared. Of these, Fraction I corresponds to a combination of the Fractions I and II of Takesue and Sato [9]. Fractions I through IV were resuspended in 0.3 M mannitol, and stored in a frozen state before use.

Preparation of purified brush borders

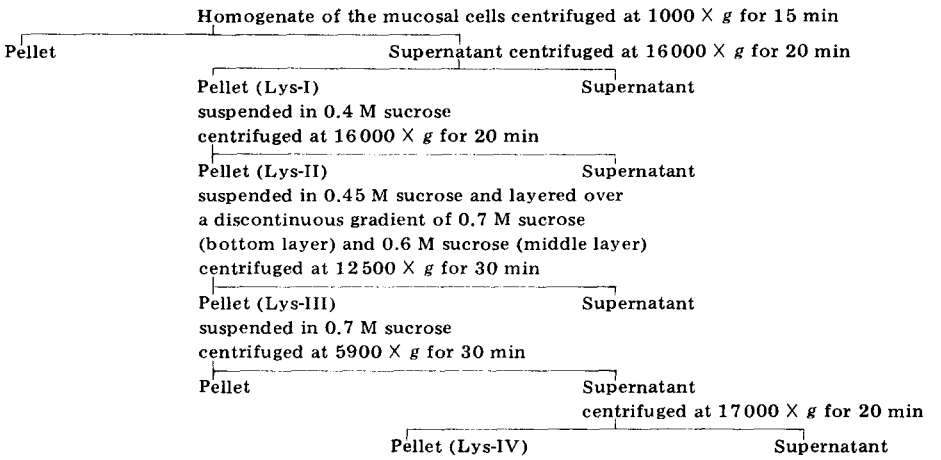
A crude brush border fraction prepared according to Miller and Crane [10] was further purified by the method of Forstner et al. [11].

Preparation of purified lysosomes

The mucosal membranes of hog small intestine were homogenized in a Waring blendor for 30 s with 10 volumes of 0.05 M sodium phosphate buffer, pH 7.4, and the lysosomes were purified from the homogenate according to Sawant et al. [12] with some modifications, as shown in Scheme 1. The lysosomes were washed with 0.3 M mannitol to remove sucrose, each time suspension and centrifugation.

Hydroxylapatite chromatography of β-galactosidases

To identify the β-galactosidases occurring in isolated subcellular fractions, chromatography on a hydroxylapatite column was carried out. The hydroxyla-



Scheme 1. Purification of lysosomes from mucosal cells of hog small intestine.

patite was prepared by the method of Siegelman et al. [13], and the columns were equilibrated with 0.01 M potassium phosphate buffer, pH 6.8.

Enzyme assays

Hetero β-galactosidase and lactase activities were assayed using *o*-nitrophenyl-β-D-galactoside (Nph-Gal) and lactose, respectively, as substrates as described previously [8]. The β-galactosidase activity towards 2-naphthyl-β-D-galactoside was assayed in 0.1 M sodium acetate buffer, pH 5.0, according to Asp [6].

Sucrase [EC 3.2.1.26] was assayed by determining the released glucose with glucose oxidase, as described by Dahlqvist [14]. Acid and alkaline phosphatases [EC 3.1.3.2 and 3.1.3.1] were assayed using glycerol-2-phosphate and *o*-nitrophenyl phosphate, respectively, as substrates. The conditions for the assays were those as described by Hubscher et al. [15]. Glucose-6-phosphatase [EC 3.1.3.9] was assayed according to Hubscher et al. [15]. Aryl esterase [EC 3.1.1.2] was assayed by the method of Nachlas and Seligman [16] using 2-naphthyl acetate as substrate. NADPH-cytochrome *c* reductase and cytochrome oxidase [EC 1.9.3.1] activities were assayed according to Sottocasa et al. [17] and Schnaitman et al. [18], respectively.

Results

Characterization of isolated subcellular fractions

Table I shows the distribution of enzymatic activities and protein in the isolated subcellular fractions (Fractions I—V).

Fraction I should consist of nuclei and brush borders according to the results of Takesue and Sato [9] for mucosal cells of rabbit small intestine. However, the sucrase activity of this fraction was very low; higher activities being found in Fractions III (lysosomal fraction) and IV (microsomal fraction). This suggested that the brush borders of the hog small intestine are less rigid than those of the rabbit small intestines, being readily fragmented to microvilli

TABLE I

INTRACELLULAR DISTRIBUTION OF VARIOUS ENZYMES IN MUCOSAL CELLS FROM HOG SMALL INTESTINE

Enzyme activities are expressed as specific activities (μmol of substrate degraded per min per mg protein) except for the last two enzymes for which values are expressed as μmol of cytochrome *c* reduced or oxidized per min per mg protein. Protein values are expressed as percentages of the protein in homogenate.

	Homogenate	I	II	III	IV	V
Nph-Gal hydrolysing activity	0.094	0.048	0.044	0.074	0.060	0.202
2-Naphthyl-β-galactosidase activity	0.017	0.008	0.015	0.015	0.007	0.029
Lactase	0.021	0.012	0.010	0.061	0.100	0.010
Sucrase	0.030	0.012	0.010	0.112	0.200	0.000
Acid phosphatase	0.021	0.018	0.016	0.032	0.020	0.025
Alkaline phosphatase	0.133	0.120	0.163	0.303	0.342	0.055
Glucose-6-phosphatase	0.043	0.047	0.035	0.073	0.043	0.031
Aryl esterase	6.57	6.60	5.90	18.2	8.10	10.1
NADPH-cytochrome *c* reductase	0.090	0.150	0.100	0.240	0.180	0.040
Cytochrome oxidase	0.285	0.470	1.930	0.890	0.090	0.000
Protein	100	50.0	3.6	6.3	8.8	31.3

through homogenization. Similar results were obtained when milder conditions, i.e. 300 rev./min through 3 strokes, were used to homogenize the mucosal cells.

Fraction II consisted mainly of mitochondria, judging from the highest specific activity of the cytochrome oxidase in this fraction. Lysosomes were concentrated in Fraction III, as seen by the highest specific activity of acid phosphatase. The activities of aryl esterase, glucose-6-phosphatase, NADPH-cytochrome *c* reductase, alkaline phosphatase and cytochrome oxidase were also considerable, indicating that Fraction III contained significant amounts of microsomes, microvilli and mitochondria. Fraction IV should be microsomal, but was heavily contaminated with microvilli and lysosomes.

The incomplete separation of the subcellular fractions from each other could be due to the presence of viscous substances secreted from the mucosal cells.

Distribution of β-galactosidases among the isolated subcellular fractions

Of the β-galactosidase activities, lactase activity essentially paralleled the distribution of sucrase activity among the isolated subcellular fractions, indicating that lactase is located in the microvilli. Nph-Gal hydrolysing activity was predominantly (66% of the total) recovered in cytosol (Fraction V) with about a 2-fold higher specific activity than that in the homogenate. Most of this Nph-Gal hydrolysing activity could be ascribed to hetero β-galactosidase since the ratio of Nph-Gal hydrolysing activity versus lactase activity was about 20; whereas, the ratio should be 0.32 for lactase and 1.25 for acid β-galactosidase, although pure hetero β-galactosidase has no lactase activity. The lactase activity in cytosol corresponded to about 15% of the total in the homogenate and appeared to differ from the bulk of the lactase activity due to lactase located in the microvilli since no sucrase activity was detected in cytosol. The detection of low but significant activities of acid phosphatase in cytosol suggested that some of the lysosomal contents, including β-galactosidase, had leaked out during cell fractionation. If it is assumed that this leaked β-galactosidase is acid

β-galactosidase, then certain 2-naphthyl-β-galactosidase activities in cytosol would be accounted for by the acid β-galactosidase which has been shown to possess the highest activity towards 2-naphthyl-β-galactoside among the intestinal β-galactosidases.

Characterization of purified brush borders

Brush borders were purified to prove the localization of lactase in this fraction. As shown in Table II, the isolated brush borders could be regarded as having high degree of purity. Microsomal and lysosomal contaminations had extensively been removed during the isolation procedure, as judged by the behaviour of the marker enzymes. Sucrase activity was very high in the purified brush borders, the specific activity being comparable to that for brush borders purified from the mucosal cells of rabbit intestine [9]. Lactase activity was concentrated about 7-fold that of the homogenate whereas Nph-Gal hydrolysing activity and 2-naphthyl-β-galactosidase activity were at lower levels than in the homogenate. The ratios of Nph-Gal hydrolysing activity/lactase activity and of 2-naphthyl-β-galactosidase/lactase were 1/3.6 and 1/0.11, respectively. These values are very close to those for purified lactase [7]. Hydroxylapatite chromatography of the β-galactosidases of the brush borders revealed that the major β-galactosidase was lactase.

Characterization of purified lysosomes

To show the lysosomal localization of acid β-galactosidase, lysosomes were purified and examined for β-galactosidase activities.

It was difficult to purify lysosomes from the mucosal cells. As shown in Table III, the most highly purified fraction, Lys-IV, had only a 9-fold higher specific activity for acid phosphatase than did the homogenate, in contrast to the 70-fold increase reported for purified lysosomes from rat liver [12]. The fraction was slightly contaminated with microvilli, as seen by the low but significant sucrase activity, and was contaminated heavily with mitochondria, as judged by the high specific activity of cytochrome oxidase.

The specific activity of Nph-Gal hydrolysing activity dropped markedly in the steps from the homogenate to Lys-IV through Lys-I, consistent with the fact that the enzyme activity in the homogenate can be predominantly accounted

TABLE II

ENZYME ACTIVITIES IN BRUSH BORDERS PURIFIED FROM MUCOSAL CELLS FROM HOG SMALL INTESTINE

Values are expressed as in Table I.

	Homogenate	Purified brush borders
Nph-Gal hydrolysing activity	0.120	0.055
Lactase	0.030	0.200
2-Naphthyl-β-galactosidase	0.060	0.006
Sucrase	0.064	0.430
Alkaline phosphatase	0.012	0.135
Acid phosphatase	0.065	0.028
NADPH-cytochrome *c* reductase	0.097	0.045

TABLE III

ENZYME ACTIVITIES IN DIFFERENT FRACTIONS OBTAINED DURING PURIFICATION OF LYSOSOMES FROM MUCOSAL CELLS OF HOG SMALL INTESTINE

Values are expressed as in Table I.

	Nph-Gal hydrolysing activity	Lactase	Sucrase	Acid phosphatase	Cytochrome oxidase
Homogenate	0.126	0.028	0.024	0.060	0.340
Lys-I	0.039	0.064	0.071	0.290	0.680
Lys-II	0.025	0.020	0.024	0.180	0.480
Lys-III	0.030	0.018	0.018	0.230	1.08
Lys-IV	0.048	0.040	0.038	0.530	2.00

for by the hetero β-galactosidase located in cytosol. The specific Nph-Gal hydrolysing activity again increased from Lys-II onward, and the level was about twice as high in the purified lysosomes as in Lys-II. In contrast, lactase activity was concentrated in Lys-I and paralleled sucrase activity, indicating that the microvilli were concentrated in this fraction. Microvilli were then removed upon purification of lysosomes through Lys-II and -III, and the specific lactase activity decreased concomitantly. The specific activity of lactase was higher in the purified lysosomes (Lys-IV) than that in the previous step (Lys-III). Sucrase activity also increased, but it appeared that more sucrase than lactase had been removed from Lys-I through Lys-IV. The Nph-Gal hydrolysing activity/lactase activity ratio decreased from 4.6 in the homogenate to 1.2 in Lys-IV which is close to the value of 1.25 for the purified acid β-galactosidase [8], indicating a lysosomal localization for acid β-galactosidase. Further proof was obtained using hydroxylapatite chromatography to distinguish the three types of β-galactosidases.

Hydroxylapatite chromatography of the β-galactosidases of isolated subcellular fractions

It has been shown that the β-galactosidases of the hog small intestine can be solubilized nearly quantitatively from the acetone powder of the tissue with a buffer containing Triton X-100 [7]. The same procedure was applied to the isolated lysosomes and brush borders to extract all the β-galactosidases contained in these fractions. The extracts were chromatographed on a column of hydroxylapatite with the results shown in Fig. 1.

Peaks I, II and III for the extract from the whole small intestine (homogenate) corresponded to lactase, hetero β-galactosidase and acid β-galactosidase, respectively, with the characteristic properties for each β-galactosidase, as shown in Table IV. It can be seen that the extract from the brush borders was primarily composed of lactase with a small peak of acid β-galactosidase. Each peak had the same characteristic properties as the respective peak from the whole tissue. Thus, the localization of lactase in the brush borders was fully established.

The extract from the lysosomes showed three peaks of the β-galactosidases. Each peak could then be assigned to each type of βgalactosidase. Acid β-galactosidase (Peak III) was remarkably concentrated in the purified lyso-

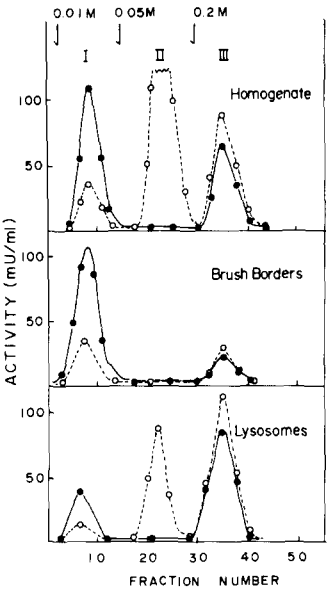


Fig. 1. Chromatographic identification of β-galactosidases using a hydroxylapatite column. Extracts (30 ml) from the acetone powder were applied to a column of hydroxylapatite (1.5 × 12 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 6.8 and eluted with the same buffer of stepwise increasing concentrations, i.e. 0.01 M, 0.05 M and 0.2 M. A flow rate of 15 ml per h was maintained, and 4.5 ml fractions were collected. The extracts contained the following amounts of protein (P), lactase (L) and Nph-Gal hydrolysing activity (O) activities: the homogenate, P, 150 mg, L, 4.0 units, O, 16 units; the brush borders, P, 11 mg, L, 2.2 units, O, 0.6 unit and the lysosomes, P, 57 mg, L, 2.3 units, O, 2.7 units. ●——●, lactase activity; ○——○, Nph-Gal hydrolysing activity.

somes, the ratio of Peak I to Peak III in terms of lactase activity was 3 : 7 whereas it was 6 : 4 in the whole tissue. If it is assumed that Peak I represents lactase derived from the brush borders contaminating the lysosomal fraction, and that mitochondrial localization of acid β-galactosidase is unlikely, then we may conclude that acid β-galactosidase is a lysosomal enzyme. However, a possibility of lysosomal localization of a small amount of hetero β-galactosidase can not be excluded.

TABLE IV

PROPERTIES OF THE THREE TYPES OF β-GALACTOSIDASES OBTAINED FROM THE HOMOGENATE, THE PURIFIED BRUSH BORDERS AND THE PURIFIED LYSOSOMES

Peak	Optimum pH*	Activity ratio Nph-Gal hydrolysing activity/lactase		Cellulase activity	*p*-Chloromercuribenzoate inhibition
I	5.5—6.0	Homogenate	1/2.8	+	—
	5.5—6.0	Brush borders	1/3.6	+	—
	5.5—6.0	Lysosomes	1/3.1	+	—
II**	5.5—6.0	No lactase activity		—	+
III	4.5—5.0	Homogenate	1/0.83	—	+
	4.5—5.0	Brush borders	1/0.81	—	+
	4.5—5.0	Lysosomes	1/0.86	—	+

* Determined as described in Ref. 7 using Nph-Gal as the substrate.
** Peak II from the homogenate and the lysosomes showed the same properties.

Discussion

Intracellular distribution of β-galactosidases in intestinal mucosal cells has been studied for human, monkey and rabbit intestines. Results have shown that lactase is localized in the plasma membranes designated as brush borders due to their unique morphology, and hetero β-galactosidase in the cytosol. No lysosomal localization of any of the β-galactosidases has definitely been demonstrated.

Cell fractionation of the intestinal mucosal cells is tricky due to the presence of mucous substances. The yield of subcellular particulates was low in the present experiments since the particulates plus the mucous substances co-precipitated with the nuclei and cell debris. The brush borders of the hog small intestine seem to be more fragile than those of rabbit intestine [9]. They underwent fragmentation, and were recovered as microvilli upon differential centrifugation together with microsomes. To obtain brush borders of high purity mucosal cells had to undergo a fractionation procedure especially designed for the isolation of brush borders. A separate method was also used to prepare purified lysosomes.

By analyzing the fractions obtained by a systematic cell fractionation, the localization of hetero β-galactosidase in the cytosol was revealed. Localization of lactase in the brush borders was then proved by analyzing the brush borders purified separately. Localization of the acid β-galactosidase, however, was difficult to determine due to the difficulty in isolating pure lysosomes. The assignment of the β-galactosidases in the isolated subcellular fractions to each of the three types of β-galactosidase based on their substrate specificity using different substrates (Nph-Gal, lactose and 2-naphthyl-β-galactoside) was difficult since lactase and acid β-galactosidase have similar specificities — both have lactase and hetero β-galactosidase activities including activity towards 2-naphthyl-β-galactoside. Hydroxylapatite chromatography of the extract of the purified lysosomes was useful in identifying the β-galactosidases contained in the lysosomes.

To quantitatively solubilize β-galactosidases from the lysosomes, it was necessary to prepare the acetone powder, then to extract the enzymes from it with phosphate buffer containing 0.2% Triton X-100. This procedure differs somewhat from that used by Alpers [2] and Asp [6] for the acid β-galactosidase of human small intestine. They reported that human acid β-galactosidase can be solubilized quantitatively by either freeze-thawing or autolysis. Acid β-galactosidase from the hog small intestine seems to bind firmly to the lysosomal membranes.

Hydroxylapatite chromatography revealed that acid β-galactosidase was the major β-galactosidase in the purified lysosomes. In view of the contamination of purified lysosomes with microvilli and unlikelihood of the mitochondrial localization of β-galactosidase, we concluded that acid β-galactosidase is a lysosomal enzyme.

The function of lactase as a digestive enzyme has definitely been established and that of acid β-galactosidase is also obvious as a catabolic enzyme. However, the function of hetero β-galactosidase is unknown since no natural substrate has yet been discovered.

Acknowledgements

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