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Properties of β-glucosidase from Carica Papaya Fruit

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ABSTRACT

The properties of β-glucosidase from Carica papaya fruit pulp, purified to homogeneity on ultrathin-layer isoelectric focusing (pI, 5·2), were studied. The molecular mass was determined to be 54 000 by gel filtration, and 27 000 by sodium dodecylsulphate polyacrylamide gel electrophoresis, respectively, indicating that the enzyme was composed of two subunits. The optimum pH and temperature for enzyme activity were at 5·0 and 50°C, respectively. The enzyme catalyzed the hydrolysis of aryl-β-D-glucosides and, to a lesser extent, alkyl-β-D-glucosides; disaccharides were hydrolyzed very slightly. Glucosyltransferase and glycosidic 'side-activities' (β-galactosidase, β-xylosidase, β-fucosidase and α-arabinosidase activities) were absent. The enzyme was activated by Ca^{2+}, Mn^{2+}, Mg^{2+} and EDTA and was strongly inhibited by Ag^{+} and Hg^{2+}. D-Glucono-1,5-lactone (K_i, 0·08 mM), 1-deoxy-D-glucose (K_i, 6 mM), 1-deoxy-1-amino-β-D-glucose and glucal (K_i, each 8 mM) as well as N-methylglucamine (K_i, 17 mM) exhibited reversible inhibitory effects. The amino acid composition, as well as sugar content and composition of the enzyme, were also determined. The carbohydrate content of 10% consisted mainly of arabinose (48%) and fucose (23%).

INTRODUCTION

As the exact physiological role of glycosidases is in most cases far from understood (Hösel, 1981), an essential prerequisite for the evaluation of glycohydrolases is thorough purification of the enzymes. Many β-D-glucosidases (β-D-glucoside glucohydrolase, E.C. 3.2.1.21) of plant origin have been purified and, in part, characterized; however, only a few of them

have been obtained as homogeneous preparations (Kleinschmidt *et al.*, 1970; Grover *et al.*, 1977; Marcinowski & Grisebach, 1978; Legler & Harder, 1978; Yagi *et al.*, 1985). Recently, we described the isolation and purification to electrophoretic homogeneity of β-glucosidase from Carica papaya fruit pulp (Hartmann-Schreier & Schreier, 1986). The enzyme of this origin had not been investigated as yet. In the course of this study, partial characterization was also carried out. Thus, β-glucosidase showed an isoelectric point of 5·2, a molecular mass of 54 000 by gel filtration and 27 000 by sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis, respectively, as well as optimum pH and temperature at 5·0 and 50°C, respectively. In this paper, the interest is focused on further properties of the enzyme, i.e. substrate specificity, influence of effectors and reversible inhibitors as well as amino acid and carbohydrate content and composition.

MATERIALS AND METHODS

Enzymes

β-Glucosidase was isolated and purified *ca.* 1000-fold from Carica papaya fruit pulp to homogeneity on ultrathin-layer isoelectric focusing (pI, 5·2) using ammonium sulphate fractionation followed by chromatography on Phenylsepharose CL-4B and Sephacryl S-200 as well as preparative isoelectric focusing (Hartmann-Schreier & Schreier, 1986).

Emulsin (Serva) was purified by gel filtration on Sephacryl S-200 as recently described (Hartmann-Schreier & Schreier, 1986).

Enzyme assay

β-Glucosidase activity was determined by measuring release of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucoside. Assays containing 20–100 μl enzyme and 1 ml 4 mM substrate in 100 mM sodium acetate buffer (pH 5·0) were incubated at 40°C. After an appropriate time (10–30 min), the reaction was stopped by the addition of 2 ml 200 mM borate buffer (pH 9·8). The reactions were linear for at least 60 min and were directly proportional to the amount of enzyme present. The A_{405} nm was measured and the amount of *p*-nitrophenol was determined from $\varepsilon = 18\,500\,\text{mol}^{-1}\,\text{cm}^{-1}$.

Substrate specificity

All substrates used were commercially available products except the monoterpene alcohol-β-D-glucosides as well as benzyl- and 2-phenylethyl-β-D-glucoside, which were synthesized using the classical Koenigs–Knorr reaction. Geranyl-β-D-glucoside was kindly provided by B.A.T., Hamburg.

Assays containing 20 μl enzyme solution (15 pmol) and 1 ml substrate (0·25 mM: 0·5 mM; 1 mM) in 100 mM sodium acetate buffer (pH 5·0) were incubated at 50°C. After 30 min the liberated glucose was determined using the common enzymatic hexokinase test (Boehringer, 1984). K_m and V values were evaluated graphically (Lineweaver & Burk, 1934). k_{cat} was calculated on the basis of a molecular mass of 54 000 for the enzyme.

Influence of effectors

The influence of a variety of effectors ($CaCl_2$, $MgCl_2$, EDTA, $MnCl_2$, NaN_3, AgCl, $HgCl_2$, D-glucono-1,5-lactone, SDS, TRIS, glucose, $CuSO_4$, $Hg(CN)_2$ and $ZnCl_2$) was studied with *p*-nitrophenyl-β-D-glucoside as substrate using standard enzyme assay (20 μl enzyme solution, 15 pmol; 30 min incubation) and concentrations of effectors from 10^{-8} to 10^{-1} M. Blank tests without enzyme addition were carried out and, if necessary, pH was corrected to 5·0.

Reversible inhibitors

All sugar and sugar derivatives used were commercially available products. Enzyme activity was determined using two substrate concentrations (0·2 mM and 1 mM *p*-nitrophenyl-β-D-glucoside) and three different inhibitor concentrations ($I_3 > I_2 > I_1$) in the range of 0·005 M to 1·5 M. The incubation time was 30 min. The determination was performed graphically (Lineweaver & Burk, 1934). The resulting K'_m values were used for a secondary Lineweaver–Burk plot leading to graphical evaluation of K_i values.

Amino acid composition

Amino acid analysis of β-glucosidase from papaya fruit pulp (3·3 mg protein) and emulsin (purified by gel filtration) (5·0 mg protein) was performed by means of ion-exchange chromatography after hydrolysis with hydrochloric acid conducted under nitrogen (Beck *et al.*, 1978).

Sugar content and composition

The sugar content of β-glucosidase from papaya fruit pulp and emulsin (purified by gel filtration) was determined according to Dubois *et al.* (1956) using arabinose as standard.

The composition of sugars of both these enzyme preparations was studied after methanolysis and trifluoroacetylation by capillary gas chromatography (HRGC) using a modification of the method described by Zanetta *et al.* (1972). The lyophilized samples (each 50 μg protein) were dried

over P_2O_5 for 24 h. Methanolysis was performed after adding 500 μl of 0·5 M methanolic HCl and heating at 80°C for 20 h. HCl was removed under N_2 at 50°C and the residue was diluted by adding 200 μl of a CH_2Cl_2/trifluoroaceticanhydride mixture (1 + 1). After heating at 110°C for 30 min, cooling and addition of internal standard (ribose, 200 μg) the samples were ready for subsequent HRGC analysis.

HRGC was carried out using a Carlo Erba Fractovap 4100 gas chromatograph with FID equipped with a J&W fused silica DB-5 capillary column (30 m, 0·25 mm inside diameter, df = 0·25 μm) and a 2-m uncoated fused silica capillary precolumn as 'retention gap'. Split injection (1:50) was employed. The temperature program was 70–110°C at 2°/min and then 110–200°C at 5°/min. The flow rates for the carrier gas were 2·5 ml/min He; for the make-up gas, 30 ml/min N_2; and for the detector gases, 30 ml/min H_2 and 300 ml/min air, respectively. The detector temperature was kept at 250°C.

Results of qualitative analyses were verified by comparison of HRGC data with those of authentic reference sugars, which were treated in the same manner as above mentioned for the samples. Quantitative HRGC determinations were carried out by standard controlled calculations using a Hewlett-Packard 3388 A laboratory data system.

Protein determinations

Protein was determined using the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

The different steps of isolation and purification of β-glucosidase from Carica papaya fruit pulp have been recently described (Hartmann-Schreier & Schreier, 1986). The enzyme used in this study to evaluate its properties and substrate specificity was homogeneous on ultrathin-layer isoelectric focusing and did not exhibit any other glycosidic 'side-activity', as found for β-glucosidase from various plant origins, e.g., almond (Helferich & Kleinschmidt, 1967; Grover & Cushley, 1977; Walker & Axelrod, 1978), 'Marianna' plum (Heuser, 1972), *Cicer arietinum* (Hösel & Barz, 1975) or *Cycas revoluta* Thunb. (Yagi *et al.*, 1985). Glucosyltransferase activity was also not detectable.

Substrate specificity

A number of disaccharides and β-D-glucosides, including monoterpene alcohol-β-D-glucosides, were used to study the substrate specificity of the

enzyme. Among the disaccharides under study, i.e. cellobiose, sophorose, gentiobiose, laminaribiose, saccharose, lactose, maltose and melibiose, enzymic hydrolysis was only found with the four first-mentioned substrates. However, incubation of 12 h or longer was necessary and only traces of substrates were hydrolyzed. Due to the low reactivity, these compounds were not further studied.

For a number of other substrates, the results of graphical determinations of K_m and V as well as the calculated k_{cat} and k_{cat}/K_m data are outlined in Table 1. As shown from Table 1, K_m values between 0·063 mM (*n*-hexyl-β-D-glucoside) and 5·0 mM (benzyl-β-D-glucoside) were determined. High V values were found, in particular, for benzyl-β-D-glucoside, but also for picein, *p*-nitrophenyl-β-D-glucoside and salicin. Very low V values were measured for the monoterpene alcohol-β-D-glucosides. The highest k_{cat}/K_m data were calculated for salicin, *p*-nitrophenyl-β-D-glucoside, picein and *n*-hexyl-β-D-glucoside (Table 1).

While β-glucosidase from papaya fruit pulp clearly differed from, for example, the common almond enzyme (emulsin) as to its specificity toward the glycan moiety, it showed similar behaviour as to the specificity toward the aglycon portion. The broad aglycon specificity, well known for β-glucosidases, was also found with the enzyme from papaya fruit pulp. The range of K_m values determined for different β-D-glucosides (Table 1) was not

TABLE 1
Kinetic Parameters of Purified β-Glucosidase from Carica Papaya Fruit Pulp for several β-D-Glucosides

Substrate	K_m (*mM*)	V (*pkat*)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/mM)
Phenyl-β-D-glucoside	0·30	19	1·27	4·2
p-Nitrophenyl-β-D-glucoside	0·11	63	4·20	38·2
β-Naphthyl-β-D-glucoside	0·16	16	1·07	6·7
Salicin	0·071	60	4·00	56·3
Arbutin	0·16	19	1·27	7·9
Picein	0·13	69	4·60	35·4
Phloridzin	1·10	19	1·27	1·1
n-Hexyl-β-D-glucoside	0·063	17	1·13	17·9
n-Octyl-β-D-glucoside	0·28	8	0·53	1·9
Benzyl-β-D-glucoside	5·0	167	11·13	2·2
2-Phenylethyl-β-D-glucoside	0·50	7	0·47	0·9
Linaloyl-β-D-glucoside	0·25	6	0·40	1·6
Neryl-β-D-glucoside	0·58	10	0·67	1·1
Geranyl-β-D-glucoside	0·66	10	0·67	1·0

uncommon; similar data have been obtained for a number of aryl-β-D-glucosidases from plant origin.

Influence of effectors

The influence of a variety of effectors was studied with *p*-nitrophenyl-β-D-glucoside as substrate. Activation of enzymic activity was observed by addition of $CaCl_2$, $MgCl_2$, EDTA, $MnCl_2$ and NaN_3. Except the last-mentioned substance, different effects were obtained depending on the concentration of effector used (Fig. 1). While increased activation was found with increasing concentration of Ca^{2+} and Mg^{2+}, increase of Mn^{2+} and EDTA $> 10^{-4}$ M led again to a decrease of enzyme activity. Activating effects caused by Mn^{2+} and Ca^{2+} have been described previously; for example, for fungal β-glucosidases (Otsuka *et al.*, 1979; Kohchi *et al.*, 1985).

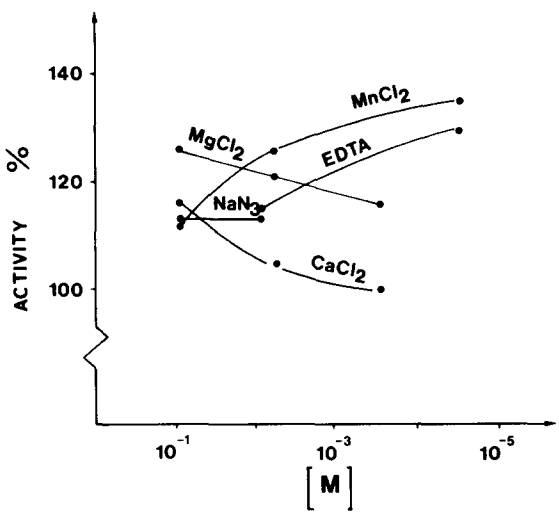

Fig. 1. Influence of concentration of various effectors of β-glucosidase from Carica papaya fruit pulp.

Inhibition of β-glucosidase was observed by addition of AgCl, $HgCl_2$, D-glucono-1,5-lactone, SDS, TRIS, glucose, $CuSO_4$, $Hg(CN)_2$ and $ZnCl_2$. As outlined in Fig. 2, the four first-mentioned substances showed pronounced effects. As to the metal ions, similar results have been reported for β-glucosidases from plant (Veibel, 1951), animal (Fisher, 1964) and microbial origin (Otsuka *et al.*, 1979; Saha *et al.*, 1981; Abe & Higashi, 1982; Ait *et al.*, 1982; Kohchi *et al.*, 1985). TRIS (2-amino-2-(hydroxymethyl)-1,3-propandiol) has been shown to be an inhibitor of β-glucosidase by early observations of Larner & Gillespie (1956).

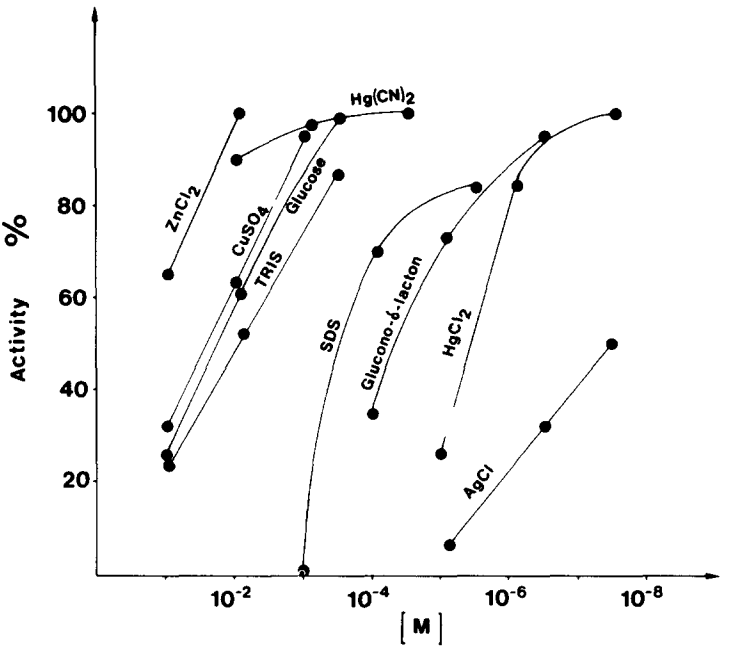

Fig. 2. Influence of concentration of various inhibitors of β-glucosidase from Carica papaya fruit pulp.

Reversible inhibitors

Reversible inhibitors are useful for probing the binding properties of enzymes and may also help to elucidate mechanisms of catalysis (Wolfenden, 1978). Table 2 summarizes the inhibition constants (K_i) of a variety of monosaccharides. These sugars were all found to be linear competitive inhibitors with respect to *p*-nitrophenyl-β-D-glucoside as substrate. The individual Lineweaver–Burk plots have been represented elsewhere (Hartmann-Schreier, 1987).

In order to evaluate the role of OH groups in the interaction of the sugar with the enzyme, a variety of D-glucose derivatives were also examined. The K_i values are outlined in Table 3; all compounds were found to be linear competitive inhibitors (Hartmann-Schreier, 1987).

From the data outlined in Tables 2 and 3 it does not appear as if any single substituent on glucose is absolutely essential for binding. It seems to be likely that the sugars can bind in a variety of orientations. Thus, for the two enantiomers of glucose, nearly identical affinity was observed, but for a number of other sugar enantiomers different values were determined (Table 2). Some additional data (Table 3) support a multiplicity of orientations. Nevertheless, there was a clear trend for enhancement of the affinity after

TABLE 2

Reversible Inhibition of β-Glucosidase from Carica Papaya Fruit Pulp by some Monosaccharides (Substrate, *p*-Nitrophenyl-β-D-Glucoside)

Inhibitor	K_i (mM)	*Inhibitor*	K_i (mM)
Aldohexoses		*Aldopentoses*	
L-Mannose	80	D-Arabinose	170
D-Glucose	200	L-Arabinose	880
L-Glucose	240	L-Xylose	1 350
D-Galactose	1 200	D-Xylose	4 800
D-Idose	1 300		
D-Mannose	2 400	*Deoxyaldohexoses*	
		1-Deoxy-D-glucose	6
Ketohexoses		6-Deoxy-D-galactose	300
D-Fructose	450	6-Deoxy-D-glucose	600
D-Tagatose	650	6-Deoxy-L-galactose	650
		2-Deoxy-D-galactose	950
		6-Deoxy-L-mannose	1 450
		2-Deoxy-D-glucose	1 450

TABLE 3

Reversible Inhibition of β-Glucosidase from Carica Papaya Fruit Pulp by D-Glucose Derivatives (Substrate, *p*-Nitrophenyl-β-D-Glucoside)

Inhibitor	K_i (mM)	*Inhibitor*	K_i (mM)
C_1-*Derivatives*		C_4-*Derivatives*	
1-Deoxy-1-amino-β-D-glucose	8	4-Methoxyglucose	550
Glucal	8		
N-Methylglucamine	17	C_6-*Derivatives*	
1-Methyl-β-D-glucopyranoside	160	D-Glucose-6-phosphate	165
Cellobiose	630	D-Glucuronic acid	4 000
1-Methyl-α-D-glucopyranoside	6 800	6-Deoxy-6-amino-β-D-glucose	6 000
Saccharose	16 800		
		Lactones	
C_2-*Derivatives*		D-Glucono-1,5-lactone	0·08
2-Deoxy-2-amino-D-glucose	250	L-Ascorbic acid	120
2-Deoxy-2-acetamido-D-glucose	3 700		
C_3-*Derivatives*			
3-Methoxyglucose	900		

substitution of a hydroxyl group by a cationic (amine) substituent. On the other hand, substitution of a OH group by an anionic substituent reduced the affinity.

Among the compounds listed in Tables 2 and 3, D-glucono-1,5-lactone was found to be the most effective reversible inhibitor of the enzyme. The K_i value of 0·08 mM approximately corresponded to data previously published for β-glucosidase in the range of 10^{-4}–10^{-5} M (Conchie *et al.*, 1967; Levvy & Snaith, 1972; Beer & Vasella, 1986).

Amino acid composition

The amino acid composition of β-glucosidase from papaya fruit pulp is shown in Table 4. In comparison with the values determined for a commercial emulsin preparation, which was further purified by gel filtration, in particular, the high content of cysteine is remarkable.

TABLE 4
Amino Acid Composition of β-Glucosidase from Carica Papaya Fruit Pulp (A) in Comparison with that of Commercial Emulsin purified by Gel Filtration (B)

Residue	*Mol residue/Mol protein*	
	A[a]	*B*[b]
Aspartic acid	63	151
Threonine	23	58
Serine	26	78
Glutamic acid	61	93
Proline	25	54
Glycine	57	106
Alanine	50	80
Valine	29	63
Cysteine	24	6
Methionine	4	4
Isoleucine	23	50
Leucine	40	100
Tyrosine	16	56
Phenylalanine	26	51
Lysine	25	58
Tryptophan	ND	ND
Histidine	7	24
Arginine	25	40

[a] M_r 54 000.
[b] M_r 135 000.
ND, not determined.

TABLE 5

Quantitative Carbohydrate Composition of β-Glucosidase from Carica Papaya Fruit Pulp (A) and a Purified Commercial Emulsin Preparation (B) determined after Methanolysis and Trifluoroacetylation by Standard-Controlled Capillary Gas Chromatography

Sugar	*% in A*[a]	*% in B*[a]
Rhamnose	4·6	7·9
Arabinose	48·3	17·9
Xylose	6·0	8·1
Fucose	23·4	13·7
Galactose	6·5	11·3
Mannose	5·0	17·2
Glucose	3·9	17·2
2-Deoxy-2-acetamidoglucose	2·2	6·6

[a] Total content of monosaccharides (= 100%); internal standard, ribose.

Sugar content and composition

Already during ultrathin-layer isoelectric focusing of β-glucosidase from papaya fruit pulp (Hartmann-Schreier & Schreier, 1986), the glycoprotein character of the enzyme was detected by alcian blue staining (Wardi & Michos, 1972). A carbohydrate content of 10% was determined using the method of Dubois *et al.* (1956).

The carbohydrate composition was analyzed after methanolysis of the enzyme. Quantitative capillary gas chromatography of trifluoroacetylated *O*-methyl glycosides revealed the composition outlined in Table 5. In parallel experiments, the carbohydrate composition of a commercial emulsin preparation, further purified by gel filtration, was also investigated. As shown in Table 5, high amounts of arabinose and fucose were found in the enzyme from papaya fruit pulp. In comparison with emulsin, low contents of galactose, mannose and glucose were observed.

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