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β-GLUCOSIDASE AND β-XYLOSIDASE OF RAT KIDNEY

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SUMMARY

1. Subcellular distribution studies of rat kidney β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21), β-xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.37) and β-galactosidase (β-D-galactoside galactohydrolase EC 3.2.1.23) using *p*-nitrophenol derivative substrates show β-glucosidase and β-galactosidase to be localized in lysosomes and the supernatant fraction. β-Xylosidase activity is mainly localized in lysosomes, however, the supernatant fraction has significant activity.

2. Lysosomal β-glucosidase and β-xylosidase are tightly bound to the membrane, whereas β-galactosidase is easily solubilized by a freeze–thaw treatment.

3. Lysosomal β-glucosidase and β-xylosidase have optimum activity at pH 5.0. The supernatant β-glucosidase and β-xylosidase have optimum activity at pH 6.4 and 5.2, respectively. The supernatant β-galactosidase has two pH optima, 2.2–3.4 and 3.6–4.8.

4. Glucono(1→4)lactone inhibits the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside; β-galactosidase activity is inhibited at pH 6.6 but not at pH 3.4. *p*-Chloromercuribenzoate inhibits all three activities.

5. The pH stabilities of lysosomal β-glucosidase and β-xylosidase activities at 37° are similar.

6. Lysosomal β-glucosidase and β-xylosidase have the same elution pattern from a DEAE-cellulose column, as does the supernatant β-glucosidase, β-xylosidase and β-galactosidase (pH 6.6).

7. The results of these studies strongly support the conclusion that a single enzyme is responsible for both β-glucosidase and β-xylosidase activity in lysosomes. β-Glucosidase and β-xylosidase, and probably β-galactosidase (pH 6.6), activities in the supernatant fraction are exhibited by a single enzyme.

INTRODUCTION

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) and β-xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.37) activities have been shown to be of a single

Abbreviation: PCMB, *p*-chloromercuribenzoate.

enzyme in rat liver lysosomes[1] and also in the supernatant portion of pig kidney[2]. PRICE AND DANCE[3] indicated that there is present in rat kidney supernatant fraction a single enzyme which has both β-glucosidase and β-glalactosidase activities. This paper describes properties of rat kidney lysosomal and supernatant β-glucosidase and β-xylosidase which show that in each fraction a single enzyme is responsible for hydrolysis of both *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside. Supernatant β-glucosidase also appears to exhibit β-galactosidase activity.

MATERIALS

p-Nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, 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, galactono(1→4)lactone, β-glycerophosphate, and sodium deoxycholate were obtained from Sigma Chemical Co.; DEAE-cellulose from Bio-Rad Laboratories; and fetuin from Grand Island Biological Co. Sugars were obtained from Nutritional Biochemical Co., and all other chemicals were of reagent grade.

METHODS

Male Sprague–Dawley rats that had been starved for 24 h were killed by decapitation. Their kidneys were removed immediately, washed with cold 0.45 M sucrose, minced, and homogenized in 0.45 sucrose with a Potter–Elvehjem homogenizer by two passes of the teflon pestle rotating at 1100 rev./min. The homogenate was centrifuged at $650 \times g$ for 3 min. After careful removal and collection of the supernatant portion, the pellet was rehomogenized and centrifuged as above. The resulting supernatant fraction was combined with the first supernatant fraction, and this combined fraction was called cytoplasmic extract; the final volume to weight ratio was 9:1. The resulting pellet was called the nuclear fraction. The cytoplasmic extract was then centrifuged at $41\,000 \times g$ for 10 min. The pellet consisted of three layers. The upper, pink layer was removed by swirling with 0.45 M sucrose and was added to the supernatant portion. The two lower layers were called the mitochondrial-lysosomal fraction. The supernatant portion was centrifuged at $100\,000 \times g$ for 90 min, yielding the microsomal pellet and the supernatant fraction.

Purified subcellular fractions were prepared from rat, beef, dog and rabbit kidneys by the method of SHIBKO AND TAPPEL[4]. Methods for solubilization of membrane bound enzymes by deoxycholate and the DEAE-cellulose column chromatography have been described previously[1].

Enzyme determinations

β-Glucosidases, β-xylosidase and acid phosphatase activities were measured as described previously[1]. Unless otherwise stated, the reaction mixture for the assay of β-galactosidase contained 4 mM *p*-nitrophenyl-β-D-galactopyranoside, 100–200 μg lysosomal or supernatant protein, and 60 mM citrate–phosphate buffer (pH 3.4) in a total volume of 1.0 ml. The incubations were for 30–60 min. The remainder of the

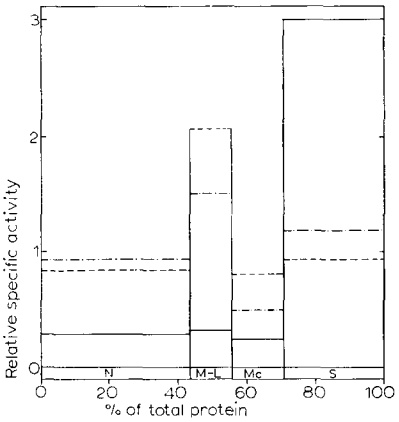

Fig. 1. Subcellular distribution of β-glucosidase (———), β-xylosidase (— — —), and β-galactosidase (— · —) in rat kidney. N, nuclear; M–L, mitochondrial–lysosomal; MC, microsomal; S, supernatant fractions. Relative specific activity = (% total activity)/(% total protein).

procedure was the same as described previously[1]. Sialidase activity was measured as described by MAHADEVAN *et al.*[5]. Protein was estimated by the method of MILLER[6].

Hydrolysis of lactose

The reaction mixture consisted of 3.5 mg of lactose and suitable diluted tissue fraction in a total volume of 1.0 ml. The final citrate–phosphate buffer concentration was 100 mM (pH 3.4 or 6.4). Incubations were done at 37° for 5–12 h. The reaction was stopped by addition of 1 ml 2% $ZnSO_4$ and 1 ml of 0.5% NaOH. The pH was adjusted to 7, if necessary. Following centrifugation, glucose in the supernatant portion was assayed by the glucose oxidase procedure with Worthington glucostat reagent.

RESULTS

Subcellular distribution of β-glucosidase and β-xylosidase in rat kidney

Fig. 1 summarizes the results of a kidney fractionation according to the procedure described in METHODS. 20% of β-glucosidase activity and 73% of β-xylosidase activity in the cytoplasmic extract were sedimentable with the mitochondrial-lysosomal and the microsomal fractions. More than 80% of the acid phosphatase, a lysosomal marker enzyme, was sedimentable with these fractions. The highest relative specific activity of β-glucosidase was in the supernatant fraction; those of β-xylosidase and acid phosphatase were found in the mitochondrial-lysosomal fraction. The subcellular localization of β-galactosidase is shown to be in the mitochondrial-lysosomal and supernatant fractions.

In purified rat kidney fractions prepared according to the procedure of SHIBKO AND TAPPEL[4], β-glucosidase in the lysosomal fraction did not show an increase in specific activity over homogenate when measured at pH 5.0 (Table I). However, when the activity was measured at pH 3.6, where the lysosomal fraction has about 60% of its maximum activity and the homogenate has only 8–10% of its maximum activity (Fig. 2), the increase in specific activity over the homogenate is 6-fold. This indicates

TABLE I

β-GLUCOSIDASE AND β-XYLOSIDASE IN PURIFIED SUBCELLULAR FRACTIONS OF RAT KIDNEY

Specific activity is expressed as nmoles of substrate hydrolyzed per mg of protein per h. Determinations of enzyme activities were done at pH 5.0 as described in METHODS.

Fraction	*Specific activity*		
	Acid phosphatase	*β-Glucosidase*	*β-Xylosidase*
Homogenate	1 910	473 (34)*	11.5
Mitochondrial	740	94	3.5
Microsomal	1 100	150	3.4
Supernatant	480	1442	14.8
Lysosomal	25 000	360 (198)*	134
Lysosomal membrane	6 200	655	260
Lysosomal soluble	45 000	63	23.6

* Activity measured at pH 3.4.

that the lysosomal β-glucosidase activity in the homogenate is obscured by the higher activity of the supernatant β-glucosidase. The 14-fold increase in specific activity of β-xylosidase is similar to the increase in acid phosphatase activity, which indicates that β-xylosidase is mainly lysosomal. Although not shown in the table, β-galactosidase also has a bimodal distribution in lysosomes and the supernatant fraction.

Distribution of β-glucosidase, β-xylosidase and β-galactosidase between soluble and membrane fractions of kidney lysosomes

It has been shown with liver[7] and kidney[4,8] lysosomes that repeated freezing and thawing usually makes their enzymes available to their substrates, but not all the enzymes are released into soluble form to the same extent. Freezing and thawing of kidney lysosomes, 10–15 times, solubilizes only 15% of the total β-glucosidase and β-xylosidase, whereas 88% of β-galactosidase activity is solubilized. Treatment with sodium deoxycholate for 5 h at 4° solubilizes all of the membrane-bound β-glucosidase and β-xylosidase activities; it also solubilizes other membrane-bound enzymes. The bound nature of lysosomal β-glucosidase and β-xylosidase, and their solubilization to

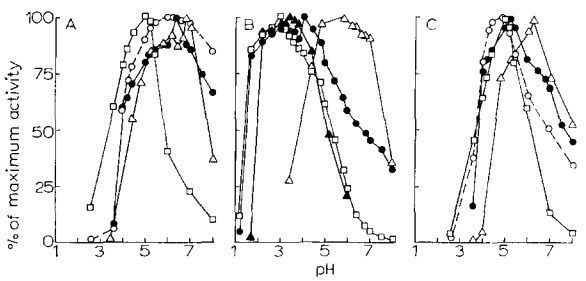

Fig. 2. β-Glucosidase (A), β-galactosidase (B), and β-xylosidase (C) activity as a function of pH. Substrates used were 4 mM *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, and *p*-nitrophenyl-β-D-xylopyranoside in 60 mM citrate–phosphate buffer. Below pH 2.6 KCl–HCl buffer was used. □, lysosomal fraction; ●, supernatant fraction; △, Peak VIII; ▲, Peak I; ○, homogenate. Peaks I and VIII are pooled fractions (Fig. 5) of the supernatant fraction on DEAE-cellulose column.

the same extent by deoxycholate treatment indicate their similarity. The ease with which lysosomal β-galactosidase is solubilized by the freeze–thaw treatment indicates that this enzyme is not identical with lysosomal β-glucosidase.

Effect of pH

As shown in Fig. 2, maximum hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside or *p*-nitrophenyl-β-D-xylopyranoside by kidney lysosomes occurs at pH 5.0. The homogenate has an optimum pH of 6.2 for β-glucosidase and pH 5.0 for β-xylosidase, whereas the supernatant fraction has a pH optimum of 6.4 for β-glucosidase and 5.2 for β-xylosidase activity. These data indicate that the homogenate is dominated by lysosomal β-xylosidase and by supernatant β-glucosidase. The different pH optima of lysosomal and supernatant β-glucosidase indicate that these activities belong to different enzymes. The pH optima of supernatant and lysosomal β-xylosidase are similar. This is probably due to contamination by lysosomal β-xylosidase activity in the supernatant fraction.

As shown in Fig. 2B, β-galactosidase activity in the lysosomal and supernatant fractions have a common optimum at pH 2.2–3.4; however, the supernatant fraction has an additional optimum at pH 4.0. The supernatant β-galactosidase has activity over a wider pH range than the lysosomal enzyme, but it is probable that the optimum at pH 2.2–3.4 may be due to contamination by lysosomal β-galactosidase. In the DEAE-cellulose fractionation of the supernatant fraction (Fig. 5) Peak VIII, the main peak exhibiting β-glucosidase and β-xylosidase activities, has a broad pH optimum for β-galactosidase activity (Fig. 2B) but the enzyme does not have activity below pH 3.0.

Lysosomal fractions prepared from beef, dog, and rabbit kidneys hydrolyzed *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside maximally at pH 5.0 (4.8–5.2). The supernatant fraction from the kidneys of these animals had broad optima, pH 5.4–6.8 for β-glucosidase and pH 5.2–6.2 for β-xylosidase activities.

Effect of time and protein concentration on β-glucosidase and β-xylosidase activity

Lysosomal β-glucosidase and β-xylosidase activities increased linearly with time up to 60 min of incubation and each activity was proportional to the amount of lysosomal membrane protein up to 400 μg. Supernatant β-glucosidase activity increased linearly with time up to 40 min, and increased proportionally with protein concentration up to 150 μg. Supernatant β-xylosidase activity increased linearly with time up to 4 h and the proportionality of activity was maintained up to 1500 μg when the incubation was for 60 min.

Effect of substrate concentration on β-glucosidase and β-xylosidase activity

From LINEWEAVER-BURK[9] plots for β-glucosidase and β-xylosidase activities, the K_m values were determined. For the lysosomal activities the K_m was 0.87 mM *p*-nitrophenyl-β-D-glucopyranoside, while it was 2.5 mM *p*-nitrophenyl-β-D-xylopyranoside. When measured with the supernatant fraction, the K_m values were 1.8 and 1.7 mM, respectively, for β-glucosidase and β-xylosidase.

Effect of some sugars and glycono lactones

Glucono(1→4)lactone has been shown by CONCHIE AND LEVVY[10] to be a specific

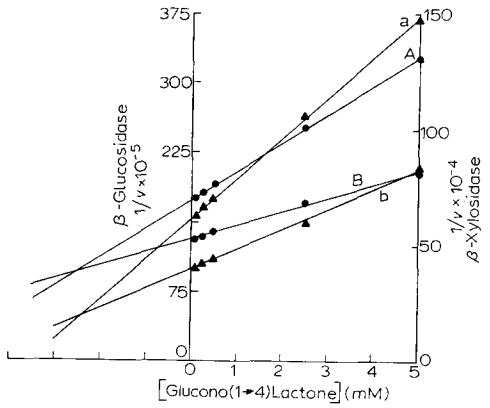

Fig. 3. Effect of glucono(1 → 4) lactone on β-glucosidase (●) and β-xylosidase (▲) activity. The reaction mixture contained 150 μg of rat kidney lysosomal membrane protein, 60 mM citrate–phosphate buffer (pH 5.2) and indicated amounts of glucono(1 → 4)lactone in a total volume of 1.0 ml. Incubations were for 30 min for β-glucosidase and 60 min for β-xylosidase. The concentrations of *p*-nitrophenyl-β-D-glucopyranoside were (A) 1.67 mM and (B) 4.17 mM, and of *p*-nitrophenyl-β-D-xylopyranoside they were (a) 1.85 mM and (b) 4.63 mM.

competitive inhibitor of β-glucosidase activity. The effects of glucono(1→4)lactone on lysosomal β-glucosidase and β-xylosidase activities, plotted according to a DIXON[11] plot, are shown in Fig. 3. The K_i values of 3.0 and 2.9 mM for lysosomal β-glucosidase and β-xylosidase, respectively are almost identical. This indicates the probability of a single catalytic site for hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside and *p*-

TABLE II

EFFECT OF SOME SUGARS AND GLYCONO LACTONES ON SUPERNATANT β-GLUCOSIDASE AND β-GALACTOSIDASE ACTIVITIES

Enzyme activities were measured as described in METHODS.

Sugar or lactone	*Final concn. (mM)*	*Per cent of control activity*		
		β-Glucosidase	*β-Galactosidase*	
		pH 6.4	*pH 3.4*	*pH 6.6*
β-D-Glucose	20	55	98	90
β-D-Xylose	20	100	102	110
α-D-Mannose	20	72	98	75
D-Arabinose	20	105	98	99
β-D-Galactose	20	94	52	103
D-Ribose	20	92	98	105
Lyxose	20	87	96	77
Melibiose	20	97	98	107
Lactose	20	110	91	107
Inositol	20	98	105	96
Glucono(1 → 4)lactone	10	27*	100	31
Galactono(1 → 4)lactone	5	30	67	36

* 0.5 mM final concentration.

nitrophenyl-β-D-xylopyranoside. The K_i values for supernatant β-glucosidase and β-xylosidase were 0.06 and 0.21 mM, respectively, when determined from the initial slopes of a DIXON[11] plot. However, at lactone concentrations higher than 0.4 mM, the slope deviated from linearity, showing less inhibition of β-xylosidase activity than would be expected if only one enzyme activity were being measured. This indicates that another enzyme(s) is contributing to the hydrolysis of *p*-nitrophenyl xylopyranoside which has a different affinity for glucono(1→4)lactone. Contamination by lysosomal β-xylosidase, which has a higher K_i for the lactone, could cause this effect. This is supported by the fact that at low substrate concentration (1.8 mM), the activity of the supernatant β-xylosidase shifts toward a less acid pH optimum (from pH 5.2 to 6.0). Lysosomal and supernatant β-glucosidase and β-xylosidase activities of beef, dog and rabbit kidney were also inhibited by glucono(1→4)lactone.

Table II shows the effect of some sugars and glycono lactones on the supernatant β-glucosidase and β-galactosidase activities. Among the sugars tested, β-glucose and lyxose inhibit the β-glucosidase and β-galactosidase (pH 6.6) activities. However, β-galactosidase (pH 3.4) activity is not affected significantly. β-Glucose and lyxose at this concentration (20 mM) had no effect on lysosomal β-glucosidase and β-xylosidase activities. β-Galactose and lactose, which inhibit β-galactosidase activity at pH 3.4 have little effect on β-galactosidase (pH 6.6) or β-glucosidase activities. Glucono(1→4)-lactone strongly inhibits both β-glucosidase and β-galactosidase (pH 6.6) and has no effect on β-galactosidase activity at pH 3.4. These effects indicate that the supernatant β-glucosidase and β-galactosidase activities at pH 6.6 may be identical. The effects of the sugars and the lactones on supernatant β-xylosidase were similar to those on the supernatant β-glucosidase, however, β-xylosidase was inhibited to a lesser degree.

Inhibition by PCMB

β-Glucosidase from *Saccharomyces cerevisiae* has been shown to be inhibited by PCMB[12]. The K_i for the strong and non-competitive inhibition of lysosomal β-glucosidase and β-xylosidase activities was 17.3 μM PCMB, as determined by aDIXON[11] plot. The supernatant β-glucosidase and β-xylosidase activities were also strongly inhibited. The strong inhibition of both the activities indicates the involvement of a –SH group at the active site, and the similarity of the activities. β-Galactosidase activity was more strongly inhibited at pH 6.6 (similar to β-glucosidase activity) than at pH 3.4. PCMB also inhibits β-glucosidase and β-xylosidase activities of beef, dog and rabbit kidneys.

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

The inhibitions of β-glucosidase by 4-methylumbelliferyl-β-D-xylopyranoside, 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[11] plot gave a K_i of 4.4 and 1.6 mM, respectively, for lysosomal β-glucosidase and β-xylosidase; the corresponding K_i values for the supernatant activities were 7.2 and 5.8 mM. The competitive inhibition of both the activities strongly indicates that the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside occurs at the same catalytic site.

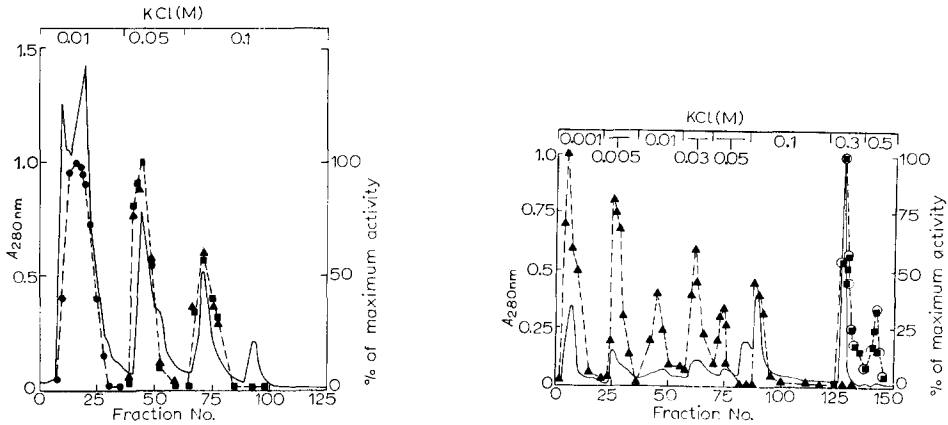

Fig. 4. Chromatography of deoxycholate-solubilized kidney lysosomal fraction on DEAE-cellulose at pH 7.6 in 10 mM potassium phosphate buffer. Protein (———) was eluted stepwise with KCl as indicated. Maximum specific activities of β-glucosidase (■— — —■) and β-xylosidase (▲— —▲) were obtained in Fraction 45 which gave 10-fold purification of β-glucosidase and 150-fold purification of β-xylosidase over homogenate. The recovery of each activity applied to the column was 23%. ●— —●, sialidase activity.

Fig. 5. Chromatography of lyophilized supernatant fraction on DEAE-cellulose at pH 6.8 in 1 mM potassium phosphate buffer. Protein (———) was eluted stepwise by increasing concentrations of KCl. Absorbance at 280 mμ from Fraction 124 to 150 is shown as one-half the actual value. Enzyme activities were as follows: ▲— ▲, β-galactosidase at pH 3.4; ■— —■, β-glucosidase; ○— —○, β-xylosidase. Recovery of each activity applied to the column was greater than 80%. The protein peaks were pooled as follows (peak numbers, fraction numbers): I, 5–10; II, 25–35; III, 40–53; IV, 60–72; V, 73–80; VI, 81–87; VII, 88–103; VIII, 126–132; and IX, 135–145.

pH stability of lysosomal β-glucosidase and β-xylosidase

Aliquots of a lysosomal membrane fraction were brought to different levels of pH in 1 mM citrate–phosphate buffer, with a final concentration of 1 mg of protein per ml. The mixtures were held at 27° for 10 h. Samples were taken at varying intervals of time and iced until the enzyme activities were measured. Both the activities exhibited a similar pH stability at 15 min and 10 h.

DEAE-cellulose chromatography

Fractionations on DEAE-cellulose columns were carried out using a deoxycholate-solubilized kidney lysosomal fraction and a lyophilized supernatant fraction. In Fig. 4, it is shown that lysosomal β-glucosidase and β-xylosidase activities have the same elution pattern. These activities were separated from sialidase, a membrane-bound enzyme. In Fig. 5, it is shown that β-glucosidase and β-xylosidase activities of the supernatant fraction have the same elution pattern and that they are separated from β-galactosidase activity at pH 3.4. However, Peaks VIII and IX, which exhibit maximum β-glucosidase and β-xylosidase activity, also hydrolyze *p*-nitrophenyl-β-D-galactopyranoside between pH 3.6 and 7.0. The β-galactosidase activity at pH 6.6 had similar elution patterns to that of β-glucosidase and β-xylosidase activities. All but Peaks VIII and IX hydrolyzed lactose. This is a further indication that supernatant β-glucosidase and β-galactosidase (pH 6.6) activities may be identical. The

relative activities of the enzyme in Peak VIII towards *p*-nitrophenyl xylopyranoside, -galactopyranoside and -glucopyranoside were 1:50:100, respectively.

DISCUSSION

Lysosomes are known to contain a large number of glycosidases and other hydrolases[13] and are known to be involved in the degradation of proteins, glycoproteins, glycosaminoglycans, and other complex substances[14–17]. β-Glucose occurs mainly in glycolipids and to some extent in glycoproteins. β-Xylose occurs in proteoglycans and glycoproteins and has been shown to form a linkage (*O*-β-xylopyranosyl-L-serine) between carbohydrate and protein moieties of proteoglycans[18–20] and glycoproteins[21]. Although the possibility that β-xylosidase activity is involved in cleaving the xylosyl–serine linkage has been suggested by ROBINSON AND ABRAHAMS[2], lysosomal preparations from rat liver and kidney failed to hydrolyze xylosyl–serine[1,15]. At this time it is not known whether xylosyl–serine is cleaved by β-xylosidase if the serine is part of a longer peptide. However, excretion of significant amounts of xylosyl–serine in human urine[22], which may arise from degradation of proteoglycans or glycoproteins, makes such a possibility appear unlikely. As suggested previously[1], structural similarity between β-glucose and β-xylose makes it possible for a single enzyme to exhibit activities toward both *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-xylopyranoside. Competitive inhibition of both the activities in lysosomes and in the supernatant fraction by glucono(1→4)lactone and by analogous substrates support this view.

Since β-glucosidase and β-xylosidase activities of beef, dog, and rabbit kidney lysosomes have similar pH optima and are inhibited similarly by glucono(1→4)lactone and PCMB, these activities are probably identical in the kidneys of these animals also; the supernatant β-glucosidase and β-xylosidase activities may also be identical.

Experimental evidence presented in this paper strongly indicates that rat kidney lysosomes have β-glucosidase activity which also exhibits β-xylosidase activity, but which is different from supernatant β-glucosidase. The supernatant β-glucosidase also has β-xylosidase activity and further studies of β-galactosidase in the supernatant fraction may show that it is identical with β-glucosidase, also ROBINSON *et al.*[23], using an extract from rat kidney homogenate, showed that the β-glucosidase activity of the extract separated on DEAE-cellulose column showed β-galactosidase activity.

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