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PURIFICATION AND PROPERTIES OF AN INDUCED β-D-GLUCOSIDASE FROM *STACHYBOTRYS ATRA*

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Summary

We have purified an induced β-D-glucosidase (β-D glucoside glucohydrolase, EC 3.2.1.21) from *Stachybotrys atra* to apparent homogeneity. The induced enzyme was clearly different from the constitutive β-D-glucosidase. The molecular weight was 65 500—69 000, the pH optimum was at 6.7 and the isoelectric point at 4.8. Carbohydrate content (related to glucose) was 14.4%. The enzyme showed β-D-glucosidase, β-D-xylosidase and β-D-thioglucosidase activity. These three activities appear to be due to the same enzyme. The enzyme was strongly inhibited by D-glucono-(1 → 5)-lactone and nojirimycin and was very sensitive to sulfhydryl reagents.

Introduction

The fungus, *Stachybotrys atra* DSL 1, produces constitutively an extracellular β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) [1]. It was demonstrated that *S. atra*, induced by phenyl 1-thio-β-D-glucopyranoside, also produced an intracellular β-D-glucosidase [2,3]. As this induced β-D-glucosidase also showed β-D-xylosidase (EC 3.2.1.37) and β-D-thioglucosidase activity, it seemed worthwhile to purify this enzyme and to determine whether the three activities could be attributed to the same catalytic site or represent three different enzyme species.

In the first case, a comparative study using 3 different glycosidic substrates, could lead to a better insight into the reaction mechanism of this glycosidase. The structure of the glycon part could indeed affect one or more aspects of the enzymic reaction and, eventually, a differentiation between several steps in the intrinsic mechanism could result.

This paper describes the induction, purification and some properties of the

induced enzyme. It also presents convincing evidence that the three activities are attributable to the same enzyme molecule.

Materials

Stachybotrys atra DSL 1, a gift from Dr. M.A. Jermyn (Melbourne, Australia), was maintained on a solid medium (unfermented beer, 8° Ball, agar 2.5%, pH adjusted to 6.5).

Following chemicals were synthesized as indicated: *p*-nitrophenyl β-D-glucopyranoside [4], *p*-nitrophenyl β-D-xylopyranoside [5], *p*-nitrophenyl 1-thio-β-D-glucopyranoside [6], phenyl 1-thio-β-D-glucopyranoside [6], 4-methylumbelliferyl β-D-glucopyranoside [7], 4-methylumbelliferyl β-D-xylopyranoside [7], *p*-chlorophenyl β-D-xylopyranoside [8], *p*-chlorophenyl 1-thio-β-D-xylopyranoside [9], *p*-acetylphenyl β-D-glucopyranoside [4], methyl 1-thio-β-D-glucopyranoside [10], *p*-nitrophenyl 6-bromo-6-deoxy-β-D-glucopyranoside and *p*-nitrophenyl 6-chloro-6-deoxy-β-D-glucopyranoside [11,4].

Yeast extract and agar were Oxoid (U.K.) products. Sephadex G-75 and G-200 were obtained from Pharmacia (Sweden) and hydroxyapatite was a Bio-Rad (U.S.A.) product. Reference proteins were purchased from Worthington (U.S.A.), Sigma (U.S.A.), Koch-Light (U.K.) and Boehringer (G.F.R.), ampholines from L.K.B. (Sweden).

D-xylose isomerase (EC 5.3.1.5) from *Lactobacillus brevis* was a generous gift from Professor K. Yamanaka (Kagawa University, Japan). D-glucitol dehydrogenase (EC 1.1.1.14) from sheep liver, D-glucose oxidase (EC 1.1.3.4) of fungal origin, and peroxidase (EC 1.11.1.7) from horseradish were purchased from Boehringer.

Methods

Growth and harvesting procedures

Precultures were grown in liquid medium containing (per l): 16 g KH_2PO_4, 30 g starch, 8 g NH_4Cl, 2.5 g yeast extract, 1 ml 1% $FeCl_3$ solution and 10 ml salt solution (2 g $CaCl_2$, 440 g $MgSO_4 \cdot 7\ H_2O$, 0.2 g $ZnSO_4 \cdot 7\ H_2O$, 0.1 g $MnSO_4$ and 1 mg Biotin per l). 20-ml aliquots of this medium were inoculated with cells from the agar slant and agitated at 30°C for 96 h.

For bulk production of mycelium, 5-ml aliquots of the preculture were added to 500-ml portions of bulk medium. Cultures were then incubated with vigorous shaking at 30°C for 48 h. The bulk medium contained (per l): 16 g NH_4Cl, 32 g K_2HPO_4, 4 ml 1% $FeCl_3$ solution, 40 ml of the salt solution and 35 g sucrose. After 48 h, mycelium of 16 h cultures was harvested by filtration and resuspended in 8 l aqueous 0.1% phenyl 1-thio-β-D-glucopyranoside solution. Maximal yield of induced β-D-glucosidase was obtained after shaking for 24 h at 30°C in the dark. The mycelium was subsequently filtered off, thoroughly washed with water, resuspended in 20 mM phosphate buffer (pH 7.2), homogenized at 0°C for 10 min in a Virtis 45 mixer and centrifuged at 8000 ×*g* for 10 min at 4°C in a Sorvall RC-2B centrifuge. The supernatant and washings were collected and used as crude extract.

Assay of enzyme activity and protein concentration

β-D-glucosidase activity was determined with *p*-nitrophenyl β-D-glucopyrano-

side: 2 mM substrate, 0.1 M phosphate buffer (pH 6.7) at 30°C, final volume 3 ml. The release of *p*-nitrophenol was measured as the increase in absorbance at 400 nm with a Beckman DB-G spectrophotometer. Under the same conditions β-D-xylosidase activity was measured with 5 mM *p*-nitrophenyl β-D-xylopyranoside as substrate. β-D-thioglucosidase was measured with 5 mM *p*-nitrophenyl 1-thio-β-D-glucopyranoside at pH 6.7 as the increase in absorbance at 430 nm. 1 unit of enzyme activity was defined as that amount of enzyme which catalyzed the hydrolysis of 1 μmol substrate per min. Specific activity is expressed as units per mg protein.

Protein concentration was determined by the method of Warburg and Christian [12].

The carbohydrate content of the enzyme was determined by the method of Dubois [13].

D-xylose isomerase was assayed spectrophotometrically as NADH oxidation in a coupled reaction with D-glucitol dehydrogenase [14]. Enzyme units were defined as described previously [14,15]. D-glucose oxidase was assayed spectrophotometrically as *o*-dianisidine oxidation in a coupled reaction with peroxidase according to Wakim [16]. The anomeric configuration of the reaction products D-glucose and D-xylose was determined by the methods of Wakim [16] and Kersters-Hilderson [17] respectively. Kinetic parameters (V, K_m and K_i) were estimated graphically by the method of Hanes [18], and then calculated on a Wang 2200 table-computer by the procedure of Wilkinson [19]. V is always expressed per unit of enzyme activity.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed according to the method of Hjérten [20] with a discontinuous buffer system of 7% acrylamide gel, 10 mM Tris · HCl buffer (pH 8.8) and run at room temperature for 2 h at 3 mA per tube. Enzyme activities were located with 1 mM 4-methylumbelliferyl β-D-glucopyranoside and 4-methylumbelliferyl β-D-xylopyranoside (in phosphate buffer, pH 6.7). Protein bands on the gels were stained with Coomassie Brilliant Blue, and scanned densitometrically with a Vitatron MPS photometer.

Sucrose density gradient centrifugation

Gradient centrifugation was carried out, by the method of Martin and Ames [21], at 4°C in a Beckmann Model L ultracentrifuge at 34 000 rev./min for 24 h, using the SW-36 rotor. Linear sucrose gradients (5—20%, w/v) were prepared in 50 mM Tris · HCl buffer (pH 7.2). The protein markers used were catalase (250 000) and alcohol dehydrogenase (150 000).

Determination of molecular weight by gel-filtration

Gel filtration on a Sephadex G-200 column (1 × 95 cm) was performed according to the method of Andrews [22]. The column was equilibrated with 50 mM Tris · HCl buffer (pH 7.2)/0.1 M KCl/1 mM EDTA. The protein markers were pyruvate kinase (237 000), aldolase (159 000), alcohol dehydrogenase (150 000), hexokinase (98 000) and malate dehydrogenase (65 000).

Determination of the isoelectric point

The isoelectric point was determined according to the method of Vesterberg

and Svensson [23] in a 110-ml column (LKB) at 4°C. Carrier ampholines (pH 4—6) were used in a sucrose gradient (24—47%, w/v). The voltage was increased from 300 to 900 V over 48 h. 2-ml fractions were collected and analyzed for pH and enzymic activity.

Determination of the absorption coefficient

1 ml purified β-D-glucosidase samples (approx. 1 mg protein) were dialyzed against 0.1 M potassium phosphate buffer (pH 6.7) and the ultraviolet absorption at 280 nm of a suitably diluted sample was recorded with a Zeiss PMQII-M4QIII spectrophotometer. The protein samples were dried on P_2O_5 at 100°C, the weight recorded and correlated with the absorbance at 280 nm.

Purification of β-D-glucosidase

To the crude extract a solution of 50% streptomycin sulfate 20 mM phosphate buffer (pH 7.2) was added dropwise to a final concentration of 4%. After 12 h at 4°C, insoluble material was removed by centrifugation at 24 000 $\times g$ for 15 min.

The supernatant solution was chilled to 4°C and acetone at —20°C was slowly added to final concentration of 40% (v/v). After standing for 1 h at 4°C, the supernatant liquid was decanted and discarded. The precipitate was dissolved in 50 mM Na_2HPO_4 solution and dialyzed at 4°C for 24 h against 4 mM potassium phosphate buffer (pH 7.2). The solution was concentrated by ultrafiltration in a Diaflo apparatus with PM 30 filter.

The concentrated extract was applied to a 2.5 × 23 cm hydroxyapatite column equilibrated at 4°C with 4 mM potassium phosphate buffer (pH 7.2). After elution of non-bonded material with the same buffer, the concentration of the buffer salts was increased discontinuously (Fig. 1) up to 0.8 M. The β-D-glucosidase and β-D-xylosidase activities, which eluted at 0.15 M phosphate, were pooled and concentrated. This solution was applied to a 3 × 93 cm Sephadex G-75 column equilibrated with 10 mM potassium phosphate buffer/0.1 M NaCl (pH 7.2). The column was eluted with the same buffer (30 ml/h,

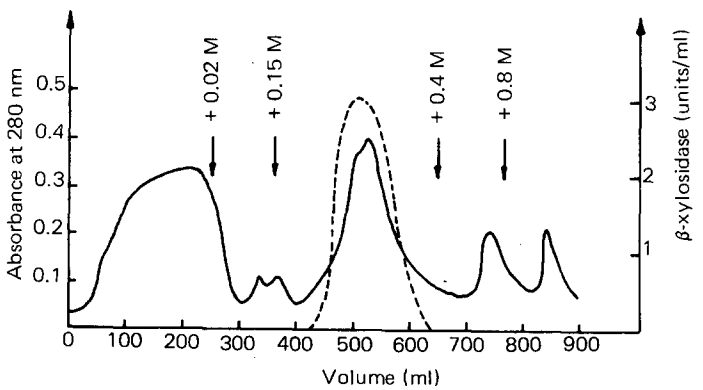

Fig. 1. Hydroxyapatite chromatography. The column (2.5 × 23 cm) was equilibrated with 4 mM phosphate buffer pH 7.2. The concentration of the buffer salt was increased discontinuously as indicated. ———, protein distribution as determined from A_{280}; - - - - -, β-D-xylosidase activity expressed as units/ml.

10-ml fractions). A β-D-glucosidase active peak appeared in fractions 50—66 and was identified with the enzyme prepared by Jermyn (see below).

A second β-D-glucosidase activity, eluted between fractions 70 and 88 (Fig. 2) was concentrated in the Amicon Diaflo apparatus (PM 10 filter); this was the induced enzyme.

Alternative methods

Affinity chromatography on *p*-aminobenzyl 1-thio-β-D-glucopyranoside [24] coupled via 6-aminocaproic acid to a matrix of Sepharose 2B [25] resulted in a separation of the two β-D-glucosidases. However, the very low yield (30%) precluded further use of this method.

The induced β-D-glucosidase was not bound on a Sepharose 4B-concanavalin A column prepared according to the method of Axen [26], in contrast to the constitutive enzyme which was strongly retained. As a consequence of the very strong binding of the latter enzyme, regeneration of the column was almost impossible and the method was not used further.

Results and Discussion

The purification procedure is shown in Table I and the specific activity of β-D-glucosidase increased 197-fold. The yield was 68% xylosidase activity and 0.35% protein. Fraction V was free of β-D-galactosidase, α-D-mannosidase, α-D-glucosidase and α-D-galactosidase activities. It could be kept as a suspension in $(NH_4)_2SO_4$solution for at least one year at 4°C without significant loss of activity.

Properties of the constitutive β-D-glucosidase

A study of the properties (Table II) of the β-D-glucosidase, which eluted in the void volume of the Sephadex G-75 column (Fig. 2), indicated that this enzyme was similar to the constitutive β-D-glucosidase prepared earlier [1]. This enzyme, which could clearly be differentiated from the induced β-D-glucosidase was not further examined.

TABLE I

PURIFICATION OF β-D-GLUCOSIDASE FROM *STACHYBOTRYS ATRA*

A, activity with *p*-nitrophenyl β-D-glucopyranoside as substrate; B, activity with *p*-nitrophenyl β-D-xylopyranoside as substrate; C, activity with *p*-nitrophenyl 1-thio-β-D-glucopyranoside as substrate.

Fraction	Volume (ml)	Total protein (mg)	Total units			Specific activity			Purification (-fold) (B)	Recovery (%) (B)
			A	B	C	A	B	C		
Crude extract	1500	9500	2480	420	42.4	0.26	0.044	0.005	1	100
Streptomycin ppt.	1700	8360	2440	416	41.6	0.29	0.049	0.005	1.1	99
Acetone ppt.	580	890	2300	390	39.6	2.58	0.438	0.045	10	93
Ultrafiltration	110	252	2100	362	36.4	8.33	1.437	0.144	33	86
Hydroxyapatite	200	106	1812	326	32.8	17.1	3.075	0.309	70	78
Sephadex G-75	170	33	1172	286	28.4	35.5	8.667	0.861	197	68

TABLE II

Properties of β-D-glucosidase eluted in the void volume of the Sephadex G-75 column and of β-D-glucosidase prepared by Jermyn compared with the properties of the induced β-D-glucosidase.

	Constitutive enzyme		Induced enzyme
	This work	Jermyn *	
pH optimum	4.7—5.0	4.7—5.0	6.7
Molecular weight	>100 000	500 000	69 000—65 500
K_m for *p*-nitrophenyl β-D-glucopyranoside	70 μM	50 μM	55.5 μM
K_i for *p*-nitrophenyl β-D-xylopyranoside	35 μM	37 μM	175 μM **

* Data taken from refs. 1 and 27.
** K_m value.

Characterisation of the activity

During the purification procedure, activity was monitored with three substrates, *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-xylopyranoside and *p*-nitrophenyl 1-thio-β-D-glucopyranoside. The ratios of the activities remain unchanged, except when the preparation was passed through the Sephadex G-75 column (Table I). At this stage, the activity ratios glucosidase/xylosidase and glucosidase/thioglucosidase (but not xylosidase/thioglucosidase) are changed by the removal of the constitutive β-D-glucosidase. This is a first indication that the three activities can be attributed to the same enzyme.

Polyacrylamide gel electrophoresis

After electrophoresis, bands of active enzyme in the gels were detected using 4-methylumbelliferyl β-D-glucopyranoside or the corresponding β-D-xylopyra-

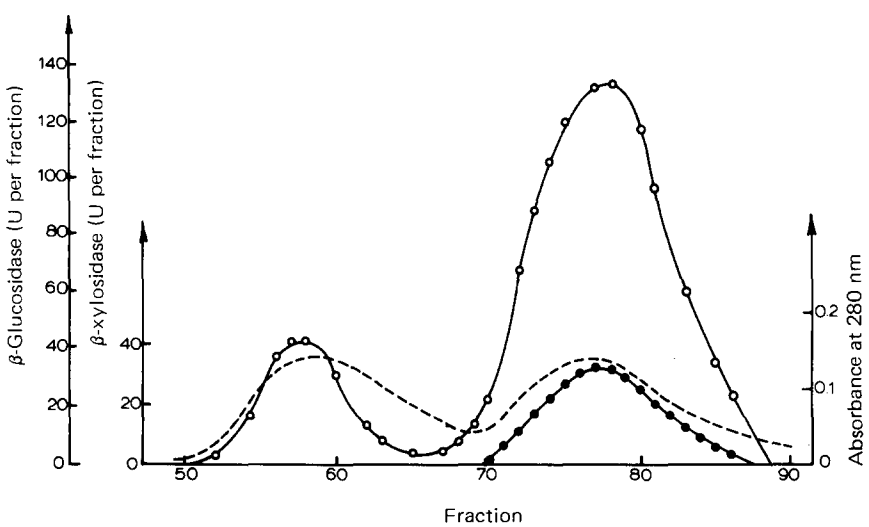

Fig. 2. Sephadex G-75 gel filtration. The column (3 X 93 cm) was equilibrated and eluted with 10 mM phosphate buffer pH 7.2 (0.1 M NaCl). Fractions of 10 ml each were collected at a flow rate of 30 ml/h. - - - - -, protein as determined from A_{280}; ○———○, β-D-glucosidase activity; ●———●, β-D-xylosidase activity.

noside. It was found that the pooled active fractions from the hydroxyapatite column contained two bands of activity. The first showed only β-D-glucosidase activity, the second showed both β-D-glucosidase and β-D-xylosidase activities (Fig. 3). Active fractions from the Sephadex G-75 column contained only one band, showing both activities. Staining with Coomassie Blue revealed two protein bands in the former material, and only one band in the latter. The protein bands correspond with the bands of enzyme activity (Fig. 3).

Isoelectric focusing

The isoelectric point was shown to be pH 4.8 (Fig. 4) for the three activities.

Molecular weight

Gel filtration on Sephadex G-200, according to the method of Andrews [22] showed that the molecular weight was 69 000. By sucrose density gradient centrifugation the molecular weight was 65 500.

Carbohydrate content

The purified β-D-glucosidase contained 14.4% carbohydrates (expressed as glucose). Their chemical composition was not further examined.

Absorption coefficient

The determination of the absorption coefficient at 280 nm was based on dry weight estimations. After appropriate corrections for non-specific absorption and buffer blanks a value of $A_{1\,cm}^{0.1\%} = 1.486$ was found.

Effect of pH and temperature

The enzyme solution (2 U/ml) was kept at 30°C for 30 min at pH 4—8 (0.1 M citrate/phosphate buffer) and the activity determined under standard

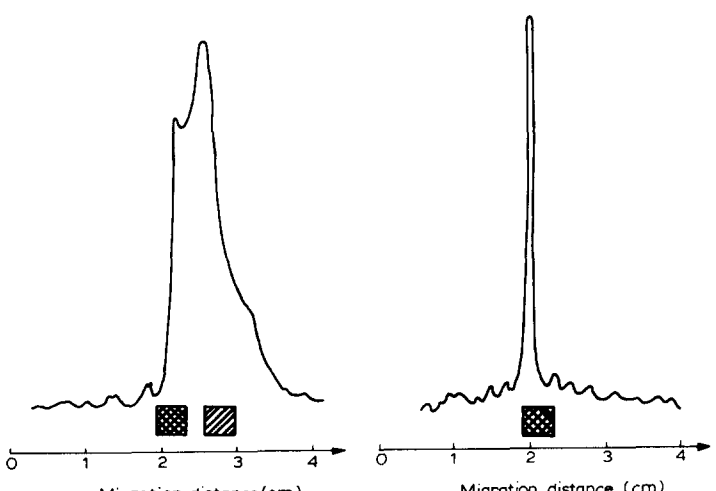

Fig. 3. Densitometric tracings of electrophoretograms on polyacrylamide gels. Left: after hydroxyapatite chromatography; right: after chromatography on Sephadex G-75. Migration of the protein is from left to right. Zone with β-D-glucosidase activity: ▩; zone with β-D-xylosidase activity: ▨.

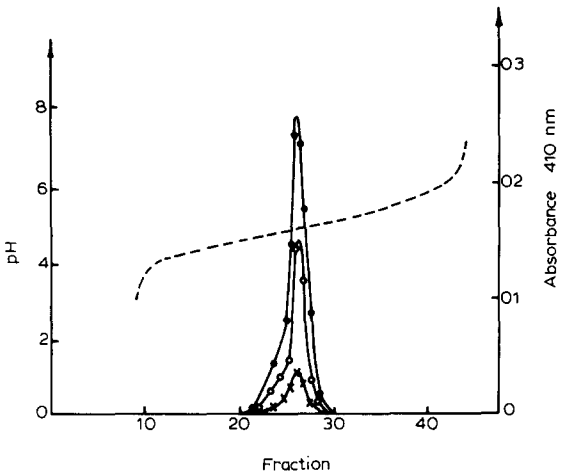

Fig. 4. Isoelectric focusing. Enzyme activities were measured with the *p*-nitrophenyl substrates and are expressed as absorbance at 410 nm. β-D-glucosidase activity: ●———●; β-D-xylosidase activity: ○———○; β-D-thioglucosidase activity: X———X.

conditions. The residual activity was expressed as a percentage of the initial activity (Fig. 5). The results indicate that the enzyme is most stable at pH 5—7, but is rapidly inactivated below pH 5.

The enzyme solution (2 U/ml) was kept at pH 6.7 for 30 min at 25—50°C. The residual activities are given in Fig. 5. The enzyme was most stable below 40°C, but was rapidly inactivated after 30 min of incubation at temperatures above 40°C. From Fig. 5 it can be seen that the inactivation patterns of the three enzyme activities are identical.

In 0.1 M phosphate buffers, the optimum pH for β-D-glucosidase and β-D-xylosidase activities was 6.7. Although the enzyme is only slightly active

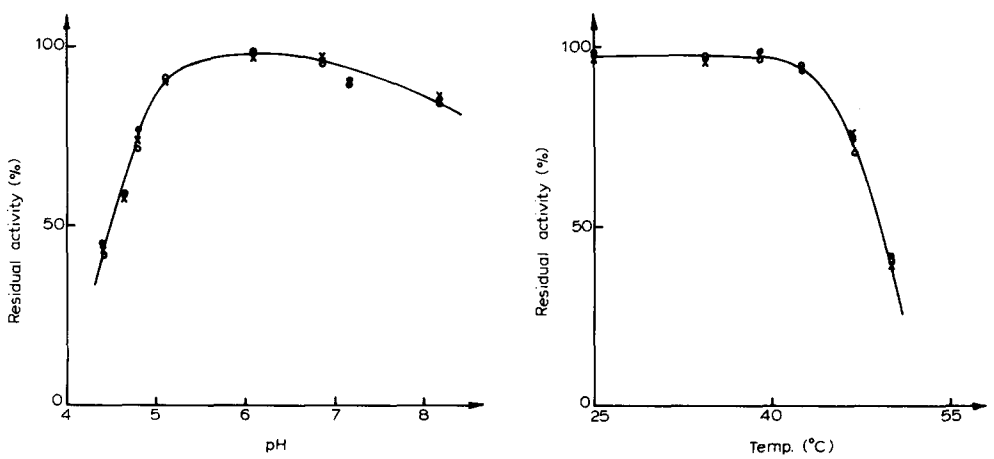

Fig. 5. Effect of pH and temperature on the enzyme stability. Left: enzyme samples were incubated (30°C) for 30 min in 0.1 M citrate/phosphate buffers of different pH. Right: enzyme samples were incubated at pH 6.7 for 30 min at different temperatures. Activity was determined at 30°C and pH 6.7 (*p*-nitrophenyl derivatives as substrates) and expressed as % activity remaining. ●———●, β-D-glucosidase; ○———○, β-D-xylosidase; X———X, β-D-thioglucosidase activity.

against 1-thio-β-D-glucopyranosides, the pH optimum for hydrolysis of the *p*-nitrophenyl derivative was estimated at approx. 6.5.

Influence of ionic environment

When the three enzymic activities were measured in the presence of large amounts (up to 0.1 M) of various salts, the reaction velocity remained unchanged. EDTA had no effect on the activity.

Substrate specificity

The following *p*-nitrophenyl glycopyranosides were not hydrolysed by the enzyme: α- and β-D-galactopyranosides, α- and β-D-mannopyranosides, β-D-ribopyranoside, and α-D-glucopyranoside. These findings indicate strict specificity requirements in the glycon moiety at C-1, C-2, C-3 and C-4. In contrast, substitution at C-5, although lowering the maximal velocity, did not prevent hydrolysis (Table III).

Aryl 1-thio-β-D-glucopyranosides behave as competitive inhibitors. They are hydrolysed only when a strong electron-withdrawing substituent (e.g. *p*-nitro group) is present in the aglycon group (Table III). Aryl β-D-glucopyranosides and β-D-xylopyranosides are good substrates. On the other hand, alkyl β-D-glucopyranosides and β-D-xylopyranosides are very poor substrates. Their 1-thio-analogues behave as competitive inhibitors.

Anomeric configuration of the reaction product

The anomeric configuration of the liberated D-glucose was determined by measuring its rate of oxidation by D-glucose oxidase [28]. To *p*-acetylphenyl β-D-glucopyranoside (0.6 mM) in 20 mM phosphate buffer (pH 6.7), enough (1.5 U/ml) β-D-glucosidase was added to give complete hydrolysis of the substrate after 1 min at 30°C. In a first experiment, the oxidation of D-glucose by the D-glucose oxidase-peroxidase-*o*-dianisidine system [16] was started immediately after hydrolysis. In a second experiment, oxidation was started after 50 min. Meanwhile mutarotation of the liberated D-glucose had taken place. As measured by the change in absorbance at 460 nm, the rate of oxidation was

TABLE III

SUBSTRATE SPECIFICITY

Enzyme units are based on β-D-glucosidase activity. K is the apparent association constant ($K = 1/K_m$). Enzyme modified with 5,5′-dithiobis-(2-nitrobenzoate) as described in the text.

Substrate, *P*-nitrophenyl derivative of	Native enzyme		Modified enzyme	
	K (M^{-1})	$10^6 \cdot V$ (mol · min^{-1} · U^{-1})	K (M^{-1})	$10^6 \cdot V$ (mol · min^{-1} · U^{-1})
β-D-glucopyranoside	18 000	1.11	18 700	0.757
β-D-xylopyranoside	5 700	0.245	6 080	0.172
1-Thio-β-D-glucopyranoside	1 360	0.0296		
6-Bromo-6-deoxy-β-D-glucopyranoside	17 800	0.276		
6-Chloro-6-deoxy-β-D-glucopyranoside	19 700	0.335		

higher in the first than in the second experiment. Since D-glucose oxidase is specific [28] for the β-anomer of D-glucose, the above findings indicate that the liberated D-glucose has the β-configuration.

The configuration of the reaction product D-xylose was determined with a coupled reaction of D-xylose isomerase and D-glucitol dehydrogenase [17,29]. β-D-Glucosidase (in such amount that hydrolysis was complete in less than 1 min) and the D-xylose isomerase system were added simultaneously to 3 mM *p*-chlorophenyl β-D-xylopyranoside in 40 mM maleate buffer (pH 6.8) at 30°C. β-D-Glucosidase and its substrate were also incubated for 30 min (to enable mutarotation of D-xylose) before the D-xylose isomerase system was added. The enzyme D-xylose isomerase is specific for α-D-xylose, converting it to α-D-xylulose. Glucitol dehydrogenase reduces α-D-xylulose to xylitol with simultaneous oxidation of NADH. The rate of oxidation of NADH (followed by measuring absorbance changes at 340 nm) thus depends on the concentration of α-D-xylose.

Whereas in the second experiment, the decrease in absorbance at 340 nm starts immediately after the addition of the D-glucitol dehydrogenase system, a lag period of several minutes was observed in the first experiment. This indicated that during the first minutes no α-D-xylose was present in the reaction mixture and thus that the anomeric configuration of the liberated D-xylose is β.

Work is now in progress to determine the anomeric configuration of reaction products when alcohols and other nucleophiles are added to the hydrolysis mixture and transferproducts are being formed.

Inhibition experiments

Using methyl 1-thio-β-D-glucopyranoside and *p*-chlorophenyl 1-thio-β-D-glucopyranoside as competitive inhibitors of respectively the β-D-glucosidase, β-D-xylosidase and thio-β-D-glucosidase activity (*p*-nitrophenyl derivatives as substrates) the inhibition constants K_i (association) were determined. These constants (27 ± 3 M^{-1} for the methyl and 2800 ± 200 M^{-1} for the *p*-chlorophenyl derivative) were independent of the activity tested, and agreed for the three activities within experimental error.

45 μM D-glucono-(1 → 5)-lactone causes a 50% inhibition of the hydrolysis of *p*-nitrophenyl β-D-glucopyranoside. However, since the inhibition pattern is complicated by the rapid hydrolysis of this lactone [30], no further quantitative measurements were performed. 10 μM Nojirimycin [30] (5-amino-5-deoxy-D-glucopyranose) caused a 50% inhibition, whereas for D-glucose a concentration of 13 mM was required for the same level of inhibition. The fact that nojirimycin and D-glucono-(1 → 5)-lactone are very potent inhibitors indicates that this enzyme is, according to Reese's criteria [30], a true β-D-glucosidase. The high affinity of the lactone and of nojirimycin, suggest that the glycon moiety of the normal substrates is bound to the enzyme in a conformation that resembles that of glucono-(1 → 5)-lactone. This lactone can assume a half-chair conformation [30] similar to that of a D-glucopyranosyl cation. Thus, it can be assumed that the hydrolysis proceeds by the generation of a glycosyl carbenium-oxonium ion-like structure, in which the pyranose ring has changed from a chair to half-chair conformation. The induction of the conformational change in the substrate should thus lead to a lowering of the free energy of activation.

Effect of group specific reagents

The enzyme is very sensitive to sulfhydryl [31] and histidine [32] reagents. Treatment with 2 mM iodoacetamide, 0.25 mM *p*-chloromercuribenzoic acid and 2.7 mM diethylpyrocarbonate for 50 min, resulted in total loss of activity. Treatment with 5,5′-dithio-bis-(2-nitrobenzoate) results in a loss of approx. 30% activity within a few minutes. The percentage inactivation was independent of the concentration (10 μM—0.5 mM) of the reagent. Thereafter, the remaining activity decreased slowly and became zero after approx. 6 h. However, when 0.1 M methyl β-D-glucopyranoside was added to the mixture, the slow inactivation reaction was not observed (Fig. 6). Treatment of the partially (30%) inactivated enzyme with 1 mM dithiothreitol reversed the inactivation and yielded a fully active enzyme.

When the fast inactivation reaction was analyzed as a pseudo-first order reaction, the plot of log[% activity] vs. time yielded a pseudo-first order rate coefficient of 0.034 min^{-1} for the β-D-glucosidase and 0.032 min^{-1} for the β-D-xylosidase activity. Using the modified enzyme, the kinetic parameters V and K were determined for *p*-nitrophenyl β-D-glucopyranoside and β-D-xylopyranoside. Comparison with the values for the unmodified enzyme (Table III) shows that the modification decreases V but has no effect on K. For both substrates the decrease in V amounts to 31%. The ratio β-D-glucosidase to β-D-xylosidase activity is 4.5 for the unmodified and 4.4 for the partially modified enzyme.

A more detailed study of the complex kinetics of these inactivation reactions is now in progress.

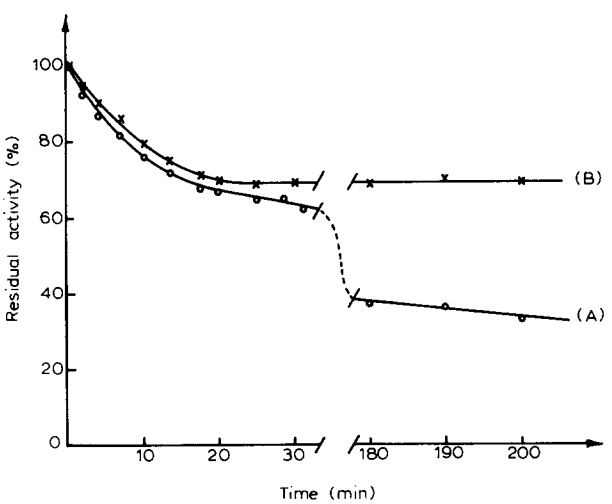

Fig. 6. Inactivation with 5,5′-dithiobis(2-nitrobenzoate). The enzyme was incubated at 30°C with excess reagent in phosphate buffer pH 7.5. (a) Without and (b) with methyl β-D-glucopyranoside (0.1 M). After different periods of incubation, the activity was measured with *p*-nitrophenyl β-D-glucopyranoside and expressed as % activity remaining.

Conclusion

This work indicates that the constitutive β-D-glucosidase from the fungus *S. atra* could be clearly differentiated from the induced β-D-glucosidase.

The study of both physical and kinetic properties of the induced β-D-glucosidase (isoelectric focusing data, inactivation patterns as a function of pH and temperature, inhibition experiments and inactivation study with DTNB) prove the identity of the three activities observed. Thus sufficient evidence has been obtained to conclude that these three activities may be attributed to the same active site.

We intend to investigate the catalytic properties of this enzyme using three series of substrates: glucosides, xylosides and the thioglucosides. In this way we hope to gain further insight to the mechanism of action of this glycosidase.

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