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IDENTITY OF β-GLUCOSIDASE AND β-XYLOSIDASE ACTIVITIES IN RAT LIVER LYSOSOMES

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SUMMARY

1. Subcellular distribution studies of rat liver β-glucosidase (β-D-glucosidase glucohydrolase, EC 3.2.1.21) and β-xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.37), using *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside as a substrate, respectively, show these enzyme activities to be localized in the lysosomes. The β-glucosidase to β-xylosidase ratio in all subcellular fractions is constant.

2. Both of the activities are more than 80% bound to the lysosomal membrane.

3. Maximum hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside occurs at pH 5.2. The K_m with *p*-nitrophenyl-β-D-glucopyranoside is 1.45 mM and with *p*-nitrophenyl-β-D-xylopyranoside it is 4.05 mM.

4. Glucono-(1→4)-lactone, a specific competitive inhibitor of β-glucosidase, inhibited the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside with a K_i of 1.7 mM. *p*-Chloromercuribenzoate also inhibits both the activities with a K_i of 12.5 μM.

5. The pH stability of the two activities at 37, 50, and 60° is similar.

6. Both the activities are eluted from DEAE-cellulose by 0.122–0.155 M NaCl.

7. The results of these studies strongly support the conclusion that a single enzyme is responsible for both of the activities.

INTRODUCTION

Previous studies[1] indicated that β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) and β-xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.37) activities of rat liver lysosomes may be of a single enzyme. ROBINSON AND ABRAHAMS[2] have shown these two activities to reside in a single enzyme in the supernatant portion of pig kidney. This paper presents properties of β-glucosidase and β-xylosidase activity of rat liver lysosomes which show that a single enzyme is responsible for hydrolysis of both *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

MATERIALS AND METHODS

p-Nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, 4-methylumbelliferyl-β-D-glucopyranoside, and 4-methylumbelliferyl-β-D-xylopyranoside were obtained from Pierce Chemical Co. *p*-Chloromercuribenzoate (PCMB) was obtained from California Foundation for Biochemicals. Glucono-(1→4)-lactone, Triton X-100, β-glycerophosphate and sodium deoxycholate were obtained from Sigma Chemical Co.; Triton WR 1339, from Ruger Chemical Co.; and DEAE-cellulose from Bio-Rad Laboratories. All other chemicals were of reagent grade.

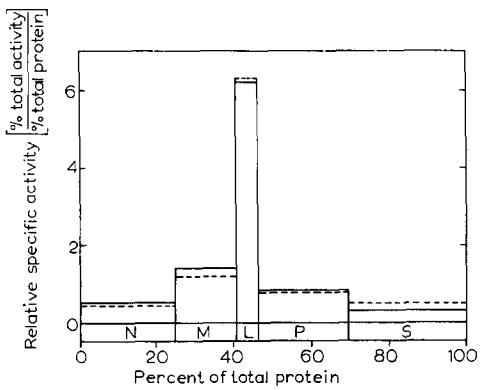


Fig. 1. Subcellular distribution of β-glucosidase (———) and β-xylosidase (- - - -) in rat liver. N, nuclear; M, mitochondrial; L, light mitochondrial; P, microsomal; and S, supernatant fractions. The specific activities of β-glucosidase and β-xylosidase in the light-mitochondrial fraction were 95.8 and 26.0 nmoles of substrate hydrolyzed per mg of protein per h, respectively.

Liver fractionation

Fractionation of rat livers into principal subcellular fractions was done according to the procedure of RAGAB *et al.*[3]. In some experiments (Fig. 1) the livers were homogenized and fractionated into nuclear, cytoplasmic extract, mitochondrial, light mitochondrial, microsomal and supernatant fractions according to the procedure of DE DUVE *et al.*[4]. Triton WR 1339-filled lysosomes were prepared as described by WATTIAUX *et al.*[5].

Separation and solubilization of membrane bound enzyme

Isolated liver lysosomes suspended in 0.25 M sucrose were frozen and thawed 10 times and were then centrifuged at 100 000 × *g* for 60 min to separate the soluble fraction from the membrane fraction. The membrane fraction was suspended in 0.25 M sucrose and homogenized in a Potter–Elvehjem homogenizer.

Since treatment by freezing and thawing solubilized only 17% of β-glucosidase and β-xylosidase activities, it was necessary to solubilize the activities by a different treatment. The membrane fraction suspended in 0.25 M sucrose was treated with 1.0 mg of sodium deoxycholate per mg of membrane protein. The mixture was stirred at 3–4° for 1.5–2 h, unless otherwise stated, and was then centrifuged at 100 000 × *g* for 60 min to separate the soluble fraction from the residue. The soluble portion is

called deoxycholate-solubilized fraction. This treatment also solubilizes other lysosomal membrane bound enzymes, *e.g.* *N*-acetyl-β-D-glucosaminidase, *N*-acetyl-β-D-galactosaminidase and sialidase.

Chromatography of deoxycholate-solubilized fraction of rat liver lysosomes on DEAE-cellulose

DEAE-cellulose was treated as described by PETERSON AND SOBER[6]. The DEAE-cellulose was equilibrated with 5 mM potassium phosphate buffer (pH 7.6) and poured into a 2.0 cm × 30 cm column. The deoxycholate-solubilized fraction was dialyzed against 5 mM potassium phosphate buffer (pH 7.6) for 8 h. A sample of the dialyzed material that contained 25 mg of protein was applied to the column. Fractions were eluted with a linear NaCl gradient and collected in 4.0 ml fractions. Protein in the eluate was estimated by absorbance at 280 nm, using bovine serum albumin as a standard. All operations were done at 4°.

Enzyme determinations

Unless otherwise stated, the reaction mixtures for the assay of β-glucosidase and β-xylosidase contained 4 mM *p*-nitrophenyl-β-D-glucopyranoside or *p*-nitrophenyl-β-D-xylopyranoside, 200–400 μg of lysosomal protein and 60 mM citrate–phosphate buffer (pH 5.2) in a total volume of 1.0 ml. The reaction mixture for measurement of *N*-acetyl-β-D-glucosaminidase contained 2.5 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, 10–15 μg lysosomal protein and 50 mM citrate–phosphate buffer (pH 4.2). Incubations were done at 37° for 30–60 min. The reaction was stopped by addition of 1.5 ml of 2.0 M NH_4OH–HCl buffer (pH 10.7). In assays which contained homogenate, the reaction was stopped by addition of 0.5 ml of 25% trichloroacetic acid. After cooling and centrifuging 1.0 ml of the supernatant portion was used for color development with 1.5 ml of 2.0 M NH_4OH–HCl buffer (pH 10.7). The absorbance of the liberated *p*-nitrophenol was measured at 420 nm. Appropriate substrate, enzyme and reagent blanks were included. Acid phosphatase activity was determined according to GIANETTO AND DE DUVE[7]. The protein was determined according to the method of MILLER[8].

RESULTS

Subcellular distribution of β-glucosidase and β-xylosidase

Two methods were used to study the intracellular distribution of these enzymes. Fig. 1 summarizes the result of fractionation according to the procedure of DE DUVE *et al.*[4]. More than 70% of β-glucosidase and β-xylosidase activities in the cytoplasmic extract was sedimentable with the mitochondrial, light-mitochondrial and microsomal fractions. The highest relative specific activities of β-glucosidase, β-xylosidase and acid phosphatase were found in the light-mitochrondrial fraction. The pattern of distribution for β-glucosidase and β-xylosidase is similar to other lysosomal enzymes studied by DE DUVE *et al.*[4]. The similarity of the distributions of these two activities is apparent. The recoveries of the total activities of acid phosphatase, β-glucosidase and β-xylosidase ranged from 93 to 105%.

Table I presents the specific activities of β-glucosidase and β-xylosidase in purified liver fractions and in Triton-filled liver lysosomes; acid phosphatase is included

TABLE I

β-GLUCOSIDASE AND β-XYLOSIDASE ACTIVITY IN PURIFIED RAT LIVER SUBCELLULAR FRACTIONS

Specific activity is expressed as nmoles of substrate hydrolyzed per mg of protein per h. Determinations of enzyme activities were done as described in MATERIALS AND METHODS.

Liver fraction	*Specific activity*			*Ratio**
	Acid phosphatase	*β-Glucosidase*	*β-Xylosidase*	*β-Glucosidase / β-Xylosidase*
Homogenate	1 290	15.5	4.2	3.7
Mitochondrial	2 320	33.4	9.6	3.5
Microsomal	1 100	30.0	7.9	3.9
Supernatant	983	4.5	1.4	3.2
Lysosomal	33 280	380	99.5	3.8
Lysosomal membrane	17 420	1150	310	3.7
Lysosomal soluble	51 600	105	27.8	3.8
Deoxycholate-solubilized membrane	33 200	1530	424	3.6
Triton homogenate	1 020	12.3	3.3	3.7
Triton lysosomes	35 500	413	103	4.0

* The values for the ratios can be multiplied by a factor of 0.68 to correct for the v_{max} and K_m for the two activities.

for comparison. The increases (24-fold) in the specific activity for both of the activities over the homogenate was almost identical. Table I also shows that the β-glucosidase to β-xylosidase ratio in the fractions remained almost constant from homogenate to deoxycholate-solubilized fraction. The constancy of the ratio in the fractions emphasizes the identity of these two activities.

Distribution of β-glucosidase and β-xylosidase between soluble and membrane fractions of liver lysosomes

Usually, repeated freezing and thawing procedures make lysosomal enzymes available to their substrates, but not all of the enzymes are released from the particulate

TABLE II

CONCURRENCE OF β-GLUCOSIDASE AND β-XYLOSIDASE ACTIVITY IN MEMBRANE AND SOLUBILIZATION BY DEOXYCHOLATE

Soluble fraction refers to the nonsedimentable portion of lysosomes obtained after freezing and thawing 10 times and centrifuging at 100 000 × *g* for 1.0 h. Membrane fraction refers to the sedimentable portion. Deoxycholate treatment is described in MATERIALS AND METHODS.

Fraction	*Percent of total activity*	
	β-Glucosidase	*β-Xylosidase*
Soluble	17	17
Membrane	83	83
Solubilized by deoxycholate treatment		
0.5 h	54	55
1.0 h	75	75
2.0 h	83	83

material into soluble form to the same extent. This has been demonstrated with liver lysosomes[3] and kidney lysosomes[9,10]. The results in Table II show that the freezing and thawing of rat liver lysosomes 10–15 times solubilized only 17% of the total β-glucosidase and β-xylosidase activities. Table II also shows the extent of solubilization by deoxycholate treatment. β-Glucosidase and β-xylosidase are solubilized to the same extent, having 83% of the activities solubilized by 2 h of treatment.

Effect of pH

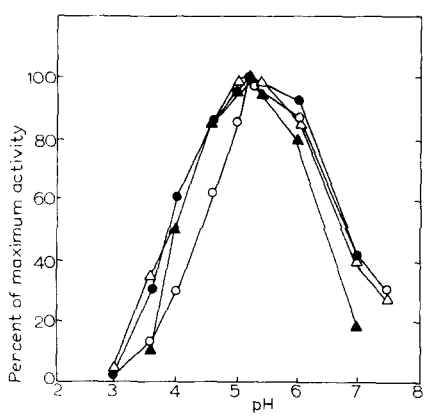
Maximum hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside or *p*-nitrophenyl-β-D-xylopyranoside by rat liver homogenate, lysosomes, lysosomal soluble fraction, lysosomal membrane fraction and deoxycholate-solubilized fraction occurred at pH 5.2. The pH–activity curves for β-glucosidase and β-xylosidase in homogenate and deoxycholate-solubilized fraction are shown in Fig. 2.

Effect of time and protein concentration

β-Glucosidase and β-xylosidase activities increased linearly with time up to 60 min of incubation when assayed in a total volume of 1.0 ml with 300 μg of lysosomal membrane protein and both of the activities were proportional to the amount of enzyme added up to 400 μg lysosomal membrane protein.

Effect of substrate concentration

From LINEWEAVER–BURK[11] plots for β-glucosidase and β-xylosidase activity, the K_m values were determined. The K_m was 1.45 mM with *p*-nitrophenyl-β-D-glucopyranoside as substrate while it was 4.05 mM with *p*-nitrophenyl-β-D-xylopyranoside



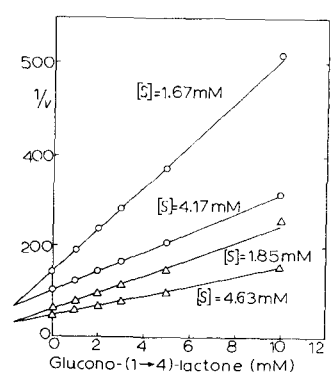


Fig. 2. β-Glucosidase and β-xylosidase activity as a function of pH. Substrate used was 4 mM *p*-nitrophenyl-β-D-glucopyranoside or *p*-nitrophenyl-β-D-xylopyranoside in 60 mM citrate–phosphate buffer. β-Glucosidase activity in homogenate (○—○) and in deoxycholate-solubilized fraction (●—●); β-xylosidase activity in homogenate (△—△) and in deoxycholate-solubilized fraction (▲—▲).

Fig. 3. Effect of glucono-(1 → 4)-lactone on β-glucosidase (○—○) and β-xylosidase (△—△) activity. The reaction mixture contained 260 μg of protein from the dialyzed deoxycholate-solubilized fraction, 60 mM citrate–phosphate buffer (pH 5.2) and the indicated amounts of substrate and glucono-(1 → 4)-lactone in a total volume of 1.0 ml. Incubations were conducted for 30 min for β-glucosidase and 60 min for β-xylosidase. $1/v$ is multiplied by 10^5 for β-glucosidase and 10^4 for β-xylosidase activity, respectively.

as substrate. The difference between the K_m values of these two activities reflects the affinities of this enzyme for these substrates.

Effect of glucono-(1→4)-lactone

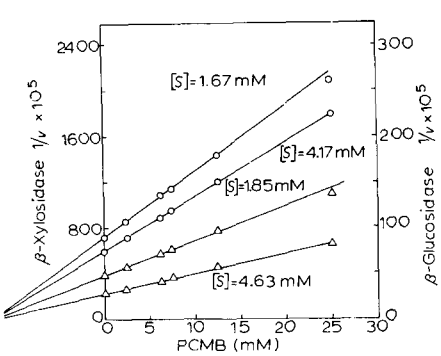
Glucono-(1→4)-lactone has been shown by CONCHIE AND LEVVY[12] to be a specific competitive inhibitor of β-glucosidase activity. The effect of glucone-(1→4)-lactone on β-glucosidase and β-xylosidase activity is shown in Fig. 3. The data are plotted by a DIXON[13] plot. The K_i, 1.7 mM, is the same for both the activities. The K_i is similar to the K_m for β-glucosidase, which indicates equal affinity for glucono-(1→4)-lactone and *p*-nitrophenyl-β-D-glucopyranoside. Since the K_i with *p*-nitrophenyl-β-D-xylopyranoside or *p*-nitrophenyl-β-D-glucopyranoside as substrate is the same, this indicates the probability of a single catalytic site for hydrolysis of the substrates.

Inhibition by PCMB

β-Glucosidase from *Saccharomyces cerevisiae* has been shown to be inhibited by PCMB[14]. The inhibition of β-glucosidase and β-xylosidase activity by PCMB is shown in Fig. 4. Both β-glucosidase and β-xylosidase activities are strongly and noncompetitively inhibited. The K_i for both the activities is the same, being 12.5 μM PCMB. The strong inhibition of both the activities by PCMB indicates the involvement of an -SH group at the active site. The fact that the K_i is the same for both of the activities strongly indicates that a single catalytic site is involved in the hydrolysis of both *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside.

Inhibition of β-glucosidase by 4-methylumbelliferyl-β-D-xylopyranoside and β-xylosidase by 4-methylumbelliferyl-β-D-glucopyranoside

The inhibitions of β-glucosidase by 4-methylumbelliferyl-β-D-xylopyranoside,



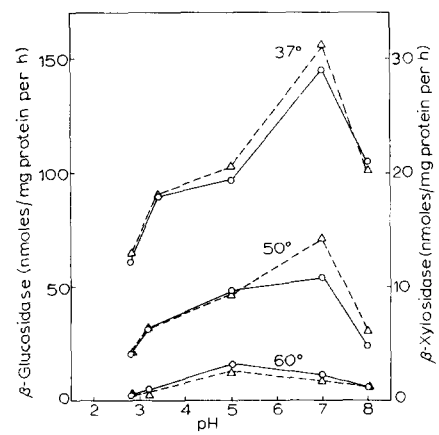


Fig. 4. Effect of PCMB on β-glucosidase (○—○) and β-xylosidase (△—△) activity. The reaction conditions are as described in Fig. 3.

Fig. 5. pH and heat stability of β-glucosidase (○—○) and β-xylosidase (△- - -△). The deoxycholate-solubilized fraction was brought to different pH levels in 1 mM citrate–phosphate buffer. Enzyme assays were done with 80 mM citrate–phosphate buffer, 4 mM substrate and 260 μg of the deoxycholate-solubilized protein. The temperature treatment is described in RESULTS.

a substrate for β-xylosidase, and of β-xylosidase by 4-methylumbelliferyl-β-D-glucopyranoside, a substrate for β-glucosidase, were found to be competitive. The data plotted by a DIXON[13] plot gave a K_i of 6.4 mM for β-glucosidase and 2.3 mM for β-xylosidase. The competitive inhibition of both the activities by these substrates very strongly suggests that the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside occurs at the same catalytic site.

pH and heat stability

Aliquots of the deoxycholate-solubilized fraction were brought to different levels of pH in 1 mM citrate–phosphate buffer, with final concentration of 1 mg of protein per ml. The mixtures were brought to 37, 50 and 60° and held for 10 min. The fractions were then cooled in a dry ice–acetone mixture (approx. −60°). The enzyme assays were done as described in MATERIALS AND METHODS except that the final buffer concentration was 80 mM. Incubations for measuring activity after the 50 and 60° treatment were 1.5–3 h for β-glucosidase and 3–6 h for β-xylosidase. The effects of pH and temperature are shown in Fig. 5. At 37 and 50° both β-glucosidase and β-xylosidase activities were most stable at pH 7.0. However, at 60° they were most stable at pH 5.0. The pH–stability curves at 37, 50 and 60° are similar for both the activities.

DEAE-cellulose chromatography

The deoxycholate-solubilized fraction was used for the fractionation. The experimental conditions are described in MATERIALS AND METHODS. The results in Fig. 6 show that both the activities are eluted between 0.122 and 0.155 M NaCl. Fig. 6 also shows the elution patterns for β-*N*-acetylglucosaminidase and acid phosphatase, which are separated from β-glucosidase and β-xylosidase activities. The similarity in elution pattern and constancy of β-glucosidase to β-xylosidase ratio throughout the column fractions emphasize their identity.

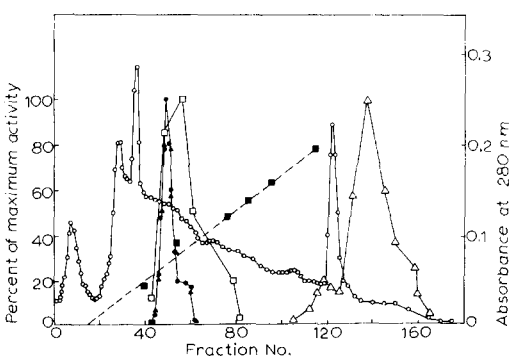


Fig. 6. Chromatography of deoxycholate-solubilized fraction of lysosomal membrane on DEAE-cellulose at pH 7.6 in 5 mM potassium phosphate buffer. Protein (○—○) was eluted with a linear gradient (■- - - -■) of 0–0.4 M NaCl at Fraction 120 and then by 0.8 M NaCl from Fractions 120–180. Enzyme activities were as follows: β-glucosidase, ●—●; β-xylosidase, ▲—▲; β-*N*-acetylglucosaminidase, □—□; and acid phosphatase, △—△. Maximum specific activity for β-glucosidase and β-xylosidase was obtained in Fraction 59. Fraction 59 gave 253-fold purification of β-glucosidase and 257-fold for β-xylosidase over homogenate. The recovery of each activity applied to the column was 15%.

DISCUSSION

Lysosomes are known to be involved in the degradation of proteins, glycoproteins and other complex substances[15–18] and are known to contain a large number of glycosidases[19]. In addition to β-glucosidase and β-xylosidase there are: β-glucuronidase (EC 3.2.1.31), β-galactosidase (EC 3.2.1.23), α-glucosidase (EC 3.2.1.20), α-mannosidase (EC 3.2.1.24), β-*N*-acetylglucosaminidase, β-*N*-acetylgalactosaminidase, lysozyme, and hyaluronidase. β-Glucose occurs in gangliosides and β-xylose occurs in proteoglycans and glycoproteins. β-Glucosidase and β-xylosidase appear to be involved in the detachment of these sugar molecules. β-Xylose has been shown to form a linkage between carbohydrate and protein moieties of proteoglycans[20–22] and glycoproteins[23]. Although the possibility that β-xylosidase activity is involved in cleaving the xylosyl–serine linkage is attractive and in fact has been suggested by ROBINSON AND ABRAHAMS[2], we have found that the lysosomal preparations from rat liver and kidney fail to hydrolyze xylosyl–serine. ARONSON AND DE DUVE[16] also found no hydrolysis of xylosyl–serine by rat liver lysosomes. These latter findings may account, in part, for excretion of significant amounts of xylosyl–serine in human urine, which may arise from degradation of proteoglycans or glycoproteins as has been demonstrated by TOMINGA *et al.*[24]. Even though hydrolysis of the dipeptide xylosyl–serine by a lysosomal enzyme has not been found, it is not known whether or not the bond is hydrolyzed if it is a part of a longer peptide. Structural similarity between β-D-glucose and β-D-xylose makes it possible for a single enzyme to exhibit activities towards *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside. The enzyme apparently does not differentiate between the $-CH_2OH$ group attached to the C-5 in the pyranose form of β-D-glucose and the –H atom on C-5 of β-D-xylose. This possibility has been suggested by BAUMANN AND PIGMAN[25] and GOTTSCHALK[26]. Competitive inhibition of both the activities by glucono-(1→4)-lactone, and analogous substrates supports this suggestion.

Other work also emphasizes the identity of β-glucosidase and β-xylosidase. We found that all subcellular fractions from dog and rabbit liver show the same ratio of β-glucosidase to β-xylosidase activity. Hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside by lysosomes from the livers of these animals occurred maximally at pH 5.2. β-Glucosidase and β-xylosidase have been shown to be identical in human liver also[27].

Experimental evidence presented in this paper strongly suggests that a single enzyme is responsible for β-glucosidase and β-xylosidase activities. It is tempting to postulate that β-xylosidase activity is always thusly associated with β-glucosidase activity in mammalian tissue. These two activities are separate in plants[28,29], which would be expected since compounds which contain β-xylose are abundant in plants. It is noteworthy that we have observed hydrolysis of xylosyl–serine by homogenates of the snail *(Helix pomatia)*. This is not surprising since snails feed on plants. FISHER *et al.*[30] reported hydrolysis of xylosyl–serine by β-xylosidase obtained from commercial almond emulsion. A preliminary report by MANNERS AND MITCHELL[31] indicates that almond β-xylosidase and β-glucosidase activities may be due to a single enzyme.

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