Kinetic studies on the broad-specificity β -D-glucosidase from pig kidney

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A broad-specificity β -D-glucosidase from pig kidney cortex was isolated and purified to homogeneity by a rapid purification procedure. The pI (5.14 ± 0.05), M_r ($59\,000 \pm 2000$) and specific activities with several *p*-nitrophenyl glycosides (galactopyranoside, glucopyranoside, arabinopyranoside, xylopyranoside) were comparable with those published previously for cytoplasmic β -D-glucosidase from other sources and organs. Mixed-substrate experiments and inhibition studies with glucono-(1 $\rightarrow$ 5)-lactone revealed that a single active centre, containing one catalytic site and one saccharide-binding site, was responsible for the splitting of all four synthetic substrates. Inhibition experiments with substrate analogues demonstrated that (i) the major binding determinant of the glycosides was the aglycone moiety, (ii) an anionic side chain of the enzyme (probably a carboxy group) interacted with the glycosidic linkages and (iii) the properties of the aglycone significantly influenced the binding of the carbohydrate moiety. The inhibition constants of the *p*-nitrothiophenyl derivatives were in good agreement with the K_m values of the corresponding substrates. Therefore the Michaelis constants could be regarded as true equilibrium constants (K_e). The 'three-point-attachment model' of the substrate splitting, proposed by Daniels [(1983) Ph.D. Dissertation, University of Pittsburgh] for the analogous liver enzyme, was applicable for β -D-glucosidase from pig kidney too. The possible nature of the 'attachments' is discussed.

INTRODUCTION

The broad-specificity β -D-glycosidase (variously named in the literature as β -D-glucosidase, β -D-galactosidase etc.) found in the cytosol of convoluted-proximal-tubule cells of the kidney has been extensively studied in recent decades (e.g. Abrahams & Robinson, 1969; Glew *et al.*, 1976; Pierce *et al.*, 1977; Chinchetru *et al.*, 1986; Paez de la Cadena *et al.*, 1986). Irrespective of the species, the enzyme has an M_r of 47 000–58 000, has an acidic pI of about 5 and possesses both a high affinity and a neutral pH optimum between 5 and 7.5 for the hydrolysis of synthetic (*p*-nitrophenyl, 4-methylumbelliferyl) β -D-glucopyranosides, β -D-galactopyranosides, β -D-fucopyranosides, β -D-xylopyranosides and α -L-arabinopyranosides. The enzyme consists of a single polypeptide chain without any carbohydrate moiety and has a common active site for all substrates mentioned above. The physiological function of cytoplasmic β -D-glycosidase is still an enigma today. The enzyme could play a role in renal re-absorption of sugars (Abrahams & Robinson, 1969; Glew *et al.*, 1976) or might be involved in the hydrolysis of several oestrogen glucosides (Mellor & Layne, 1971).

The kinetic properties of the enzyme have already been studied by several authors (Abrahams & Robinson, 1969; Glew *et al.*, 1976; Pierce *et al.*, 1977; Paez de la Cadena *et al.*, 1986), but their investigations were limited almost exclusively to substrate specificity and inhibition by glucono- and galactono-lactones in the case of some substrates. On the other hand, inhibition experiments with phloridzin (Abrahams & Robinson, 1969) and 2,5-dihydroxymethyl-3,4-dihydropyridine (Chinchetru *et al.*, 1986) are rather of physiological and analytical

significance respectively. Thus relatively little is known about the mechanism of the action of neutral renal β -D-glycosidase. Therefore our goals were (i) to obtain reliable information on the formation of enzyme-substrate complexes and the splitting of substrate molecules and (ii) to compare the kinetic characteristics with those obtained for the analogous liver enzyme (Chester *et al.*, 1976; Daniels *et al.*, 1981; LaMarco & Glew, 1985, 1986).

MATERIALS AND METHODS

Chemicals

Polybuffer exchanger (PBE 94), Polybuffer 74, Sephacryl S-200 (superfine grade) and Blue Dextran 2000 were from Pharmacia (Uppsala, Sweden). *p*-Nitrophenyl β -D-glucopyranoside, *p*-nitrophenyl β -D-galactopyranoside, *p*-nitrophenyl β -D-xylopyranoside, M_r gel-filtration calibration kit (MS-II) and bovine serum albumin were purchased from Serva Feinbiochemica (Heidelberg, Germany). *p*-Aminothiophenyl β -D-glucopyranoside, *p*-aminothiophenyl β -D-galactopyranoside and Coomassie Brilliant Blue R were bought from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glucono-(1 $\rightarrow$ 5)-lactone, *p*-nitrophenol, thiophenol and *p*-nitrothiophenol were from Fluka AG (Buchs, Switzerland). Fructose-bisphosphate aldolase, SDS and other chemicals, unless otherwise stated, were purchased from Reanal (Budapest, Hungary).

The following compounds were prepared in our own laboratory: *p*-nitrophenyl α -L-arabinopyranoside, m.p. 199–201 °C, lit. m.p. 201–202 °C (Snyder & Link, 1952); thiomethyl β -D-galactopyranoside, m.p. 174–175 °C,

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lit. m.p. 174–175 °C (Heferich *et al.*, 1953); thiophenyl β -D-glucopyranoside, m.p. 131–132 °C, lit. m.p. 135 °C (Fischer & Delbrück, 1909); thiophenyl β -D-galactopyranoside, m.p. 100–101 °C, lit. m.p. 99–100 °C (Janaki *et al.*, 1969); thiophenyl β -D-xylopyranoside, m.p. 139–140 °C, lit. m.p. 144 °C (Purves, 1939); *p*-nitrothiophenyl β -D-glucopyranoside, m.p. 162–164 °C, $[\alpha]_D^{22} - 109.6^\circ$ (*c* 0.30 in methanol), lit. m.p. 148–149 °C (Grover & Cushley, 1977); *p*-nitrothiophenyl β -D-galactopyranoside, m.p. 173–174 °C, $[\alpha]_D^{22} - 101.6^\circ$ (*c* 0.28 in methanol), lit. m.p. 173–174 °C, lit. $[\alpha]_D^{22} - 103.4^\circ$ (*c* 0.8 in methanol) (Shah & Bahl, 1974). *p*-Nitrothiophenyl β -D-xylopyranoside was prepared by adaptation of the method used by Shah & Bahl (1974) for the synthesis of *p*-nitrothiophenyl β -D-galactoside. A 180 mg yield of three-times-crystallized chromatographically pure *p*-nitrothiophenyl β -D-xylopyranoside was obtained from 5 g of 2,3,4-tri-*O*-acetyl- β -D-xylopyranosyl bromide (Newth & Phillips, 1953) (yield 4%), m.p. 146–148 °C, $[\alpha]_D^{22} - 79.1^\circ$ (*c* 0.18 in methanol), ^1H n.m.r. δ (p.p.m.) ($^2\text{H}_2\text{O}$) 5.08 (1 H, d, *J* = 9 Hz, *H*-1), ^{13}C n.m.r. δ (p.p.m.) ($^2\text{H}_2\text{O}$) 86.1 (*C*-1). (Rotations were measured on a Perkin–Elmer 241 polarimeter; n.m.r. spectra were recorded on a Bruker WP-200SY FT instrument, operating at 200.1 MHz frequency for protons and at 50.3 MHz for ^{13}C nuclei.)

Tissue fractionation

The first step in the preparation of the enzyme was essentially the same as that described previously by Price & Robinson (1966). Kidneys from 11 pigs of different breeds, ages and sexes were obtained within 30 min after slaughter (Állatforgalmi és Húsipari Vállalat, Debrecen, Hungary). After removal of capsules, adrenal glands and pelvises, kidney cortices were homogenized in cold acetone (–20 °C) with an MSE Ato-Mix homogenizer at high speed (3 × 30 s). Following that the homogenate was filtered out and air-dried at room temperature. The fine powder could be stored at –20 °C for 6 months without any loss of activity.

Partial purification

A 2–3 g portion of the acetone-dried powder was extracted by gentle stirring in 30 ml of 0.15 M-sodium phosphate/citrate buffer, pH 6.5, for 3 h at 0–1 °C, then the extraction mixture was subjected to centrifugation at 10000 *g* for 15 min. After that the supernatant was adjusted quickly to pH 4.3 with 1 M-citric acid and held for 2 h at 1 °C. The agglutinated protein was centrifuged off and the supernatant (about 20 ml) was dialysed against 25 mM-imidazole/HCl buffer, pH 7.3, for 2 days at 4 °C.

Chromatofocusing

The dialysed supernatant (about 30 ml, containing 120–160 mg of protein) was re-centrifuged and applied directly to a 1 cm × 18 cm column of PBE 94 previously equilibrated with 25 mM-imidazole/HCl buffer, pH 7.3. During the sample application the pH of the solution leaving the column was continuously checked, but it was always less than 7.8. The pH gradient was developed by 9-fold-diluted Polybuffer 74, pH 4.3, at a flow rate 30 ml/h. The change in the pH of the effluent was monitored with a Radelkis OP-211/1 pH-meter equipped with an OP-0808P type combined electrode. The pH of each fraction (2.5 ml) containing more than 5 nkat

of β -D-galactosidase activity was adjusted to 6 with 0.2 M- Na_2HPO_4 . After activity measurements and protein determination the fractions were pooled and directly subjected to gel filtration.

Gel filtration

Samples (4 ml) of the enzyme preparation from the chromatofocusing column were loaded on a Sephacryl S-200 column (1.5 cm × 90 cm) that had been equilibrated in eluting buffer (10 mM-sodium phosphate buffer, pH 6.0; flow rate 30 ml/h). Fractions (3.0 ml) having the most intense β -D-glycosidase activity were combined (6–9 ml) and taken for kinetic experiments. The enzyme preparation could be stored at –20 °C with a relatively small (20–30%) loss of activity for some days.

M_r determination was carried out by comparison with marker proteins as described by Andrews (1965).

SDS/polyacrylamide-gel electrophoresis

Gel electrophoresis was performed at pH 8.8 according to the method of Laemmli (1970) with the exception that 1 mm-thick × 14 cm-long slabs were used instead of rods.

Protein determination

Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard, or by monitoring the u.v. absorption at 280 nm (Warburg & Christian, 1941).

Enzyme assays

All measurements were carried out in 0.15 M-sodium phosphate/citrate buffer, pH 5.5, at 37 °C. The total reaction volume was 1 ml, 5 min preincubation was used and the reaction was initiated by addition of the enzyme in all cases. To quench the reaction 2 ml of 0.2 M-sodium borate buffer, pH 10.0, was added to the reaction mixture and the concentration of *p*-nitrophenolate ions was determined spectrophotometrically at 400 nm (Beckman DB) by the use of calibration curves. Blank solutions always consisted of the same components except the enzyme.

In substrate-specificity investigations the reaction velocity was determined at six different substrate concentrations, which were chosen from the range of 0.5–5.0 K_m for each substrate. All these assays were performed in quintuplicate for hexosides and in triplicate for pentosides. The incubation time was varied in each replicate determination, namely it was altered between 5 and 15 min for hexosides and between 20 and 35 min for pentoside substrates. All of the reaction rate values obtained were unified for each substrate and handled as a single set of data points in the further evaluation procedures.

Inhibition experiments were carried out at four different substrate concentrations by the application of three or four different inhibitor concentrations (0.5–3.5 K_i), i.e. 12–16 experimental data points were gained for each substrate–inhibition pair.

To minimize experimental error only freshly made solutions were used. Glucono-(1→5)-lactone solutions were always applied within 3 min after preparation (Shimahara & Takahashi, 1970).

Estimation of kinetic parameters

In the case of hexoside substrates and mixed-substrate experiments the decrease in substrate concentrations never exceeded 15% of the total for any substrate and the formation of product was linear with time. On the other hand, Selwyn's (1965) test of inactivation was always negative. Therefore the steady-state assumption was considered to be valid in processing of the data. At first the Lineweaver-Burk (1934) linearization was used to determine the types of the investigated reactions and to gain a crude estimation of the corresponding kinetic parameters (K_m , V_{max} , and K_i). As has been proved previously (Tommasini *et al.*, 1985), the enzyme kinetic constants calculated from the classic linearizations are very imprecise and misleading in most cases. For this reason the graphically gained constants were further refined by a computer program based on a newly developed non-linear least-squares procedure (Zékány & Nagypál, 1985). For more details, e.g. the definition of the standard deviation (s.d.) and the standard errors (s.e.) of the fitted parameters, see the Appendix.

In the case of pentoside substrates both K_m and V_{max} values were rather low. Hence, to optimize the product detection, both the enzyme concentration and the incubation time were increased. Under these conditions significant (15–45%) decreases in the initial substrate concentrations (0.5 – $5.0 K_m$) were observed. Therefore kinetic parameters were gained by linearization of the integrated reaction equations.

RESULTS

Purification procedure

Cytoplasmic β -D-glucosidase from the kidney cortices of 11 pigs was isolated and purified to homogeneity by a rapid procedure, consisting of five steps: preparation of acetone-dried powder, extraction, acidic precipitation, chromatofocusing and gel filtration. All four observed activities, namely *p*-nitrophenyl β -D-glucosidase, *p*-nitrophenyl β -D-galactosidase, *p*-nitrophenyl α -L-arabinosidase and *p*-nitrophenyl β -D-xylosidase, co-purified throughout the purification scheme, as demonstrated by the elution profiles in Figs. 1 and 2 and by the purification parameters summarized in Table 1. The purification data show that the procedure resulted in an overall yield of activity of 15–20%, and the specific activity increased about 80-fold for hexoside substrates. With *p*-nitrophenyl α -L-arabinopyranoside and *p*-nitrophenyl β -D-xylopyranoside a slightly lower purification was achieved, but this difference was comparable with the experimental error.

Criteria of purity

The final enzyme preparation migrated as a single protein band on polyacrylamide-slab-gel electrophoresis in the presence of SDS (Fig. 3).

pI and M_r determination

The kidney of each animal contained only one broad-specificity β -D-glucosidase, having a pI of 5.14 ± 0.05 (mean $\pm$ s.d., calculated from five independent measurements, as exemplified by Fig. 1).

The M_r of the enzyme was estimated by gel filtration on Sephacryl S-200, previously calibrated with standard proteins, as indicated in Fig. 2. This method yielded a

Fig. 1. Chromatofocusing of β -D-glucosidase from pig kidney cortex

●, A_{280} ; ○, pH. The following activities were measured: Δ , β -D-galactosidase; ■, β -D-glucosidase; □, α -L-arabinosidase; ○, β -D-xylosidase. For experimental details see the Materials and methods section.

Fig. 2. Gel filtration of partially purified pig kidney-cortex β -D-glucosidase on Sephacryl S-200

●, A_{280} . The following activities were measured: Δ , β -D-galactosidase; ■, β -D-glucosidase; □, α -L-arabinosidase; ○, β -D-xylosidase. Calibration for M_r determination: A, V_0 ; B, aldolase (M_r 160000); C, bovine serum albumin (M_r 67000); D, chymotrypsinogen A (M_r 25000); E, cytochrome *c* (M_r 12300); F, V_r . For experimental details see the Materials and methods section.

Table 1. Purification of cytoplasmic β -D-glucosidase from pig kidney cortexAbbreviations: PNP-Gal etc., *p*-nitrophenyl β -D-galactopyranoside etc.

Purification step	Volume (ml)	Protein (mg)†	Activity (nkat)§ [yield (%)]				Specific activity (mkat/kg) [purification (fold)]			
			PNP-Gal	PNP-Glc	PNP-Ara	PNP-Xyl	PNP-Gal	PNP-Glc	PNP-Ara	PNP-Xyl
Extraction*	21	424.2	238 [100]	131 [100]	22.9 [100]	14.4 [100]	0.56 [1]	0.31 [1]	0.054 [1]	0.034 [1]
Acidic precipitation, dialysis	29	123.8	181 [76]	102 [77]	14.9 [65]	10.3 [72]	1.46 [2.60]	0.82 [2.65]	0.120 [2.22]	0.083 [2.44]
Chromatofocusing	10	11.5	90.9 [38]	49.5 [38]	8.2 [36]	4.8 [33]	7.9 [14.1]	4.3 [13.9]	0.71 [13.1]	0.42 [12.4]
Sephacryl S-200 chromatography†	15	0.87	38.6 [16]	22.2 [17]	3.1 [14]	2.0 [14]	44.4 [79.3]	25.5 [82.2]	3.6 [66.7]	2.3 [67.6]

* A 2 g portion of acetone-dried powder was extracted.

† A 8 ml portion of the enzyme preparation from the chromatofocusing column was loaded on the Sephacryl S-200 column in two 4 ml aliquots and the most active fractions of both runs were pooled.

‡ In the case of chromatofocusing protein was determined by monitoring the u.v. absorbance at 280 nm; in all other cases the method of Lowry *et al.* (1951) was used.

§ The standard reaction mixture contained 2.0 mM of hexoside substrates or 0.2 mM of pentoside ones.

value of 59000 ± 2000 (mean $\pm$ s.d., coming from three independent determinations).

Substrate specificity

Hydrolysis of all four substrates obeyed Michaelis-Menten kinetics. The appropriate kinetic constants for *p*-nitrophenyl β -D-glucopyranoside and *p*-nitrophenyl β -D-galactopyranoside (K_m and V_{max}) were calculated by non-linear least-squares fit of experimental data to eqn. (1):

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]} \quad (1)$$

where [S] represents the substrate concentration.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of pig kidney-cortex β -D-glucosidase preparations obtained in successive purification steps

Lane 1, extraction; lane 2, acidic precipitation; lane 3, chromatofocusing; lane 4, gel filtration. For experimental details see the Materials and methods section.

For pentoside substrates kinetic parameters were gained from the Foster-Niemann (1953) linearization of the integrated form of eqn. (1), as shown by eqn. (2):

$$\frac{1}{t} \cdot \ln \left(\frac{[S]_0}{[S]_0 - [Y]} \right) = \frac{V_{max}}{K_m} - \frac{[Y]}{K_m \times t} \quad (2)$$

where $[S]_0$ stands for the initial substrate concentration and [Y] represents the concentration of released *p*-nitrophenolate ions. In the linearization $(1/t) \cdot \ln\{[S]_0/([S]_0 - [Y])\}$ was plotted against $[Y]/t$ and the parameters were calculated by linear regression. The results are summarized in Table 2.

Mixed-substrate experiments

In order to determine if all the enzyme activities could be attributed to a single catalytic site, mixed-substrate experiments were carried out. The experimental protocol was the same as in substrate-specificity investigations except that every sample also contained a certain amount of another substrate in addition to the standard one.

Table 2. Investigation of the substrate specificity of pig kidney-cortex β -D-glucosidaseAbbreviations: PNP-Glc etc., *p*-nitrophenyl β -D-glucopyranoside etc.

Substrate	K_m (M)	V_{max} (kat/kg)	V_{max}/K_m ($\text{kat} \cdot \text{kg}^{-1} \cdot \text{M}^{-1}$)
PNP-Glc*	$9.0(\pm 1.0) \times 10^{-5}$	$2.5(\pm 0.1) \times 10^{-2}$	278
PNP-Gal*	$2.3(\pm 0.1) \times 10^{-4}$	$4.6(\pm 0.1) \times 10^{-2}$	200
PNP-Ara	1.7×10^{-5}	3.7×10^{-3}	218
PNP-Xyl	3.3×10^{-5}	2.7×10^{-3}	82

* The s.e. values of the fitted parameters are shown (in parentheses); the s.d. values were 0.7 mkat/kg for PNP-Glc and 0.4 mkat/kg for PNP-Gal.

Fig. 4. Typical series of mixed-substrate experiments with pig kidney-cortex β -D-glucosidase

The thick continuous line originating from the zero point represents the rectangular hyperbola for *p*-nitrophenyl β -D-galactopyranoside as a standard. The theoretical curves in the presence of *p*-nitrophenyl β -D-galactopyranoside and another substrate (other continuous lines) were calculated from the kinetic parameters given in Table 2. Experimental points are: $\circ$, 0.10 mM-*p*-nitrophenyl β -D-glucopyranoside; $\bullet$, 0.075 mM-*p*-nitrophenyl α -L-arabinopyranoside; $\square$, 0.10 mM-*p*-nitrophenyl β -D-xylopyranoside. For further details see the text.

Theoretical curves, expected for a single site and calculated on the basis of eqn. (3), as well as observations, are shown in Fig. 4:

$$v = \frac{V_{\max,1} \cdot [S_1] \cdot K_{m2} + V_{\max,2} \cdot [S_2] \cdot K_{m1}}{K_{m1} \cdot K_{m2} + [S_1] \cdot K_{m2} + [S_2] \cdot K_{m1}} \quad (3)$$

where the indices stand for the different substrates. As can be seen, the theoretical and measured rate values are in good accordance, i.e. there is a competition between the substrate molecules on the splitting site of the enzyme. Similar results were also obtained when any other substrate was used as standard.

Inhibition by glucono-(1 $\rightarrow$ 5)-lactone

Aldono-(1 $\rightarrow$ 5)-lactones are potent, highly specific, so-called transition-state-analogue inhibitors of glycosidases (Levy *et al.*, 1962; Conchie *et al.*, 1967; Leaback, 1968; Walaszek *et al.*, 1982). Although the microscopic binding modes are probably rather different, the carbohydrate moiety of a substrate and the lactone ring of corresponding configuration bind competitively to the same site of the active centre (Tanaka *et al.*, 1986). In order to verify that only one saccharide-binding site exists for all substrates, inhibition by glucono-(1 $\rightarrow$ 5)-lactone was extensively studied. The observed inhibition types and K_i values are shown in Table 3.

In the case of hexoside substrates inhibition constants (K_i) were evaluated by fitting to eqn. (4):

$$v = \frac{V_{\max} \cdot [S] \cdot K_i}{K_m \cdot K_i + [S] \cdot K_i + [I] \cdot K_m} \quad (4)$$

Table 3. Studies on the inhibition of pig kidney-cortex β -D-glucosidase by glucono-(1 $\rightarrow$ 5)-lactone

Abbreviations: PNP-Glc etc., *p*-nitrophenyl β -D-glucopyranoside etc.

Substrate	Type of inhibition	K_i (M)
PNP-Glc*	Competitive	$3.3(\pm 0.1) \times 10^{-6}$
PNP-Gal*	Competitive	$2.8(\pm 0.1) \times 10^{-6}$
PNP-Ara	Competitive	3.6×10^{-6}
PNP-Xyl	Competitive	4.3×10^{-6}

* The S.E. values of the fitted parameters are shown (in parentheses); the S.D. values were 0.5 mkat/kg for PNP-Glc and 0.8 mkat/kg for PNP-Gal.

Fig. 5. Inhibition of α -L-arabinosidase activity of pig kidney-cortex β -D-glucosidase by glucono-(1 $\rightarrow$ 5)-lactone

The inhibitor concentrations were: —, no inhibitor; $\bullet$, 2.5 μ M; $\square$, 5.0 μ M; $\blacksquare$, 10 μ M. For linearizations see the text.

where $[I]$ represents the inhibitor concentration and both K_m and $V_{\max}$ were determined from independent measurements.

For pentoside substrates a linearization of the integrated form of eqn. (4) was used as shown by eqns. (5) and (6) and exemplified by Fig. 5:

$$\frac{1}{t} \cdot \ln \left(\frac{[S]_0}{[S]_0 - [Y]} \right) = \frac{V_{\max}}{K'_m} - \frac{[Y]}{K'_m \cdot t} \quad (5)$$

where

$$K'_m = K_m + \frac{[I]}{K_i} \cdot K_m \quad (6)$$

Inhibition by substrate analogues

To elucidate the role of the aglycone in the substrate binding, a series of substrate-analogue inhibitors was applied. Inhibition experiments were carried out with both hexoside substrates. Only the competitive type of

Table 4. Studies on the inhibition of pig kidney-cortex β -D-glucosidase by substrate analogues

Abbreviations: PNP-Glc etc., *p*-nitrophenyl β -D-glucopyranoside etc.; TM-Gal, thiomethyl β -D-galactopyranoside; PATP-Glc etc., *p*-aminothiophenyl β -D-glucopyranoside etc.; TP-Glc etc., thiophenyl β -D-glucopyranoside etc.; PNTP-Glc etc., *p*-nitrothiophenyl β -D-glucopyranoside etc.

Inhibitor*	K_i (M)	
	PNP-Glc†	PNP-Gal†
TM-Gal	$6.9(\pm 0.2) \times 10^{-2}$	$5.0(\pm 0.2) \times 10^{-2}$
PATP-Glc	$9.0(\pm 0.6) \times 10^{-4}$	$1.1(\pm 0.1) \times 10^{-3}$
PATP-Gal	$1.2(\pm 0.3) \times 10^{-3}$	$1.8(\pm 0.1) \times 10^{-3}$
TP-Glc	$8.4(\pm 0.3) \times 10^{-4}$	$6.2(\pm 0.3) \times 10^{-4}$
TP-Gal	$6.7(\pm 0.4) \times 10^{-4}$	$7.2(\pm 0.3) \times 10^{-4}$
TP-Xyl	$8.8(\pm 0.3) \times 10^{-4}$	$6.9(\pm 0.4) \times 10^{-4}$
PNTP-Glc	$2.4(\pm 0.1) \times 10^{-4}$	$1.9(\pm 0.1) \times 10^{-4}$
PNTP-Gal	$1.2(\pm 0.1) \times 10^{-4}$	$8.9(\pm 0.5) \times 10^{-5}$
PNTP-Xyl	$3.3(\pm 0.1) \times 10^{-5}$	$3.7(\pm 0.3) \times 10^{-5}$

* The S.E. values of the fitted parameters are shown (in parentheses); the S.D. values did not exceed 2 pkat in any case.

† The K_m (M) and V_{max} (kat/kg) values were determined from independent measurements.

inhibition was observed, so the K_i values were calculated as described for glucono-(1 $\rightarrow$ 5)-lactone. The results are summarized in Table 4.

DISCUSSION

Comments on the purification

In the present paper we have described a rapid purification procedure that yielded homogeneous broad-specificity β -D-glucosidase preparations from pig kidney in milligram quantities. The overall yield of activity and the specific activities were comparable with those published for cytoplasmic β -D-glycosidases from other sources, namely from monkey small intestine (Swaminathan & Radhakrishnan, 1969), rat kidney (Glew *et al.*, 1976), human liver (Daniels *et al.*, 1981) and rabbit kidney (Paez de la Cadena *et al.*, 1986). However, the purification scheme described by LaMarco & Glew (1985, 1986) for the analogous enzyme from human liver and guinea-pig liver seems to be more potent.

Abrahams & Robinson (1969) found and characterized two forms of neutral β -D-glucosidase in kidneys of pigs, which were named ' β -D-glucosidase 1' and ' β -D-glucosidase 2' in order of decreasing electrophoretic mobility. In our investigations we could observe the presence of only one cytoplasmic β -D-glucosidase, though this, of course, does not exclude the existence of other isoenzyme(s) in animals of other breeds. On the basis of the pI (5.14 ± 0.05) and the kinetic characteristics our enzyme might be identified as ' β -D-glucosidase 2'. The M_r of the enzyme (about 59000) is larger than the most literature data for renal β -D-glycosidase (Abrahams & Robinson, 1969; Pierce *et al.*, 1977; Paez de la Cadena *et al.*, 1986) but it agrees with that published by Glew *et al.* (1976).

Comments on the kinetic studies

As shown in Table 2, the pentoside substrates attached more tightly to the active centre of the enzyme than did the hexosides, and the V_{max} values were higher for *p*-nitrophenyl β -D-galactopyranoside and *p*-nitrophenyl β -D-glucopyranoside. The catalytic efficiency was highest for *p*-nitrophenyl β -D-glucopyranoside, less for *p*-nitrophenyl β -D-galactopyranoside and *p*-nitrophenyl α -L-arabinopyranoside and the poorest for *p*-nitrophenyl β -D-xylopyranoside. On the other hand, mixed-substrate experiments persuasively proved that the enzyme has a common splitting site for all substrates. Our observations are in good accordance with most literature data obtained for β -D-glycosidases from different sources (Abrahams & Robinson, 1969; Chester *et al.*, 1976; LaMarco & Glew, 1985, 1986).

As the data in Table 3 show, glucono-(1 $\rightarrow$ 5)-lactone inhibited competitively the hydrolysis of each substrate, and moreover the K_i values were in very good agreement in all cases. Therefore we can conclude that the carbohydrate moiety of all substrates bound to the enzyme at the same site. It is noteworthy that the inhibition constants are the lowest values yet described for the inhibition of neutral β -D-glycosidases by lactones.

Inhibition experiments with several substrate analogues provided further information on the active centre. As shown in Table 4, the inhibition constants were essentially the same with both hexoside substrates, which also confirms the existence of a single glycoside-binding site.

Comparing the K_i values for thiomethyl β -D-galactopyranoside and *p*-nitrothiophenyl β -D-galactopyranoside a deviation of the order of 10^3 can be observed. In the case of thiomethyl β -D-galactopyranoside the aglycone plays a negligible role in the formation of the enzyme-inhibitor complex (Grover & Cushley, 1977), and hence it is likely that the major binding determinant of the aryl glycosides is the aglycone. In other words, the active centre also contains a hydrophobic binding region and the interactions between the aglycone and the non-polar amino acid side chains of the enzyme are the most important factors in the binding.

From the data in Table 4 it can also be established that the substitution at position 4 of the aglycone considerably influenced the binding of the inhibitor. Namely, the stronger the electron-withdrawing effect of the substituent, the more tightly the glycoside bound to the enzyme. This observation indicates that an anionic group interacting with the glycosidic sulphur atom must be present near the aglycone-binding site. We assume that this anionic group (perhaps a carboxy group) also plays a crucial role in the splitting of the substrates, but verification of this assumption needs further investigation. It is remarkable that the observations concerning the aglycone-binding are in good agreement with those published by Dale *et al.* (1985) for sweet-almond β -D-glucosidase.

A surprising result coming from the inhibition experiments was that in the case of both thiophenyl and *p*-aminothiophenyl glycosides the inhibition constants were substantially identical irrespective of the carbohydrate moiety. That is, the more loosely the glycosides bound to the active centre, the less the structural properties of the carbohydrate moiety

influenced the binding. Because the strength of the enzyme-inhibitor binding basically depends on the nature of the aglycone, we can conclude that the aglycone also significantly affects the observed carbohydrate-specificity of the enzyme.

The K_m values of the substrates (Table 2) are in good agreement with the inhibition constants of the corresponding *p*-nitrothiophenyl derivatives (Table 4) in order of magnitude, which permits us to conclude that (i) an oxygen/sulphur exchange at the glycosidic position does not basically alter the strength and the nature of the binding and (ii) the Michaelis constants can be regarded as true equilibrium constants (K_s). Nevertheless, *p*-nitrothiophenyl β -D-galactopyranoside bound somewhat more tightly than *p*-nitrothiophenyl β -D-glucopyranoside, which is the reverse of what we observed with *p*-nitrophenyl β -D-galactopyranoside and *p*-nitrophenyl β -D-glucopyranoside. The explanation for this observation may be that an atomic exchange at C-1 results in a steric change around the glycosidic linkage (e.g. in the angular arrangement), which also influences the binding of the saccharide moiety to a certain extent.

Daniels (1983) has proposed a model for the action of broad-specificity β -D-glucosidase from liver. This so-called 'three-point-attachment' model appears to be applicable for the β -D-glucosidase from the pig kidney too. An attachment between the glycosidic linkage and an anionic side chain of the enzyme seems to be proved by substrate analogues. The existence of other two attachments at the positions C-4 and C-6 are also supported by substrate-specificity investigations, but it is unlikely that these interactions would result in a firmer fitting to the binding domain. Presumably, at these 'attachment points' certain bulky enzymic groups strive to compress the fixed glycoside molecule, which causes a significant strain and deformation in the pyranoside ring. Consequently these interactions may play a crucial role (i) in the orientation of the glycosidic linkage to the catalytic site and (ii) in the formation and stabilization of the half-chair conformation of the transition state.

Of course, these considerations can be applied only to *p*-nitrophenyl and 4-methylumbelliferyl derivatives, but in the case of other substrates involving the naturally occurring ones the mechanism of the splitting might be significantly different.

Because the kinetic properties of broad-specificity β -D-glucosidases from different organs are surprisingly similar in some cases, a common physiological function, e.g. in detoxication (LaMarco & Glew, 1986), cannot be excluded.

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APPENDIX

The relative deviations between the measured and calculated rate values ($v_{\text{obs.}}$ and $v_{\text{calc.}}$) can be expressed as eqn. (7):

$$D = z \cdot I \quad (7)$$

where $D_i = \ln(v_{\text{calc.},i}/v_{\text{obs.},i})$, $z_{ij} = (v_{\text{obs.},i})^{-1} \cdot (\partial v_{\text{calc.},i} / \partial P_j)$, $i = 1, 2, \dots, n$, $j = 1, 2, \dots, k$, and n is the number of the experimental points; k stands for the number of the fitted parameters (P) and I_j represents the improving value of the j th parameter. The I vector was calculated by the Cholesky algorithm in the standard Gauss-Newton

procedure. In every iteration step the standard deviation (S.D.) and the standard error of the j th fitted parameter (S.E. _{j}) were obtained as shown by eqns. (8) and (9):

$$\text{S.D.} = \sqrt{n \times A^2 / (n - k)} \quad (8)$$

$$\text{S.E.}_j = \sqrt{\text{S.D.}^2 \times G_{jj}^{-1}} \quad (9)$$

where A is the average of the relative difference in the measured and calculated rate values and G^{-1} represents the inverse of the Gauss matrix.

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