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RAT-LIVER LYSOSOMAL β-GLUCOSIDASE: A MEMBRANE ENZYME

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SUMMARY

1. Intracellular distribution studies of rat-liver β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21), using *p*-nitrophenyl-β-D-glucopyranoside as substrate, show it to be localized in the lysosomal fraction.

2. The enzyme is well resolved from β-galactosidase (EC 3.2.1.23) and other lysosomal enzymes, due to its retention on the membrane. The action of β-galactosidase with pH optimum of 3.0 is demonstrated upon lactose and both *o*- and *p*-nitrophenyl substrates. β-Glucosidase is also distinguished from β-acetylglucosaminidase (EC 3.2.1.30), another membrane enzyme.

3. β-Glucosidase has maximum activity at pH 5.0 and a K_m (app) of 0.17 mM *p*-nitrophenyl-β-D-glucopyranoside. The activity is linear with protein and linear with time for at least 60 min.

4. Activity upon *p*-nitrophenyl-β-D-xylopyranoside is also found in the lysosomal membrane fraction.

5. The significance of enzyme binding to the membrane is discussed.

INTRODUCTION

Recently, FISHER, WHITEHOUSE AND KENT[1] have suggested that β-glucosidase (β-D-glucoside glycohydrolase, EC 3.2.1.21) is lysosomal and they have done inhibition and pH studies on the enzyme from rat liver. Previously, FURTH AND ROBINSON[2] had found no β-glucosidase activity in rat-liver homogenates. Historically, β-glucosidase has been difficult to resolve from β-galactosidase (EC 3.2.1.23); however, several workers have succeeded in preferential purification of one or both of the enzymes[3–7].

This paper presents evidence that rat-liver β-glucosidase is a lysosomal enzyme which is firmly bound to the membrane. Its properties were studied and compared with β-galactosidase, a soluble lysosomal enzyme, and with β-acetylglucosaminidase (EC 3.2.1.30), another membrane enzyme.

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MATERIALS AND METHODS

Liver fractionation

A modified method of SAWANT *et al.*[8] was used to prepare rat-liver fractions from male Sprague–Dawley rats (400–500 g). This method, based on differential centrifugation, has an average protein yield in the final lysosomal fraction of 0.6 mg protein/g wet liver, and an average increase in specific activity, over that in the homogenate, of 24 for acid phosphatase (EC 3.1.3.2). The average yield of this enzyme was 6.6%. Each of the subcellular fractions was frozen and thawed 10 times to release the soluble enzymes. The lysosomal fraction was centrifuged at 100 000 × *g* for 1 h in a Spinco Model L centrifuge to sediment the membranous material.

Chromatography

CM-cellulose obtained from Bio Rad Laboratories was treated as described by SGARBIERI *et al.*[9], and was equilibrated in 0.01 M sodium acetate buffer (pH 5.25). The CM-cellulose was poured into a 20 mm × 450 mm jacketed column which was maintained at 5°. The buffer and NaCl gradient were pumped at a rate of 22.4 ml/h and 7.7-ml fractions were collected. A dialyzed sample of the soluble lysosomal fraction containing 35 mg of protein was applied to the column.

Enzyme determinations

Protein was measured according to MILLER[10]. Acid phosphatase was measured according to GIANETTO AND DE DUVE[11], the liberated phosphate being determined by the FISKE–SUBBAROW method[12]. Determinations in which liberated nitrophenol was measured were all of the same type: 0.5 ml enzyme, 0.5 ml substrate, and 0.5 ml McIlvaine's citrate–phosphate buffer[13] were incubated at 37° for about 30 min. The reaction was stopped with 1.5 ml of 2.0 M NH_4OH (pH 10.7) and the color was read at 420 mμ. The conditions for each determination were as follows: (enzyme—concentration in the reaction mixture and name of substrate, source of substrate, buffer pH); β-glucosidase—1 mM *p*-nitrophenyl-β-D-glucopyranoside, General Biochemicals, pH 5.0; β-galactosidase—1 mM *o*- or *p*-nitrophenyl-β-D-galactopyranoside, Calbiochem, pH 3.0; β-acetylglucosaminidase—2.4 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, Koch-Light Labs. Ltd., pH 4.2., this reaction mixture was also 0.1 M in NaCl; β-xylosidase (EC 3.2.1.37)—2.5 mM *o*- or *p*-nitrophenyl-β-D-xylopyranoside, Pierce Chemical Co., pH 5.0; α-L-fucosidase (Class EC 3.2.1)—2.5 mM *p*-nitrophenyl-α-L-fucopyranoside, Pierce Chemical Co., pH 6.0. Whenever protein caused the reaction mixture to be turbid, the reaction was stopped with 0.5 ml of 40% (w/v) trichloroacetic acid and then centrifuged; 1 ml of the supernatant portion was added to 1.5 ml of the NH_4OH stopper, and the absorbance was read as described. β-Galactosidase activity was also determined using 3 mM lactose as substrate. Activity was obtained by measuring liberated glucose with glucoseoxidase (Worthington, special glucostat).

RESULTS

Subcellular distribution

Rat liver was fractionated into the major subcellular fractions; the β-glucosidase activities of these fractions are given in Table I. Distributions of activities for

TABLE I

INTRACELLULAR DISTRIBUTION OF RAT-LIVER ACID PHOSPHATASE, β-GALACTOSIDASE AND β-GLUCOSIDASE

Specific activities of acid phosphatase, β-galactosidase and β-glucosidase in homogenate were, respectively, 30, 3.4 and 0.22 mμmoles substrate hydrolyzed/mg protein per min. Determinations were done as described in the text with the exception that the hydrolysis of 1.0 mM *p*-nitrophenyl-β-D-glucopyranoside was at pH 3.0. Data are listed as mean ± S.D. Number of determinations is given in parentheses.

Rat-liver fraction	*Fold increase in specific activity*		
	Acid phosphatase	*β-Galactosidase*	*β-Glucosidase*
Homogenate	1.00	1.00	1.00
Mitochondrial	1.1 ± 0.32 (2)	3.7 ± 2.2 (3)	2.0 ± 1.2 (4)
Microsomal	0.91 ± 0.38 (5)	0.70 ± 0.45 (3)	1.9 ± 0.87 (5)
Supernatant	0.61 ± 0.22 (5)	0.59 ± 0.20 (3)	0.28 ± 0.14 (5)
Lysosomal	24 ± 12 (9)	26 ± 6.4 (4)	26 ± 7.0 (3)
Lysosomal membrane	16 ± 6.3 (4)	4.2 ± 4.8 (3)	114 ± 59 (5)
Lysosomal soluble	33 ± 3.2 (4)	79 ± 30 (3)	27 ± 34 (4)

acid phosphatase and β-galactosidase are given for comparison. The highest increases in specific activity for each enzyme was in the lysosomal fraction.

Characterization of β-glucosidase

Using the lysosomal membrane fraction the pH profile of β-glucosidase activity was determined with different buffer systems; results are shown in Fig. 1. The pH optimum with pyridine–HCl buffer was slightly higher than the pH 5 optimum obtained with citrate–phosphate buffer. The activity with sodium acetate buffer was the same as that with citrate–phosphate buffer.

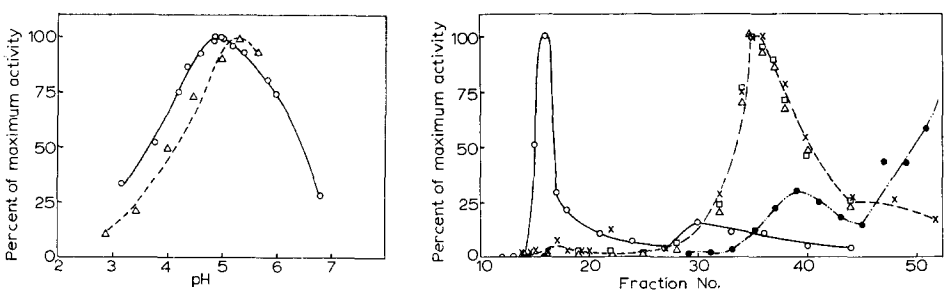

Fig. 1. pH–activity profile for β-glucosidase. Substrate used was 1 mM *p*-nitrophenyl-β-D-glucopyranoside in citrate–phosphate buffer (○—○), and in pyridine–HCl buffer (△— — —△). The lysosomal membrane fraction of rat-liver lysosomes was employed as the enzyme source. Incubations were at 37° for 30 min.

Fig. 2. Chromatography of the soluble portion of rat-liver lysosomes on CM-cellulose at pH 5.25 in 0.01 M sodium acetate buffer. Enzymes were eluted with a linear NaCl gradient, 0 M in Fraction 18 to 0.12 M in Fraction 50. Enzyme activities were as follows: ○—○, β-glucosidase; □, △, ×, β-galactosidase with *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside and lactose, respectively; ●— · · —●, β-acetylglucosaminidase. Maximum activity for β-acetylglucosaminidase was obtained in Fraction 61. Fractions contained 7.7 ml. Maximum specific activities for β-glucosidase and β-galactosidase were about 500 while β-acetylglucosaminidase was greater than 7000 mμmoles/mg protein per min.

The effect of substrate concentration on enzyme activity was studied and the K_m (app.) was found to be 0.17 mM *p*-nitrophenyl-β-D-glucopyranoside. Studies of the effect of enzyme concentration showed that the rate of hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside was linear with enzyme protein increasing up to 0.6 mg/assay. The linearity of activity with increasing protein (lysosomal membrane fraction) argues against endogenous inhibitors of β-glucosidase residing on the membrane. The enzyme from the lysosomal membrane fraction was stable under assay conditions for longer than 60 min.

On the average, less than 15% of the activity in the lysosomal fraction could be solubilized with either water or 0.5 M NaCl. When the soluble lysosomal fraction was chromatographed on CM-cellulose in 0.01 M sodium acetate buffer (pH 5.25) the β-glucosidase (a small porportion of the lysosomal enzyme) had a different distribution from that of β-galactosidase or β-acetylglucosaminidase. The chromatogram is shown in Fig. 2. Although the soluble β-glucosidase could be different from the membrane bound enzyme, the predominance of bound enzyme and the sharpness of the single peak in the chromatogram indicate equivalence. Further, soluble and bound β-glucosidase have the same pH optimum.

Studies of β-galactosidase show that it is not found in the lysosomal membrane fraction (Table I) and that the soluble enzyme hydrolyzes *o*- or *p*-nitrophenyl-β-D-galactopyranoside or lactose, each at pH 3.0. Activity profiles for β-galactosidase were similar with 0.067 M sodium acetate buffer, McIlvaine's citrate–phosphate buffer and 0.017 M pyridine–HCl buffer (see also FURTH AND ROBINSON[2] and SELLINGER *et al.*[14]).

Other glycosidases were studied for localization within lysosomes and for membrane binding. Activity was found in the soluble lysosomal fraction for hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside and *p*-nitrophenyl-α-L-fucopyranoside. Membrane bound activity was observed against both *o*- and *p*-nitrophenyl-β-D-xylopyranoside. More than 3 times as much specific activity was observed for β-glucosidase as for β-xylosidase in the membrane. β-Xylosidase had the same pH optimum, pH 5.0, as β-glucosidase.

DISCUSSION

Since the discovery of lysosomes by DE DUVE *et al.*[15], a large number of glycosidases has been shown to be localized within lysosomes; VAES[16] has listed β-glucuronidase (EC 3.2.1.31), β-galactosidase, β-acetylglucosaminidase, hyaluronidase (EC 3.2.1.35) and lysozyme (EC 3.2.1.17). Other glycosidases have been implicated as being lysosomal or have been shown to be lysosomal. α-Acetylgalactosaminidase (Class EC 3.2.1)[17], α-acetylglucosaminidase (Class EC 3.2.1)[17], β-xylosidase[18] and α-L-fucosidase[19] have been shown to have the same particulate distribution as other mammalian glycosidases. Recently, a lysosomal sialidase (EC 3.1.1.18) has been reported[20].

The data presented here clearly show β-glucosidase to be a lysosomal enzyme and that the enzyme remains firmly bound to the membrane after the particles are ruptured. This finding is quite similar to that of WEISSMANN *et al.*[17] who showed that β-acetylglucosaminidase is completely membrane bound. Non-specific esterase has been associated with the lysosomal membrane; its non-latent characteristic indicates that this enzyme resides on the outside of the membrane[21]. Other enzymes have been

shown to be less firmly associated with the membrane. About 30% of acid phosphatase remains bound to the membrane as shown here and in previous studies[22]. UGAZIO AND PANI[23] studied enzymes retained on the membrane after exposure to several levels of Triton X-100 and concluded that arylsulfatase A was retained to a greater degree than arylsulfatase B, β-glucuronidase, or acid phosphatase. The release of enzymes from the membrane of rat-liver lysosomes was studied by varying sucrose concentration, osmotic pressure, pH, temperature, divalent cations, *etc.*, and it was concluded that electrostatic interaction was a major factor in binding[24]. CONCHIE AND HAY[25] studied 4 mammalian glycosidases and found the degree of particle binding to be an individual property of each enzyme. As all of this evidence is drawn together, more support can be rendered for organization of the lysosomal particle. Examples of membrane binding indicate that at least some of the lysosomal enzymes are membrane bound; perhaps all are bound, but most are easily released. Such a possibility would be consistent with a previously reported[9,26] two-stage enzyme release which was studied by following available activity, the sedimentability of kidney lysosomal fractions and lysosomal morphology by electron micrography. Firstly, the enzymes became available to the substrate but remained sedimentable. Secondly, amounts of soluble enzymes increased until they were almost equal to total available enzymes.

In earlier studies on the specificity of β-glucosidase it was stated that this enzyme should also possess β-xylosidase activity and that this activity should be greater than that for the glucoside[3,27]. Like β-glucosidase, β-xylosidase was observed to have membrane bound activity and to have optimum activity at pH 5; however, the activity against the xyloside was only one-third that for the glucoside. The particulate nature of β-xylosidase is known[18] and this study shows it to be linked with the lysosomal membrane. In agreement with ROBINSON AND ABRAHAMS[28], these results suggest that β-xylosidase and β-glucosidase activities may be of a single enzyme. Chromatographic analyses by ROBINSON AND ABRAHAMS[28] and that performed in this study show that β-glucosidase is not identical with membrane bound β-acetylglucosaminidase.

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