Appl Microbiol Biotechnol (1992) 37:590–593

Applied and Microbiology Biotechnology

Properties of β-glucosidase purified from *Aspergillus niger* mutants USDB 0827 and USDB 0828

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Received 8 January 1992/Accepted 9 April 1992

Summary. Two extracellular β-glucosidases (EC 3.2.1.21) were isolated from *Aspergillus niger* USDB 0827 and *A. niger* USDB 0828, and their physical and kinetic properties studied. Both enzymes were very similar in terms of molecular size (230000 Da), pH optimum (pH 4.6), temperature optimum (65° C), stability at high temperatures and substrate preferences. They were capable of hydrolysing β-linked disaccharides, phenyl β-D-glucoside, *p*-nitrophenyl β-D-glucoside (PNPG), *o*-nitrophenyl β-D-glucoside, salicin and methyl β-D-glucoside but lacked activity towards α-linked disaccharides, a range of *p*-nitrophenyl monoglycosides and *p*-nitrophenyl diglycosides. Both β-glucosidases were better at hydrolysing cellobiose than cellotriose, cellotetraose or cellopentaose. For both enzymes, glucose showed competitive inhibition with PNPG as substrate but had no effect with cellobiose. However, the two β-glucosidases differed in inhibition by glucono-1,5-lactone and affinity for cellobiose. β-Glucosidase from *A. niger* USDB 0827 also gave lower specific activity, and was more susceptible to metal ions (Ag^+, Fe^{2+} and Fe^{3+}) inhibition than that of *A. niger* USDB 0828.

Introduction

β-Glucosidase (cellobiase) is an important component of the cellulase complex. Not only does it catalyse the final step of cellulose degradation, i.e. the hydrolysis of cellulose-derived cellobiose to glucose, but it also removes the inhibitory effect of cellobiose on the other components of the cellulase complex (Woodward and Wiseman 1982). In view of its potential application in cellulose waste recycling, we have screened many local strains of fungi for cellulase and β-glucosidase activities (Tan et al. 1986; Yeoh et al. 1988). As reported elsewhere, we also found that *Aspergillus* spp. are good β-glucosidase producers. In this paper, we describe the characteristics of β-glucosidase (cellobiase) purified from two *A. niger* mutants, USDB 0827 and USDB 0828.

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Materials and methods

Organisms. A. niger USDB 0827 (Met^-) and *A. niger* USDB 0828 (Arg^-, His^-) were maintained on minimal agar medium supplemented with Met (200 μg/ml), and Arg and His (200 μg/ml each), respectively. The minimal medium contained in grams per litre of distilled water: $NaNO_3$, 6; $MgSO_4 \cdot 7H_2O$, 0.52; KCl, 0.52; KH_2PO_4, 1.52; glucose, 10; agar, 15; $FeSO_4 \cdot 7H_2O$, 0.001 and $ZnSO_4 \cdot 7H_2O$, 0.001 (Pontecorvo et al. 1953). For mycelia production, conidia of strains USDB 0827 and USDB 0828 (final conidium concentration, 1×10^6/ml) were separately cultured in minimal medium broth supplemented with Met (200 μg/ml), and Arg and His (200 μg/ml each), respectively, in a shaker incubator (130 rpm) at 30° C for 48 h. The mycelia, after washing with glucose-free minimal medium, were then grown in 2% (w/v) α-cellulose medium (Zhu et al. 1982) for 7 days at 30° C with shaking at 130 rpm for β-glucosidase production.

Purification of β-glucosidase. β-Glucosidase was purified as described in Yeoh et al. (1988) with one modification. The initial gel filtration step involving Sephadex G-200 was replaced with Sephacryl S-300 chromatography. Fractions collected were assayed for β-glucosidase and cellobiase activities. Protein content was assayed according to the method of Lowry et al. (1951). The purity of the enzyme preparation and estimation of its molecular size were carried out by electrophoresis in different polyacrylamide gel concentrations (Hedrick and Smith 1968).

Enzyme assays. β-Glucosidase activities of enzymes from strains USDB 0827 and USDB 0828 were measured using 4.5 mM and 4.0 mM *p*-nitrophenyl β-D-glucoside (PNPG), respectively. The reaction mixture was prepared in 50 mM sodium citrate (pH 4.6) and incubated at 60° C for 15 min (Yeoh et al. 1986). Cellobiase activity was determined using 5 mM cellobiose in 50 mM sodium citrate, pH 4.6, at 60° C for 15 min and the reaction terminated by boiling in a water bath for 5 min. The glucose released was determined by the D-glucose oxidase/peroxidase method (Raabo and Terkildsen 1960).

Kinetic studies. These were carried out using both PNPG and cellobiose as substrates in 50 mM sodium citrate, pH 4.6. The effect of pH on enzyme activity was studied for the pH range 3.0–6.0 using 50 mM sodium citrate-citric acid buffer. The temperature optimum was determined over the range 30–70° C at pH 4.6,

whereas thermal stability of the enzyme was investigated for the range 50–70° C at pH 4.6 over a period of 3 h. The ability of the enzyme to hydrolyse different disaccharides, β-glycosides, soluble cello-oligosaccharides and *p*-nitrophenyl (PNP) β-glycosides was carried out at 5 mM. K_m values were determined using PNPG (up to 4.5 mM for USDB 0827 and up to 4 mM for USDB 0828), cellobiose (up to 10 mM), phenyl β-D-glucoside (up to 8.5 mM), *o*-nitrophenyl β-D-glucoside (ONPG) (up to 8.5 mM), salicin (up to 25 mM) and methyl β-D-glucoside (up to 65 mM). The K_m values were calculated as in Wilkinson (1961). Inhibition constant values (K_i for competitive inhibition; K_{iE} and K_{iES} for mixed inhibition) were determined using the above substrate concentrations and in the presence of 1–10 mM glucose or 0.1–1 mM glucono-1,5-lactone. The effects of metal ions at concentrations of 1 to 5 mM on enzyme activity were also studied.

Results and discussion

Strains USDB 0827 and USDB 0828 are mutants requiring Met, and Arg and His respectively for growth. These two mutants were morphologically different and, when cultured in α-cellulose medium, the β-glucosidase activity of USDB 0827 was about twice that of USDB 0828. Thus, it would be interesting to examine if there were other differences in the β-glucosidases of these mutants. We purified from both mutants the β-glucosidase fraction that exhibited cellobiase activity and characterized them.

For both mutants, two forms of β-glucosidases were separated by Sephacryl S-300 chromatography but only one form exhibited cellobiase activity. The higher-molecular-mass β-glucosidase representing 77% of total enzyme activity could hydrolyse PNPG, cellobiose and soluble cello-oligosaccharides whereas the lower-molecular-mass β-glucosidase (the minor fraction) exhibited activity only with PNPG. The presence of two β-glucosidases in these mutants was not unexpected because multiple β-glucosidases have been observed in many fungi, including *Aspergillus* spp. (Deshpande et al. 1978; Sharma et al. 1991).

In this work, we concentrated our effort on characterizing the β-glucosidase with cellobiase activity from both mutants as this enzyme is relevant to cellulose hydrolysis. The enzymes purified from both sources were homogeneous in polyacrylamide gel electrophoresis and exhibited the same molecular mass of 230000 Da. A wide range of molecular sizes from 47000 to 340000 Da has been reported for β-glucosidases from different fungi (Shewale 1982). By comparison, the molecular size of USDB 0827 and USDB 0828 β-glucosidase is similar to that reported for the enzyme from *Macrophomina phaseolina* (Saha et al. 1981) but smaller than those isolated from *Botryodiplodia theobromae* (Woodward and Wiseman 1982) and *A. niger* USDB 0355 (Yeoh et al. 1988).

The β-glucosidases of both mutants showed a similar pH optimum value of 4.6 with cellobiose and PNPG as substrates. For both enzymes, 95% of maximum activity was still seen at pH 4.3 and 5.3. This pH optimum is similar to those reported for other *Aspergillus* spp. but is lower than those of *Saccharomyces* and *Candida* spp. (Woodward and Wiseman 1982).

Table 1. Substrate specificity of β-glucosidases of *Aspergillus niger* USDB 0827 and *A. niger* USDB 0828

Substrate	Specific activity (nkat/mg protein)	
	USDB 0827	USDB 0828
Laminaribiose	2408	2416
Cellobiose	2114	2326
PNPG	1917	1940
β-Gentiobiose	1696	1728
Phenyl β-D-glucoside	1213	1355
ONPG	742	622
Salicin	327	385
Methyl β-D-glucoside	248	215

PNPG, *p*-nitrophenyl β-D-glucoside; ONPG, *o*-nitrophenyl β-D-glucoside

The enzymes from both mutants also showed a similar temperature optimum value of 65° C. However, the enzymes were not stable at this temperature and lost 15% and 40% of their activities after 10 min and 30 min, respectively. At 60° C, the enzymes maintained 95% of their activities for up to 3 h. The enzymes were very stable at 50° C; they retained 85% of their activities even after 72 h. These features were also seen in *A. wentii* (Srivastava et al. 1984) and *A. niger* USDB 0355 (Yeoh et al. 1988).

β-Glucosidases from strains USDB 0827 and USDB 0828 showed similar substrate preferences although the relative activities were different (Table 1). Both enzymes were able to hydrolyse a variety of β-linked disaccharides, namely cellobiose, β-gentiobiose and laminaribiose, and not the α-linked disaccharides, namely sucrose, maltose, D(+)trehalose, α-D(+)melibiose and isomaltose. Thus, the enzymes were not restricted to only the β-D-(1→4) linkage, but could easily hydrolyse β-D-(1→3) and β-D-(1→6) linkages. The nature of the aglycone moiety is important for enzyme activity. Both enzymes were good at hydrolysing the β-linkage of phenyl or nitrophenyl groups but poor at hydrolysing that of (hydroxymethyl)phenyl (in salicin) or methyl groups (Table 1). The position of substitution of a nitro group in the benzene ring of phenyl β-D-glucoside also appeared to influence the rate of hydrolysis. The rate of hydrolysis was lower with the substrate bearing the nitro group in the *ortho* position (in ONPG) than that in the *para* position (in PNPG). Both enzymes showed no activity towards the β-linkage of a hydroquinone (in arbutin) or a phloretin (in phloridzin) group. The nature of the glycone moiety is also important for enzyme activity. Although both enzymes hydrolysed PNPG, they were inactive on PNP β-D-xyloside, PNP β-D-galactoside, PNP β-D-fucoside and PNP β-D-mannoside. The enzymes also did not hydrolyse glycosides with a disaccharide structure, namely PNP β-D-cellobioside, PNP β-D-gentiobioside and PNP β-D-lactoside. This is in contrast to the β-glucosidase of *A. niger* 15, which was reported to hydrolyse PNP β-D-xyloside, PNP β-D-galactoside, PNP β-D-cellobioside and PNP α-L-arabinoside (Tavobilov et al. 1984). It can be seen here that the na-

Table 2. Kinetic parameters of β-glucosidases of *A. niger* USDB 0827 and *A. niger* USDB 0828

Property	USDB 0827	USDB 0828
K_m values (mM)		
PNPG	0.75±0.05	0.89±0.08
Cellobiose	1.23±0.05	1.64±0.06
β-Gentiobiose	0.96±0.06	1.13±0.09
Phenyl β-D-glucoside	1.41±0.08	1.47±0.07
ONPG	2.18±0.14	2.39±0.19
Salicin	7.01±0.75	7.10±0.76
Methyl β-D-glucoside	14.22±0.89	14.60±0.66
V_{max}/K_m (nkat/mg protein mM)		
PNPG	3067	3629
Cellobiose	2274	2091
β-Gentiobiose	2094	1841
Phenyl β-D-glucoside	1099	1197
ONPG	472	368
Salicin	203	197
Methyl β-D-glucoside	68	58

ture of the aglycone and glycone moieties of the glycoside is important for enzyme activity although it was less stringent on the aglycone moiety.

With cellobiose and PNPG as substrates, β-glucosidases from both mutants showed typical Michaelis-Menten kinetics. PNPG concentrations greater than 4.5 mM were inhibitory to mutant USDB 0827 β-glucosidase, while for the USDB 0828 enzyme it was greater than 4 mM. However with cellobiose, no substrate inhibition was observed even at 10 mM. In both cases, the enzymes showed a greater affinity for PNPG than for cellobiose (Table 2). These K_m values were quite similar to those previously reported for *A. ornatus* (Yeoh et al. 1986) and *A. niger* USDB 0355 (Yeoh et al. 1988). A range of K_m (PNPG) and K_m (cellobiose) values has been reported from different fungi but this may be attributed to different conditions employed in its determination. A comparison of K_m (cellobiose) indicated that USDB 0827 β-glucosidase had a greater affinity for cellobiose than the USDB 0828 enzyme. Differences were also seen in the specific activities of the enzymes: USDB 0827 β-glucosidase gave specific activities that were lower by 25–30% than those of the USDB 0828 enzyme. The corresponding kinetic parameters were: $V_{max} = 2302 \pm 56$ versus 3233 ± 100 nkat/mg protein for PNPG; $V_{max} = 2566 \pm 40$ versus 3425 ± 10 nkat/mg protein for cellobiose.

The kinetic parameters for hydrolysis of other substrates are also presented in Table 2. It appears that there is some correlation between the K_m values and the rate of hydrolysis (see also Table 1). This suggests that the rate of hydrolysis is due, at least in part, to the respective K_m values. A comparison of V_{max}/K_m for the various substrates reaffirmed that both β-glucosidases showed a greater preference for structures having an aryl group and that substitution of a nitro group in the *para* position of a benzene ring is important for catalysis (Table 2).

Both enzymes were better at hydrolysing cellobiose than cellotriose, cellotetraose or cellopentaose (Table 3).

Table 3. Hydrolysis of cellobiose and soluble cellooligosaccharides by β-glucosidases of *A. niger* USDB 0827 and *A. niger* USDB 0828

Substrates	Specific activity (μmol glucose/min/mg protein)	
	USDB 0827	USDB 0828
D-(+)-Cellobiose	254	279
D-(+)-Cellotriose	153	158
D-(+)-Cellotetraose	132	140
D-(+)-Cellopentaose	116	121

The preferential hydrolysis of cellobiose by these enzymes would expedite the removal of the inhibitory effect of cellobiose on the other components of the cellulase complex, thus increasing the efficiency of cellulose degradation. This feature is similar to that observed for *Trichoderma viride* (Berghem and Pettersson 1974) but is different from those of *A. aculeatus* (Sakamoto et al. 1985) and *Schizophyllum commune* (Lo et al. 1990). β-Glucosidases isolated from *A. aculeatus* and *S. commune* have been reported to carry out more efficient hydrolysis of soluble cello-oligosaccharides than of cellobiose.

Glucose and glucono-1,5-lactone inhibited β-glucosidase activity of strains USDB 0827 and USDB 0828. With PNPG as substrate, glucose inhibition was competitive and K_i values for USDB 0827 and USDB 0828 were 3.22 mM and 3.78 mM, respectively. No glucose inhibition (up to 10 mM glucose) of both enzymes was observed when cellobiose was used as the substrate. The differential effects of glucose on the hydrolysis of cellobiose and PNPG are unexpected and it is unclear what they signify. Glucose has also been reported to inhibit β-glucosidase non-competitively (Gong et al. 1977). Glucono-1,5-lactone inhibition was of non-competitive (mixed) type for enzymes from both mutants. Using PNPG was substrate, the K_{iE} and K_{iES} values were 0.24 mM and 3.78 mM respectively for USDB 0827, and 0.29 mM and 16.31 mM, respectively, for USDB 0828. With cellobiose as substrate, the K_{iE} and K_{iES} values were 0.23 mM and 1.39 mM, respectively, for USDB 0827, and 0.20 mM and 6.69 mM, respectively, for USDB 0828. A comparison of K_{iES} (glucono-1,5-lactone) indicated that the affinity of the inhibitor for enzyme-substrate complex was markedly greater for USDB 0827 than for USDB 0828. Glucono-1,5-lactone has also been reported to inhibit β-glucosidase activity competitively (Sanyal et al. 1988).

The effects of metal ions on β-glucosidase activity was studied using both cellobiose and PNPG as substrates. Enzymes from both mutants were inhibited by Ag^+, Fe^{2+} and Fe^{3+}, with Ag^+ showing greater inhibition, followed by Fe^{2+} and Fe^{3+}. With PNPG as substrate, the percentage inhibition by Ag^+, Fe^{2+} and Fe^{3+} at 5 mM was 51%, 23% and 14%, respectively, for USDB 0827; and 46%, 20% and 5%, respectively, for USDB 0828. Using cellobiose as substrate, the percentage inhibition by Ag^+, Fe^{2+} and Fe^{3+} at 1 mM was

50%, 25% and 5%, respectively, for USDB 0827; and 34%, 11% and 3%, respectively, for USDB 0828. Thus, β-glucosidase from USDB 0827 was relatively more susceptible to metal ion inhibition than that of USDB 0828. Both enzymes were relatively unaffected by Ca^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}. β-Glucosidase from *A. ornatus* (Yeoh et al. 1986) has also been shown to be inhibited by Ag^{+} and Fe^{2+} whereas β-glucosidase from *A. terreus* was relatively unaffected by metal ions (Workman and Day 1982).

Although USDB 0827 and USDB 0828 differed in their ability to produce β-glucosidase in α-cellulose medium, the purified enzymes from both mutants have many properties in common, such as similar molecular mass, pH and temperature optima, stability at high temperatures, glucose inhibition, and range of substrates. Nonetheless, they also differed in some respects. They had different specific activities, K_m (cellobiose), K_{iES} (glucono-1,5-lactone) and susceptibility to metal ion inhibition. It would appear that the catalytic site of both enzymes might be similar and subtle structural differences are reflected in the different activities on different substrates, and in the different effects of glucono-1,5-lactone as an inhibitor.

Acknowledgements. This work was supported by a National University of Singapore research grant (RP 860333). HYK is the recipient of a National University of Singapore Research Scholarship.

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