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Kinetic Properties of β-glucosidase from *Aspergillus ornatus*

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Summary. Kinetic properties of extracellular β-glucosidase from *Aspergillus ornatus* were determined. The pH and temperature optima for the enzyme were found to be 4.6 and 60° C, respectively. Under these conditions, the enzyme exhibited a K_m (p-nitrophenyl-β-glucoside) value of 0.76 ± 0.11 mM. The activation energy for the enzyme was 11.8 kcal/mol. Several divalent metal ions inhibited β-glucosidase activity, some of which showed inhibition of enzyme activity only at higher concentrations. Ag^{2+} was the most potent inhibitor. A metal chelating agent, EDTA, also inhibited β-glucosidase activity. Except for trehalose, glucose, glucono-δ-lactone, cellobiose, gentiobiose, laminaribiose, maltose and isomaltose inhibited β-glucosidase activity. Glucose was found to be a competitive inhibitor, whereas glucono-δ-lactone and other β-linked disaccharides were noncompetitive (mixed) inhibitors of the enzyme.

Introduction

β-glucosidase (E.C. 3.2.1.21), an essential component of the cellulase complex, is responsible for the final step in cellulose degradation, namely, the hydrolysis of cellulose-derived glucoside cellobiose to glucose. Much attention has been given to this enzyme primarily because the efficiency of this step, which is governed not only by the amount of enzyme present but also by other kinetic parameters, is critical to the overall breakdown of cellulose (Woodward and Wiseman 1982; Parr 1983).

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It is interesting that *Trichoderma* species, seemingly the best source of cellulose solubilising activities, are poor producers of β-glucosidase (Ryu and Mandels 1980). On the other hand, *Aspergillus* species have been shown to be better producers of β-glucosidase (Woodward and Wiseman 1982). In view of the potential uses of this enzyme in cellulose waste recycling, it may be desirable to study this enzyme from as many different sources. In this respect, we have looked at the production of this enzyme from several fungal species (Yeoh et al. 1984; Tan et al. 1985). In this paper we describe our study on the kinetic properties of β-glucosidase from *Aspergillus ornatus* USDB 0313 and discuss its relevance to cellulose hydrolysis.

Materials and methods

Organisms. Aspergillus ornatus USDB 0313 stock cultures were maintained on 4.5% (w/v) Czapek Dox Agar slants. One ml of spore suspension (0.5×10^9 spores) was inoculated into 100 ml of glycerol medium for mycelia production (Zhu et al. 1982). After three days of incubation in a reciprocating shaker at 29° C in continuous darkness, the mycelia were harvested. About 1 g fresh weight mycelia was then transferred into 100 ml 1% (w/v) cellulose medium (Zhu et al. 1982) and further incubated in a reciprocating shaker for 14 days at 29° C in continuous light.

Preparation of β-glucosidase. The fungal culture was filtered to remove the mycelia and the filtrate stored frozen. When needed the filtrate was thawed. One ml filtrate was eluted through a Sephadex G-25 column (1.5 × 28 cm) with 50 mM Na citrate pH 4.6, previously equilibrated with the same buffer. Fractions of 1.3 ml each were collected and assayed for β-glucosidase activity. Fractions active in enzyme activity were pooled and used for kinetic studies.

Enzyme assay. β-glucosidase was measured with p-nitrophenyl-β-glucopyranoside (PNPG). The reaction mixture containing 125 µl 6.6 mM PNPG, 250 µl 50 mM Na citrate and 100 µl

distilled water was equilibrated at 60° C for 10 min before 25 µl of enzyme preparation was added. After 30 min, it was stopped by the addition of 2.5 ml 100 mM Na_2CO_3. The absorbance of the resulting mixture was then read at 420 nm. Appropriate enzyme and substrate blanks were included. A calibration curve was also prepared using p-nitrophenol as standard.

Protein assay. Protein was determined using the modified Lowry's method with bovine serum albumin as a standard (Miller 1959).

Kinetic studies. The kinetic properties of β-glucosidase were investigated using the assay procedure described above, but with different substrate concentrations, or in the presence of metal ions or organic compounds. K_m value was calculated using the method of Wilkinson (1961). Where applicable, the inhibition kinetics of β-glucosidase were studied using a range of substrate concentrations (0.17 to 16.6 mM PNPG) and in the presence of different inhibitor concentrations (0.1 to 10 mM). Inhibition types were determined by plotting the Lineweaver-Burke (LB) plots and the K_i's were calculated from plots of slopes/intercepts of LB plots against inhibitor concentrations (Engel 1981).

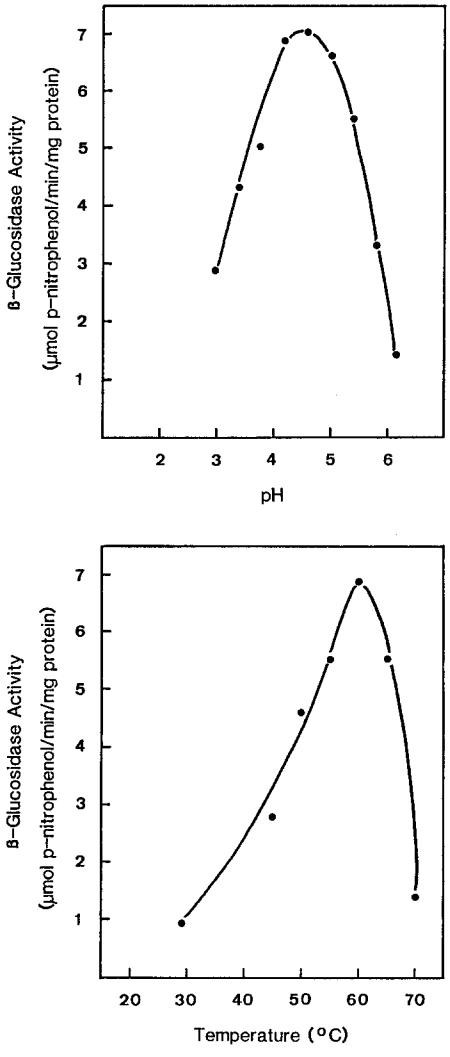

Fig. 1. Effect of pH and temperature on β-glucosidase activity of *A. ornatus*

Results and discussion

Figure 1 shows the pH and temperature optima for β-glucosidase activity from *A. ornatus* to be 4.6 and 60° C, respectively. The optimum pH for β-glucosidase is within the range of values reported for this enzyme from other *Aspergillus* species, some *Trichoderma* species, *Coriolus versicolor* and *Penicillium funiculosum* and is much lower than that of enzyme from *Saccharomyces* and *Candida* (Workman and Day 1982; Woodward and Wiseman 1982; Parr 1983; Evans 1985). The temperature optimum for this enzyme, however, is higher than those reported for the enzyme from *Saccharomyces* species, *Candida guilliermondii* and *Coriolus versicolor,* but lower than that for *Thermoascus aurantiacus* (Woodward and Wiseman 1982; Evans 1985). The activation energy of β-glucosidase activity calculated from Arrhenius plot was 11.8 kcal/mol, this being lower than the value reported for a *Saccharomyces* β-glucosidase (Hu et al. 1960).

Under the optimum conditions, β-glucosidase exhibited the Michaelis Menten type kinetics and its K_m value using PNPG as substrate was 0.76 ± 0.11 mM (Fig. 2). This value is closely similar to that of the enzyme assayed directly from the culture filtrate ($K_m = 0.84 \pm 0.12$ mM), suggesting that media composition employed in the this study has little influence on the affinity of the enzyme for its substrate. It was also observed that at PNPG concentration in excess of 7 mM, there was some inhibition of enzyme activity. Substrate inhibition of β-glucosidase activity has also been reported for the enzyme from different sources (Parr 1983; Evans 1985). K_m (PNPG) values ranging from 0.10 to 44 mM PNPG have been reported for β-glucosidase from different fungal sources, including those from *Aspergillus* species (Workman and Day 1982; Woodward and Wiseman 1982; Parr 1983; Evans 1985). Such variations in K_m values could be attributed to different conditions of enzyme assay.

Table 1 shows the effect of eight divalent metal ions and EDTA on β-glucosidase activity. This was carried out to assess whether metal ions would activate or inactivate β-glucosidase activity and if the low enzyme activity in the culture filtrate could be due to the presence of such ions.

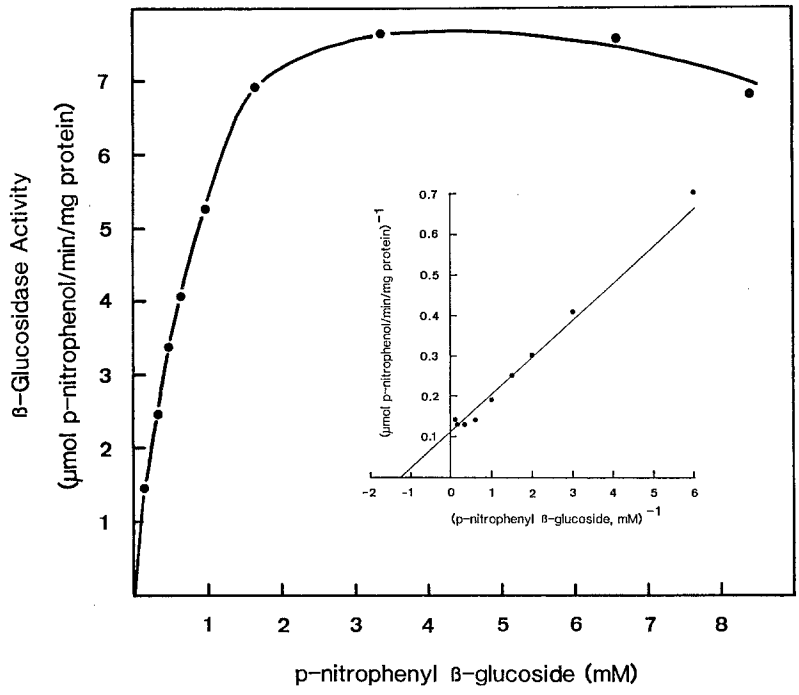

Fig. 2. Effect of substrate concentration on β-glucosidase activity of *A. ornatus*. Inset is the Lineweaver-Burke plot of β-glucosidase activity

Table 1. Effect of metal ions and EDTA on β-glucosidase activity

Metal ions	Percent activity retained				
	Concentration (mM)				
	0.1	1.0	5.0	10.0	25.0
Ag^{2+}	78	42	0	—	—
Ca^{2+}	100	100	89	94	—[a]
Co^{2+}	100	100	100	94	78
Cu^{2+}	100	100	100	64	16
Fe^{2+}	94	78	72	56	0
Mg^{2+}	100	100	100	94	90
Mn^{2+}	92	92	97	89	—[a]
Zn^{2+}	100	91	92	92	—[a]
EDTA	100	100	74	61	23

[a] β-glucosidase activity not taken due to interference with assay procedure

Table 2. Effect of glucose, glucono-δ-lactone and different disaccharides on β-glucosidase activity

Effector molecules	Percent activity retained			
	Concentration (mM)			
	0.1	1.0	5.0	10.0
Glucose	92	73	42	45
Glucono-δ-lactone	73	54	18	7
Trehalose	100	102	102	102
Maltose	97	97	97	85
Isomaltose	85	91	85	79
Laminaribiose	97	95	58	38
Cellobiose	70	61	38	22
Gentiobiose	79	61	36	21

Ag^{2+} and Fe^{2+} completely inhibited enzyme activity at 5 mM and 25 mM, respectively whereas Cu^{2+} and EDTA, at 25 mM, showed 84% and 77% inhibition of enzyme activity, respectively. Up to 10 mM, Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} had little inhibitory effect on β-glucosidase activity.

Metal inhibition of enzyme activity has also been observed for β-glucosidase from other fungal sources. *A. niger* β-glucosidase has been reported to exhibit partial inhibition by Ag^{2+} and Fe^{2+}, whereas *A. terreus* β-glucosidase was relatively unaffected by metal ions (Okada 1985; Workman and Day 1982). β-glucosidase from a *Saccharomyces* hybrid was inhibited by Zn^{2+}, Cu^{2+} and Fe^{2+} but not by EDTA and that from *Candida* was also inhibited by Zn^{2+}, Cu^{2+} and Co^{2+} (Hu et al. 1960; Kohchi et al. 1985). Since many of these metals ions are included either as mineral ions or trace elements in the growth medium, it is important that these levels should not reach concentrations inhibitory to β-glucosidase activity.

Table 2 shows the effect of glucose, glucono-δ-lactone and some disaccharides on β-glucosidase activity. At 10 mM, glucono-δ-lactone inhibited 93% of enzyme activity whereas both cellobiose and gentiobiose inhibited about 80% of enzyme activity. Laminaribiose and glucose inhibited about 60% and 55% of enzyme activity, respectively. By contrast, the α-linked disaccharides showed little inhibition on β-glucosidase activity.

Since the various compounds chosen for study are and could be possible constituents of both native and degradation products of cellulose and starch, their effect on β-glucosidase activity would be relevant to the hydrolysis of cellulosic wastes, such as spent brewery and soy bean wastes. Inhibition of β-glucosidase activity by glucose, gluconolactone and cellobiose has been reported for the enzyme from different sources (Woodward and Wiseman 1982; Parr 1983; Uziie et al. 1985). However, little is known of the inhibitory effect of laminaribiose and gentiobiose on other β-glucosidases. It is interesting to note that some of these disaccharides have been reported to regulate the synthesis of fungal β-glucosidase (Sternberg and Mandels 1982).

In view of the above, the inhibition kinetics of β-glucosidase by glucose, glucono-δ-lactone and the various β-linked disaccharides were studied with respect to PNPG as substrate. Table 3 summarises the different mechanisms of inhibition and their respective kinetic constants. Glucose showed competitive inhibition of β-glucosidase and the K_i value was 5.13 mM. Glucono-δ-lactone, an oxidized product of glucose however, gave non-competitive (mixed) inhibition and the two inhibition constants were Kie = 0.24 mM and Kies = 13.70 mM. These values reflect the higher affinity between the inhibitor and the free enzyme (Kie) than that between the inhibitor and the enzyme-substrate complex (Kies). The high binding affinity between glucono-δ-lactone and the enzyme explains the effectiveness of the molecule as an inhibitor.

The various β-linked disaccharides, namely cellobiose, gentiobiose and laminaribiose, all showed non-competitive (mixed) inhibition and their respective Kie and Kies values are given in Table 3. In all cases, the Kie values were lower than the Kies values, suggesting somewhat similar mechanism of inhibition as that of glucono-δ-lactone. In terms of their effectiveness as inhibitors, glucose is the weakest and glucono-δ-lactone the strongest inhibitor, with the other β-linked disaccharides as intermediates. Inhibition of other fungal β-glucosidase activity by glucose has been reported to be both competitive and noncompetitive and a variation in Ki values was also observed (Woodward and Wiseman 1982; Parr 1983). Gluconolactone has also been reported to be a competitive inhibitor of fungal β-glucosidase (Uziie et al. 1985).

Table 3. Inhibition kinetics of β-glucosidase

Inhibitors	Inhibition type	Dissociation constants (mM)		
		Ki	Kie	Kies
Glucose	Competitive	5.13	na	na
Glucono-δ-lactone	Noncompetitive (mixed)	na	0.24	13.70
Laminaribiose	Noncompetitive (mixed)	na	1.62	24.38
Cellobiose	Noncompetitive (mixed)	na	2.74	16.34
Gentiobiose	Noncompetitive (mixed)	na	3.27	24.56

na = not applicable

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