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LYSOSOMAL β-D-MANNOSIDASE OF RAT LIVER

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SUMMARY

1. β-Mannosidase (β-D-mannoside mannohydrolase, EC 3.2.1.25) is localized in the lysosomes of rat liver. The enzyme is a distinct activity from lysosomal α-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24), has an acid pH optimum and is stable to pH 10 incubation at 4 °C. Rat liver contains 0.37 unit of β-mannosidase per g of tissue.

2. Purified lysosomal preparations were able to hydrolyse a trimannoside, β-D-Man-(1→4)-β-D-Man-(1→4)-D-Man, at a rate proportional to the *p*-nitrophenyl-β-mannosidase activity of the preparations.

3. Rat liver β-mannosidase exhibits substrate specificity, hydrolysing trimannoside at a faster rate than dimannoside.

4. Purified lysosomal preparations extensively hydrolyse the carbohydrate portion of glycopeptides obtained from ovalbumin.

INTRODUCTION

Glycosidases have been demonstrated in a wide variety of biological sources such as leukocytes, liver, kidney, oviduct, fungi, plant stems and seeds[1]. These enzymes are generally localized in lysosomes or have characteristics such as an acidic pH optimum suggesting their localization is lysosomal[2]. In man, lysosomal storage diseases have been associated with reduced or absent lysosomal glycosidase activity. The A form of *N*-acetyl-β-D-glucosaminidase (hexosaminidase) is missing in Tay-Sachs patients[3], and this deficiency leads to an accumulation of gangliosides in the brain tissue. It appears that the abnormal lysosome is unable to degrade these glycolipids despite the presence of another enzyme, hexosaminidase B, possessing similar specificity toward artificial aryl glycoside substrates. Despite the need to determine the specificities of complete lysosomal preparations or lysosomal glycosidases for naturally occuring biological substrates, few reports of this nature have appeared.

There have been several recent publications describing β-mannosidases isolated from a variety of sources[4–8]. Such enzymes have been used to characterize the presence of a β-D-mannosyl residue near the carbohydrate to peptide linkage region

in several glycoproteins[5,6]. This paper reports that rat liver lysosomes contain, in addition to a previously described α-D-mannosidase[9,10], an activity specific for β-D-mannoside linkages. This enzyme has the capability of hydrolyzing β-mannoside bonds in naturally occuring biological substrate, as well as artificial aryl β-mannoside substrates. A preliminary report of this work has been presented[4].

MATERIALS AND METHODS

Isolation of lysosomes

175–275-g male albino rats of the Wistar strain were provided by the Pennsylvania State University Small Animal Facility. After 18 h of fasting, animals were decapitated and the livers were removed and immediately perfused with cold 0.25 M sucrose prior to homogenization in the same medium. The differential centrifugation procedure used in the fractionation of liver homogenates was that of de Duve *et al.*[11]. Detergent filled lysosomes were isolated from rats that had been injected with Triton WR-1339 (Ruger Chemical Co.) 3–5 days prior to killing as described by Leighton *et al.*[12].

Enzyme assays

α- and β-D-mannosidase (EC 3.2.1.24 and 3.2.1.25, respectively), 2-acetamido-2-deoxy-β-D-glucosaminidase and acid phosphatase were assayed at pH 4.6 as previously described[13] using the following *p*-nitrophenyl substrate concentrations: α- and β-mannoside, 10 mM; β-*N*-acetylglucosaminide and phosphate, 5 mM. 1 unit of activity is that amount of enzyme which catalyses the hydrolysis of 1 μmole of substrate per min.

Cytochrome *c* oxidase was assayed as previously described[14]. Non-specific esterase was assayed in 1 ml of 0.05 M citrate–phosphate buffer, pH 6.8, containing 1 μmole α-naphthylacetate and 0.1% (w/v) Triton X-100. After a 10-min incubation at 37 °C the reaction was stopped with 1 ml of 95% ethanol. A color reagent containing 2 mg fast red ITR salt (Sigma Chemical Co.) in 1 ml of 1 M sodium acetate buffer, pH 4.5, in 2% Triton X-100 was added, and after 10 min the color was read at 540 nm and compared to a standard of α-naphthol treated under similar conditions. The final color is stable up to 1 h. Because of the high activities present, considerable dilution (1000–10 000-fold for a 100-μl aliquot of the microsomal fraction) of liver fractions is required. The color reagent is stable at 4 °C for approximately 1 week.

Analytical methods

Total neutral sugars were analyzed by the phenol–H_2SO_4 reaction[15] using mannose as a standard. Amino acid and amino sugar composition of ovalbumin glycopeptides was determined on a Beckman amino acid analyzer (these analyses were kindly provided by Dr E. A. Davidson). *N*-Acetyl-D-glucosamine was determined by the Morgan–Elson reaction using a published micro procedure[16] scaled up to 3.3 ml. Reducing sugar was quantitated using the Park–Johnson method[17]. Protein was determined by the method of Miller[18] using bovine serum albumin as a standard.

Gas chromatography of mannose oligosaccharides

As reported by Bhatti *et al.*[19], the trimethylsilyl ethers of di- to pentasaccharides

may be separated at high temperatures by gas–liquid chromatography. A modification of this method was used for the analysis of the products of mannotriose degradation by lysosomal extracts. Chromatography was performed on a Varian Model 2100 gas chromatograph using 2 mm (internal diameter) × 100 cm columns of 3% Dexsil 300 GC on Supelcoport 100–120 mesh (all gas chromatography supplies were obtained from Supelco, Inc., Bellefonte, Pa. 16823). The carrier gas used was dry N_2 at a flow rate of 15–20 ml/min. Peak areas were obtained from a Vidar Model 2600 electronic integrator, penta-*O*-trimethylsilyl ribitol being used as an internal standard. Routinely the temperature was programmed from 120 to 320 °C at 6 °C/min (see Fig. 1). When better resolution of the monosaccharide region of the chromatogram was required due to the presence of other hexoses, the column could be lengthened to 250 cm and the program rate reduced to 1 °C/min until 180 °C was reached, at which point the program was continued to 320 °C at the increased rate of 10 °C/min.

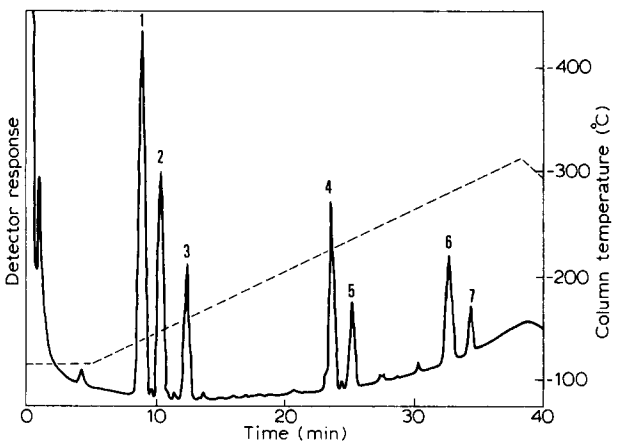

Fig. 1. Gas chromatographic separation of substrate and products of a β-linked mannotriose hydrolysis. 100-μl sample of hydrolysate was treated as described in text. Peaks corresponding to the trimethylsilyl ether derivatives of the various sugars are identified as follows: ribitol (internal standard), 1; free mannose, 2, 3; mannobiose, 4, 5; mannotriose, 6, 7. – – –, Temperature.

Samples of a hydrolysis mixture containing 1 μmole/ml ribitol as an internal standard were dried in 6 mm × 50 mm tubes under a stream of N_2 with gentle heating. Tubes were placed in a vacuum dessicator over P_2O_5 for at least 24 h, 100 μl of Sylon HTP were added, and the samples were suspended with a glass rod to aid dissolution and left covered in a dessicator for a minimum of 3 h before injection. Dessicated, derivitized samples showed no change in component analysis for at least one month. 1–4-μl injections were used.

Undialysed enzyme preparations contained residual sucrose which was hydrolysed to glucose and fructose during the incubation[13]. Of these carbohydrates only fructose overlapped either of the mannose peaks. This interference could not be resolved but could be corrected for by assuming fructose release from the sucrose was equal to glucose release.

RESULTS

Subcellular localization

The fractionation of rat liver homogenates by differential centrifugation yields five fractions enriched in various subcellular organelles, nuclear (N), heavy mitochondrial (M), light mitochondrial (also enriched in lysosomes) (L), microsomal (P), and soluble (S). Table I shows the distribution of β-mannosidase as well as a number

TABLE I

SUBCELLULAR DISTRIBUTION OF RAT LIVER ENZYMES

Relative specific activity, specific activity of the enzyme in the fraction/specific activity of the enzyme in the homogenate. Values in parentheses are the number of observations. Where the number of observations is not indicated the values are averages of 4 or more separate experiments. Purified lysosomal fraction refers to detergent-filled lysosomes isolated by the flotation method of Leighton *et al.*[12].

Enzyme	*Relative specific activity*						
	Nuclear	*Mitochondrial*		*Microsomal*	*Soluble*	*Recovery*	*Purified lysosomal fraction*
		Heavy	*Light*				
	(N)	*(M)*	*(L)*	*(P)*	*(S)*	*(%)*	
Esterase	0.5	0.6	1.4	4.2	0.1	97	0.25 (2)
Cytochrome *c* oxidase	1.6	3.3	1.8	0.1	0.03	81	*
β-*N*-Acetylglucosaminidase	0.6	1.4	10.2	0.4	0.2	100	49 (9)
Acid phosphatase	0.6	1.1	6.9	0.7	0.4	98	38 (3)
α-Mannosidase	0.6	0.8	6.2	0.8	0.2	84	51 (6)
β-Mannosidase	0.7	1.4	8.8	0.4	0.2	96	47 (7)
Protein (% of homogenate)	17.4	18.2	6.4	33.8	20.1	95	0.34

* Not determined.

of subcellular marker enzymes obtained upon the fractionation of rat liver homogenates by such a procedure. The distribution of each marker in the various fractions is characteristic of its subcellular localization. β-Mannosidase exhibits a distribution similar to the lysosomal markers acid phosphatase, *N*-acetyl-β-D-glucosaminidase, and α-mannosidase, having the highest relative specific activity (8.8) in the L fraction.

The further purification of lysosomes from the M + L fraction by the flotation of Triton WR-1339-filled lysosomes through a discontinuous sucrose gradient[12] also results in a further purification of β-mannosidase. The relative specific activity obtained by performing such a fractionation on rat liver homogenates was 47 for β-mannosidase, similar to the relative specific activity of the three lysosomal marker enzymes (Table I, purified lysosomal fraction). In twelve experiments, the average concentration of β-mannosidase in rat liver was 0.37 unit/g (0.25–0.50) with a specific activity of 1.8 units/g protein (1–2.8). The purified lysosomal fraction contained 16% of the total β-mannosidase activity with an average specific activity of 84 units/g

protein. These data conclusively demonstrate that β-mannosidase is a lysosomal enzyme.

Characteristics of rat liver β-mannosidase

Recently, the α- and β-mannosidases of hen oviduct were well characterized by Maley and co-workers[5]. Since this report was the first description of a β-mannosidase from a higher phylogenetic form, we have compared some of the properties of rat liver lysosomal α- and β-mannosidase to their results for the corresponding enzymes from hen oviduct.

Using *p*-nitrophenyl-β-mannopyranoside as a substrate in Tris–acetate buffer, the liver β-mannosidase has maximal activity at pH 4.6 and exhibits better than 50% of this activity between pH 3.5 and 5.5. Above pH 7 the activity is less than 5% of the maximum. Lysosomal β-mannosidase (as well as α-mannosidase) is strongly inhibited by salts of heavy metals, a 90–95% loss of activity being observed at 2 mM concentrations of either $AgNO_3$ or $HgCl_2$. The Ag^+ inhibition is contrary to the characteristics of hen oviduct β-mannosidase[5] which is virtually unaffected at approximately 1 mM Ag^+. Despite reports indicating that a number of α-mannosidases are zinc-requiring enzymes[20], no activation of rat liver α-mannosidase was found by including zinc acetate in the assay. A stabilization of the enzyme during dialysis at pH 5 however resulted from the inclusion of 1 mM zinc acetate in the dialysate. At least 90% recovery was obtained under these conditions compared to a loss of more than 50% of the activity in the absence of zinc. β-Mannosidase was stable to dialysis between pH 4.5 and 7.5 with or without zinc in the medium.

Unlike the hen oviduct enzyme, rat liver β-mannosidase is not greatly inactivated by an incubation for 1 h at 65 °C (Table II). Purified lysosomal preparations, both dialyzed (pH 4.6, 1 mM zinc acetate) and undialyzed, showed a loss of only 30–40% of β-mannosidase activity when subjected to a 1-h 65 °C incubation. Sukeno *et al.*[5] utilized the heat stability and alkali lability (see below) of α-mannosidase and the opposite properties of β-mannosidase in the purification of the respective enzymes from hen oviduct. Lysosomal α-mannosidase did not exhibit the same heat stability of the hen oviduct preparation but some stabilizing effect of increasing zinc acetate concentration was noted with the lysosomal enzyme.

The α- and β-mannosidases of rat liver lysosomes and hen oviduct are more

TABLE II

ALKALI AND HEAT STABILITY OF LYSOSOMAL MANNOSIDASES

Purified lysosomal preparations containing 0.25–0.5 mg protein in a total volume of 0.5 ml were treated for 1 h as indicated. Enzyme activity was measured with *p*-nitrophenyl glycosides

pH	*Temp. (°C)*	*Zn^{2+} concn*	*No. of observations*	*% Original activity*	
				α-Mannosidase	*β-Mannosidase*
10.0	4	—	3	5–10	80–95
11.0	4	—	3	<5	50–75
12.0	4	—	2	0	0
6.5	65	—	1	8	61
6.5	65	0.2 μM	1	19	58
6.5	65	0.2 mM	1	40	59

similar in their sensitivities to alkali treatment. A 1-h incubation at pH 10 and 4 °C effects a 90% inactivation of α-mannosidase (Table II) with little loss of the β-activity. Similar treatment at pH 11 is almost completely effective in destroying the α-activity but also reduces the β-mannosidase 30–40%.

At low *p*-nitrophenylglycoside concentrations typical Michaelis–Menten kinetics are observed for the two lysosomal mannosidases (Fig. 2). For the β-activity,

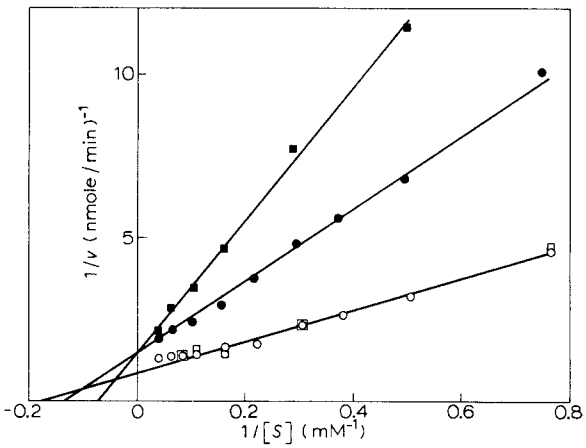

Fig. 2. Effect of methyl α-D-mannopyranoside on lysosomal mannosidases. Assays were performed as described in text with varying *p*-nitrophenyl glycoside substrate concentrations. α-Mannosidase without (●) and with (■) 100 mM methyl α-D-mannoside. β-Mannosidase without (○) and with (□) 100 mM methyl α-D-mannoside.

however, deviations are noted at higher substrate concentrations (>10 mM). Apparent K_m values of 8 and 6 mM, respectively, are obtained for α- and β-mannosidase from Lineweaver–Burk plots. Methyl-α-D-mannopyranoside is hydrolysed slowly by α-mannosidase .This glycoside therefore acts as a competitive inhibitor of the *p*-nitrophenyl α-mannosidase activity but has no effect on the *p*-nitrophenyl β-mannosidase.

Rat liver lysosomes therefore may be considered to have two mannosidase activities, one specific for α-D-linkages, the other for the β-anomer.

Hydrolysis of ovalbumin glycopeptides by isolated rat liver lysosomes

A number of workers have found it difficult to obtain complete hydrolysis of ovalbumin glycopeptides using either lysosomal extracts or purified glycosidases[13,21–24]. The recent finding that ovalbumin[6] and a number of other glycoproteins, *e.g.* pancreatic ribonuclease[5] and Taka-amylase[6], contain one β-mannosidic linkage combined with the observation of a β-mannosidase in rat liver lysosomes has led us to reinvestigate the lysosomal degradation of the carbohydrate portion of ovalbumin.

Ovalbumin glycopeptides were isolated essentially as described by Huang *et al.*[21] by Sephadex G-25 chromatography of pronase-digested ovalbumin. The final product contained aspartic acid, mannose, and *N*-acetylglucosamine in a ratio of 1.0:5.0:3.2. Aspartic acid accounted for better than 50% of the total amino acid content. Another 30% was contributed by threonine, proline, cysteine, and phenylalanine.

Free mannose and *N*-acetylglucosamine gave similar reducing equivalents in the Park–Johnson assay and thus mannose release from the glycopeptide was calculated as the difference in reducing sugar and free *N*-acetylglucosamine as determined by the Morgan–Elson reaction.

The time course of a typical hydrolysis of ovalbumin glycopeptide by lysosomes is shown in Fig. 3. During the first 100 h of the reaction mannose and *N*-acetylglucosamine are released at approximately the same rate. Although free reducing

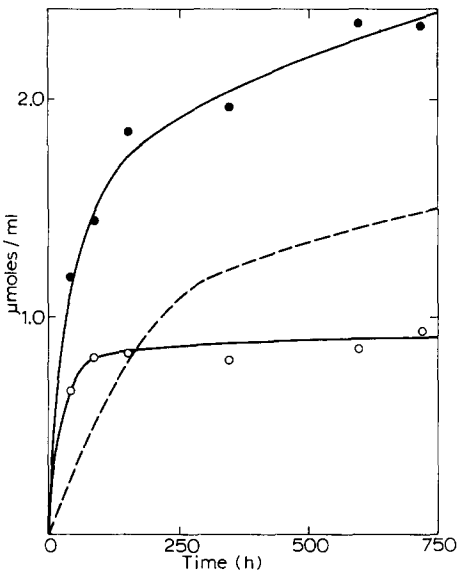

Fig. 3. Hydrolysis of ovalbumin glycopeptides by a purified lysosome preparation. Ovalbumin glycopeptide (5.5 mg) containing 15 μmoles mannose and 9.5 μmoles *N*-acetylglucosamine was incubated in a total volume of 5 ml (0.1 M acetate, pH 5) with purified lysosomes (1.7 mg lysosomal protein) containing 0.25 unit α-mannosidase, 0.20 unit β-mannosidase and 0.90 unit β-*N*-acetylglucosaminidase. Mannose release (– – –) was calculated at various times by the difference between free reducing sugar (●) and free *N*-acetylglucosamine (○). Corrections were made for controls of substrate and enzyme incubated separately under the same conditions. The reaction was layered with toluene to prevent microbial growth.

sugar continues to increase after this, the hydrolysis of amino sugar nearly halts for the remainder of the incubation (725 h). As ovalbumin contains only *N*-acetylglucosamine and mannose the continued increase in reducing sugar levels is due to the release of mannose from the glycopeptide. At 700 h approximately 50% of both mannose and *N*-acetylglucosamine had been released.

At various times, samples of the hydrolysate were assayed for enzyme activity with *p*-nitrophenyl glycosides as substrate (Table III). The observed stability of lysosomal hexosaminidase has been noted previously[13]. The cessation of *N*-acetylglucosamine release is therefore presumably due to the internal location of the remaining amino sugar[21]. The activity of α-mannosidase, the enzyme responsible for the release of the majority of the mannose residues, decreased by 300 h to 10% of its original level, with 5% remaining at 600 h. β-Mannosidase exhibits greater stability throughout the incubation but cannot function until the α-linked mannose moieties

TABLE III

LYSOSOMAL GLYCOSIDASE STABILITY DURING OVALBUMIN GLYCOPEPTIDE HYDROLYSIS

Aliquots were removed from the incubation mixture that is described in the legend to Fig. 3, and glycosidases were assayed with *p*-nitrophenyl glycoside substrate.

Time (h)	*Enzyme activity (munits/ml)*		
	β-N-Acetylglucosaminidase	*α-Mannosidase*	*β-Mannosidase*
0	180	46	44
60	—	24	33
330	83	5	10
625	—	2	7

which are distal to the single β-linked mannose are removed[5]. The rate of *in vitro* digestion of ovalbumin glycopeptide appears to be limited by the level of lysosomal α-mannosidase activity. The latter enzyme is also the least stable of the three glycosidases required for complete digestion.

Hydrolysis of mannotriose by dialysed lysosomes

A trisaccharide of mannose isolated from an acid hydrolysate of ivory nut mannan was kindly donated by Dr G. O. Aspinal. This β-(1→4)-linked mannotriose

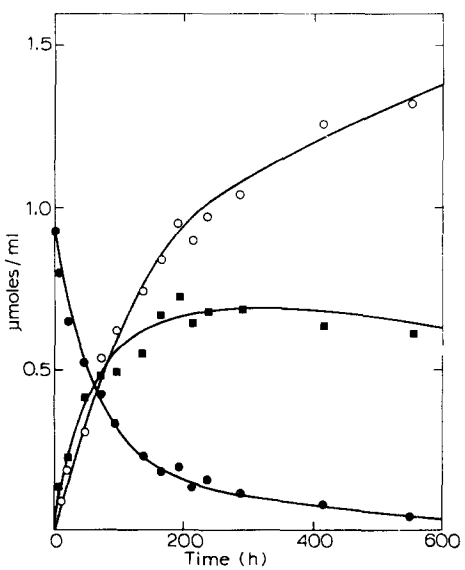

Fig. 4. Hydrolysis of β-linked trisaccharide of mannose by purified lysosomes. 3.7 μmoles of trisaccharide were incubated at 37 °C with 1 mg lysosomal protein (dialysed against 1 mM Zn^{2+}, 10 mM acetate, pH 4.6) containing 0.1 unit β-mannosidase. Final volume of hydrolysis mixture was 4 ml containing 1 μmole/ml ribitol as internal gas chromatography standard and 0.1 M acetate, pH 4.6. Samples (100 μl) were taken at indicated time points and analysed by gas chromatography. Response factors for mannotriose (●) and mannose (○) were determined on authentic samples. The relative response factor for mannobiose (■) was calculated from the relation $\text{mannose}_{(\text{total})} = 3 \times \text{mannotriose} + 2 \times \text{mannobiose} + \text{free mannose}$. Neither dialysed lysosome preparations nor mannotriose samples which were incubated alone showed significant release of di- or monosaccharides.

TABLE IV

SUBSTRATE SPECIFICITY OF β-MANNOSIDASE

During the hydrolysis of mannotriose the ratio of the concentrations of β-linked mannose di- and trisaccharide was compared with the relative rates of their hydrolysis. Molar concentrations were determined from Fig. 4 and the rates of hydrolysis were calculated as the net change in concentration occurring during the 25-h period following the indicated time.

Time (h)	*Substrate ratio* (Man_2/Man_3)	*Hydrolysis ratio* (Man_2/Man_3)
100	1.8	0.3
200	3.7	0.7
300	6.2	0.9
400	8.3	1.7
500	9.3	1.9

was incubated with purified lysosome preparations and the substrate concentration and product release were analyzed at various times by gas chromatography (Fig. 4). The stability of β-mannosidase in these experiments was similar to that shown in Table III for ovalbumin glycopeptide hydrolysis, as 45 and 10% of the initial activity remained at 300 and 1200 h, respectively.

A 50% release of the mannose contained in the original trisaccharide was observed in 600 h. Further time points (not shown) were taken to 1250 h. By 1000 h the level of mannotriose had been reduced below detectable limits (approximately 0.5% of the initial levels), however, free mannose levels had reached only 65% of theoretical, the remaining product being disaccharide. Differences in the specificity of lysosomal β-mannosidase for the di- and trisaccharide may be responsible for the slow hydrolysis of mannobiose. Mono- and disaccharide are produced in a 1:1 stoichiometry during the first 75–100 h. The free mannose may therefore be completely accounted for by hydrolysis of trisaccharide during this period. In Table IV the ratio of mannobiose to mannotriose concentrations and the rates at which each is being hydrolysed at various times are noted .If both oligosaccharides were equally good substrates the ratio of their hydrolysis rates should equal their concentration ratio. From the data in Table IV it appears that mannotriose is the better substrate by a factor of 5–7.

TABLE V

COMPARISON OF THE RATE OF β-LINKED MANNOTRIOSE HYDROLYSIS TO MANNOSIDASE ACTIVITIES IN UNTREATED, ALKALI-TREATED, AND HEAT-TREATED, PURIFIED LYSOSOMAL PREPARATIONS

1-ml aliquots of a dialysed, purified lysosomal preparation (Fig. 4) containing 1 mg of protein were either alkali-treated (pH 11, 4 °C, 1 h) or heat-treated (pH 4.6, 65 °C, 1 h). After treatment the portions were incubated with the respective *p*-nitrophenyl mannoside substrates and with mannotriose under the conditions noted in Fig. 4. Hydrolysis of the latter substrate was analysed by gas chromatography. Data were obtained at 22 h in order to approximate initial hydrolysis rate. Similar results were also obtained at later times.

Substrate	*Substrate hydrolysis (% of untreated enzyme)*	
	Alkali-treated	*Heat-treated*
p-Nitrophenyl α-D-mannopyranoside	10	30
p-Nitrophenyl β-D-mannopyranoside	65	43
Mannotriose	61	45

In order to determine whether the hydrolysis of mannotriose was catalysed by the α- or the β-mannosidase activity of rat liver, hydrolyses of the trisaccharide were performed with alkali-treated and heat-treated purified lysosomal preparations. Enzyme activities and the rate each preparation released mannose were determined at 25 h and the results were compared to those obtained with untreated lysosomal enzyme (Table V). The mannotriose hydrolysis rate paralleled the level of β-mannosidase activity rather than that of the α-mannosidase.

DISCUSSION

The existance of two mannose hydrolysing enzymes of differing specificities in rat liver lysosomes is confirmed by the following criteria: the differential effects of inhibitors, of heat-treatment and of alkali-treatment; the loss of α-activity during dialysis; the stabilizing effect of zinc on α-mannosidase; and by the observation that a β-linked trisaccharide of mannose is hydrolysed at a rate proportional to β-mannosidase activity measured with *p*-nitrophenyl-β-D-mannoside. The finding of a β-mannosidase in rat liver lysosomes further expands our knowledge of the already large complement of glycosidases localized in this organelle.

Although complete hydrolysis of mannotriose and ovalbumin glycopeptide was not achieved under our experimental conditions, the results support the conclusion that mammalian liver has the capacity to hydrolyse to a considerable extent polysaccharides and glycoproteins that may gain access to its lysosomes through endocytosis or autophagy.

Few studies have been conducted to determine the normal capacity of lysosomes to degrade glycolipids and glycoproteins. Biological materials of all classes are degraded in lysosomes but the extent of *in vivo* degradation is poorly understood. Data accumulated through the use of artificial substrates indicate that lysosomes contain a vast array of hydrolytic enzymes directed against virtually every type of glycosidic linkage known to occur in glycoproteins[1]. The use of artificial glycosides has facilitated the detection of a great number of glycosidases but the assumption that these enzymes act with similar specificity on natural substrates is unwarranted.

The complete enzymatic degradation of the carbohydrate chains of glycoproteins is difficult to accomplish. Aronson and de Duve[13] studied the ability of isolated lysosomes to hydrolyze a variety of glycoproteins and oligosaccharides. Using a preparation containing approximately 0.5 unit of α-mannosidase, they were able to release 1 of 3.6 μmoles of mannose from ovalbumin glycopeptides in 22 h. Their results also indicate that both the polypeptide and the carbohydrate portions of orosomucoid, fetuin, and submaxillary mucin were degraded to a considerable extent by lysosomal preparations, from 30 to 80% of the total carbohydrate being released. The authors did not determine whether these values represented maximum release or whether hydrolysis would continue to completion. In a similar study Mahadevan *et al.*[24] were able to show that lysosomes of kidney and liver degrade the glycoprotein fetuin in a stepwise manner removing sialic acid, galactose, and *N*-acetylglucosamine from the non-reducing terminus. The hydrolysis of the more internally located mannose residues was more difficult since the release of mannose was undetectable by paper chromatography after 132 h.

Other workers, using purified enzyme preparations from a variety of sources,

have also found the complete hydrolysis of glycoproteins or glycopeptides requires long incubations and large amounts of enzyme .Crystalline α-mannosidase and *N*-acetyl-β-glucosaminidase, 20–30 units each, were used by Huang *et al.*[21] to release the terminal sugar residues of ovalbumin L-β-aspartamido-oligosaccharides in incubations lasting up to 800 h. Conchie and co-workers[22] utilizing ovalbumin as a substrate for 1500 units of hexosaminidase and 50 units of α-mannosidase incubated the protein for periods of 48 and 116 h, respectively. Other mannose-containing oligosaccharides also seem difficult to hydrolyze. 5 of 6 residues of mannose were removed from 1 μmole of ribonuclease B glycopeptide by 306 units of α-mannosidase in 8 h[25]. Our results indicate that during long incubations, ovalbumin glycopeptides are hydrolysed to a large extent by lysosomal preparations containing low levels of enzyme.

Long *in vitro* incubations such as we have conducted may be physiologically significant in view of the following points: (1) Unlike the situation in our *in vitro* studies the *in vivo* mechanism of primary–secondary lysosome fusion allows for frequent replenishment of lysosomal enzymes. (2) This process occurs over a major portion of the functional half-life of lysosomes, estimated to be 15–30 days[26]. (3) Degradation products would diffuse from the lysosome or be transported through the membrane lowering the product concentration and allowing lysosomal enzymes to operate at maximal efficiency. (4) According to Baudhuin[27] the intact liver contains approximately 4 μl of lysosomes per g of tissue. Based on this figure the *in vivo* concentration of protein and enzyme is respectively 1000 and 3000 times greater than our *in vitro* incubation conditions. Little is known concerning the effects such concentrations may have on enzyme rates.. Rates may be influenced by possible structural organization of enzymes in the lysosomal matrix, by the association of enzymes with the lysosomal membrane, or by reduced water activity within the lysosome. The effect that compartmentalization of enzymes in a membrane-bound organelle such as the lysosome has on enzyme activity and specificity has not been deduced.

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REFERENCES

1 Aronson, Jr, N. N. (1972) in *Glycoproteins* (Gottschalk, A., ed.), Part B, 2nd ed., pp. 1211–1227, Elsevier, Amsterdam
2 Barrett, A. J. (1972) in *Lysosomes: a laboratory handbook* (Dingle, J. T., ed.), pp. 46–135, North-Holland, Amsterdam
3 Wenger, D. A., Okada, S. and O'Brien, J. S. (1972) *Arch. Biochem. Biophys.* 153, 116–129
4 LaBadie, J. H. and Aronson, Jr, N. N. (1972) *Fed. Proc.* 31, 281 Abstr.
5 Sukeno, T., Tarentino, A. L., Plummer, Jr, T. H. and Maley, F. (1972) *Biochemistry* 11, 1493–1501
6 Li, Y.-T. and Lee, Y. C. (1972) *J. Biol. Chem.* 247, 3677–3683
7 Sugahara, K., Okumura, T. and Yamashina, I. (1972) *Biochim. Biophys. Acta* 268, 488–496
8 Reese, E. T. and Shibata, Y. (1965) *Can. J. Microbiol.* 11, 167–183
9 Conchie, J. and Hay, A. J. (1963) *Biochem. J.* 87, 354–361
10 Bowers, W. E. and de Duve, C. (1967) *J. Cell Biol.* 32, 339–348
11 de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) *Biochem. J.* 60, 604–617

12 Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S. and de Duve, C. (1968) *J. Cell Biol.* 37, 482–513
13 Aronson, Jr, N. N. and de Duve, C. (1968) *J. Biol. Chem.* 243, 4564–4573
14 Appelmans, F., Wattiaux, R. and de Duve, C. (1955) *Biochem. J.* 59, 438–449
15 DuBois, M., Gilles, K., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Chem.* 28, 350–356
16 Ghuysen, J.-M., Tipper, D. J. and Strominger, J. L. (1966) in *Methods in Enzymology* (Neufeld, E. F. and Ginsberg, V., eds), Vol. VIII, pp. 685–699, Academic Press, New York
17 Park, J. T. and Johnson, M. J. (1949) *J. Biol. Chem.* 181, 149–155
18 Miller, G. L. (1959) *Anal. Chem.* 31, 964
19 Bhatti, T., Chambers, R. E. and Clamp, J. R. (1970) *Biochim. Biophys. Acta* 222, 339–347
20 Snaith, S. M. and Levvy, G. A. (1968) *Nature* 218, 91–92
21 Huang, C.-C., Mayer, H. E. and Montgomery, R. (1970) *Carbohyd. Res.* 13, 127–137
22 Conchie, J., Hay, A. J., Strachman, I. and Levvy, G. A. (1969) *Biochem. J.* 115, 717–723
23 Okumura, T. and Yamashina, I. (1970) *J. Biochem.* 68, 561–571
24 Mahadevan, S., Dillard, C. J. and Tappel, A. L. (1969) *Arch. Biochem. Biophys.* 129, 525–533
25 Tarentino, A., Plummer, Jr, T. H. and Maley, F. (1970) *J. Biol. Chem.* 245, 4150–4157
26 de Duve, C. (1963) in *Lysosomes, Ciba Foundation Symposium* (de Reuck, A. V. S. and Cameron, M. P., eds), pp. 1–35, Little, Brown and Company, Boston
27 Baudhuin, P. (1968) *L'analyse morphologique quantitative des fractions subcellulaires*, Thesis, Louvain