Purification and properties of an extracellular α -glucosidase from the thermophilic fungus *Malbranchea sulfurea*

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An extracellular α -glucosidase from the thermophilic fungus *Malbranchea sulfurea* was purified 31·6-fold with a yield of 11·68 %. The size of the enzyme was 110 kDa. The optimum pH and temperature for the enzyme activity were pH 4·8 and 60 °C, respectively; the half-life of the activity was 45 min at 65 °C, 125 min at 60 °C, and 6 h at 55 °C. The substrate preference of the α -glucosidase was *p*-nitrophenyl- α -D-glucoside > sucrose > maltose > maltotriose. No activity was recorded against isomaltose, methyl- α -D-glucoside and starch.

Introduction

α -Glucosidase (α -D-glucoside glucohydrolase, EC 3.2.1.20) hydrolyses 1,4- α -glucosidic linkages in oligosaccharides rapidly, relative to such linkages in polysaccharides, which are hydrolysed either slowly or not at all (Saha & Zeikus, 1991). α -Glucosidase is the final enzyme involved in the metabolism of starch to glucose, and is used with α -amylase for the saccharification of starch. α -Glucosidases are produced by micro-organisms in response to growth on starch-containing medium, and vary widely in their substrate specificity (Kelly & Fogarty, 1983). Bacterial and fungal α -glucosidases are reported to be specific for maltose hydrolysis (Kelly *et al.*, 1987) whereas yeast α -glucosidases hydrolyse *p*-nitrophenyl- α -D-glucoside at a higher rate than maltose (Kelly & Fogarty, 1983; Kelly *et al.*, 1987).

The enzymes of thermophilic micro-organisms are of potential industrial importance due to their thermostability. There are many advantages in the use of thermostable enzymes in the starch processing industry, e.g. increased reaction rates and decreased contamination risk (Kelly *et al.*, 1986). High activity and stability at high temperatures are the required features for an ideal amylolytic enzyme; thermophilic micro-organisms are therefore often the first-choice source when looking for a new enzyme (Melasniemi, 1987). Various reports have appeared on the production and characterization of α -glucosidase from the thermophilic and hyperthermophilic archaeobacteria (Giblin *et al.*, 1987; Costantino *et al.*, 1990; Saha & Zeikus, 1991); but no α -glucosidases

from the thermophilic fungi have yet been described. We recently described the production of extracellular and mycelial α -glucosidases, and other amylases, by the thermophilic fungus *Malbranchea sulfurea* (Gautam & Gupta, 1992; Gupta & Gautam, 1992). The present communication reports the purification and properties of an extracellular α -glucosidase from *M. sulfurea*.

Methods

Organism and culture conditions. *Malbranchea sulfurea* HDBJ AG 30 is a thermophilic fungus, originally isolated from cow dung. It was maintained on the slopes of potato dextrose agar (PDA) medium at 45 °C. The medium for α -glucosidase production contained (g l⁻¹): soluble starch, 10; yeast extract, 4; K₂HPO₄, 3; MgSO₄·7H₂O, 0·5. The medium was adjusted to pH 7·0, dispensed in 50 ml portions into 250 ml Erlenmeyer flasks, and sterilized by autoclaving at 15 lb for 15 min. The medium in each flask was inoculated with 10⁶ conidia of *M. sulfurea*, harvested from 7-d-old cultures grown on PDA medium. The flasks were incubated for 9 d at 45 °C without shaking. Mycelia were removed from the culture medium by filtration through Whatman no. 42 filter paper and then Sartorius membrane filter (0·45 μ m). The cell-free culture filtrate was assayed for α -glucosidase activity.

α -Glucosidase assay. α -Glucosidase activity was assayed by measuring the release of glucose from maltose and *p*-nitrophenyl- α -D-glucoside (pNPG). The substrates (1%, w/v) for the assay were prepared in 0·05 M-citrate/phosphate buffer, pH 4·8. Assay mixtures contained 1 ml substrate solution and 1 ml enzyme sample. After incubation for 30 min at 50 °C, the reaction was stopped by keeping the tubes in a steam bath for 15 min and the liberated glucose was analysed by the glucose-oxidase-peroxidase-*O*-dianisidine method (Hugget & Nixon, 1955). One unit of α -glucosidase activity was defined as the amount of enzyme that liberated 1 μ mol glucose or glucose equivalent min⁻¹ from the appropriate solutions under the above conditions. Protein was measured by the Lowry method using BSA as the standard.

Purification of α -glucosidase. All purification steps were carried out at 4 °C. Where needed, the enzyme sample was concentrated by

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Table 1. Summary of purification of α -glucosidase from *Malbranchea sulfurea*Data are means $\pm$ SEM of four determinations.

Step no.	Procedure	Protein (mg)	Enzyme activity (IU)	Specific activity (IU mg ⁻¹)	Purification (fold)	Yield (%)
1.	Cell-free enzyme sample	224 $\pm$ 12.1	28 $\pm$ 1.74	0.125 $\pm$ 0.011	—	100
2.	Vacuum concentration and dialysis	48.004 $\pm$ 3.4	17.325 $\pm$ 1.21	0.3609 $\pm$ 0.041	2.876 $\pm$ 0.484	61.87 $\pm$ 6.7
3.	Sephadex G-200 gel filtration	8.5 $\pm$ 0.4	10.9 $\pm$ 1.04	1.23 $\pm$ 0.101	10.293 $\pm$ 1.3	38.9 $\pm$ 3.6
4.	HPLC of fraction 1 of Sephadex G-200 gel filtration	0.83 $\pm$ 0.05	3.26 $\pm$ 0.021	3.95 $\pm$ 0.038	31.6 $\pm$ 4.6	11.68 $\pm$ 0.811

vacuum evaporation at 4 °C. The cell-free enzyme sample was concentrated ten-fold and dialysed for 24 h against 0.05 M-citrate/phosphate buffer, pH 5.0. The concentrated and dialysed enzyme sample was applied to a Sephadex G-200 column (1.56 $\times$ 50 cm, void volume 28.2 ml) and eluted at 6 ml h⁻¹ with degassed and filtered 0.05 M-citrate/phosphate buffer, pH 5.0. Fractions of 2.2 ml volume were collected. The active α -glucosidase fractions were pooled and concentrated by vacuum evaporation to a final volume of 2.0 ml. The concentrated enzyme sample thus obtained was applied to a Waters Protein Pack HPLC P 300 column (20 μ l injection), and eluted with 0.05 M-citrate/phosphate buffer, pH 5.0. The HPLC separation was repeated until sufficient purified enzyme was obtained. The active α -glucosidase fractions were pooled and concentrated by vacuum evaporation.

SDS-PAGE and molecular mass determination. The homogeneity of the α -glucosidase preparation obtained after HPLC was determined by SDS-PAGE on 7.5% (w/v) gel (Weber & Osborn, 1975). The molecular mass of the purified α -glucosidase was also determined by SDS-PAGE. The following proteins were used for calibration: phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), chicken egg albumin (45 kDa), carbonic anhydrase (29 kDa), and lactalbumin (14.2 kDa).

The experiments were repeated at least four times and the data presented here are the arithmetic mean of the observations.

Chemicals were of analytical reagent grade, purchased from Sigma, BDH, Hi-media (India) and Loba (India). Sephadex G-200 (superfine) was from Pharmacia; dialysis bags were from Sigma.

Results and Discussion

Purification of the α -glucosidase

A summary is presented in Table 1. Gel filtration of the concentrated and dialysed enzyme sample through Sephadex G-200 resulted in two protein peaks. α -Glucosidase activity was recorded in the fractions corresponding to protein peak I, while protein peak II showed no α -glucosidase or maltase activity (Fig. 1). HPLC of the concentrated α -glucosidase fractions obtained after passage through Sephadex G-200 resulted in four protein peaks. The α -glucosidase activity was

Fig. 1. Gel filtration of the concentrated and dialysed α -glucosidase preparation from Sephadex G-200 column (1.56 $\times$ 50 cm). The protein was eluted with 0.05 M-citrate/phosphate buffer, pH 5.0, and 2.2 ml fractions were collected. ●, Absorbance at 280 nm; ○, α -glucosidase activity. Error bars are SEM from at least four observations.

Fig. 2. HPLC of the α -glucosidase fractions obtained from the Sephadex G-200 column, pooled and concentrated by vacuum evaporation. Twenty μ l of the concentrated enzyme preparation was injected, and eluted with 0.05 M-citrate/phosphate buffer, pH 5.0. Proteins were monitored as A_{280} (—). ○, α -Glucosidase activity. Error bars are SEM from at least four observations.

detected in fractions corresponding to protein peak II (Fig. 2). The α -glucosidase was purified 31.6-fold with 11.68 % yield. Considering that the enzyme production

Fig. 3. Estimation of molecular mass of the *M. sulfurea* α -glucosidase by SDS-PAGE. Protein standards were: I, lactalbumin (14.2 kDa); II, carbonic anhydrase (29 kDa); III, chicken egg albumin (45 kDa); IV, bovine serum albumin (66 kDa); V, phosphorylase b (97.4 kDa); X, α -glucosidase from *M. sulfurea* (110 kDa). 50 μ l purified enzyme (25 μ g protein) was applied to the gel. Electrophoresis was for 4 h at a constant current of 8 mA. The gel was stained with Coomassie brilliant blue stain. Error bars are SEM of four observations.

medium contained 4 g l⁻¹ yeast extract, the purification achieved here is very low. The low purification factor may be due to the fact that the fungus was grown in the medium for 9 d, during which time the fungus is expected to utilize most of the supplemented yeast extract. The crude α -glucosidase thus obtained may have very low amounts of the yeast extract and other proteins to be separated, which would be a possible reason for the low purification factor achieved. SDS-PAGE of the preparation obtained after HPLC resulted in only one protein band, indicating homogeneity of the purified enzyme. The molecular mass of the purified enzyme was estimated as 110 ± 0.15 kDa by SDS-PAGE (Fig. 3). The molecular masses of most reported microbial α -glucosidases are below 70 kDa (Kelly & Fogarty, 1983); however, α -glucosidases of high molecular mass (125 kDa, 130 kDa and 140 kDa) have been reported in *Aspergillus awamori* (Yamasaki *et al.*, 1977).

Activity and stability of the enzyme

The α -glucosidase showed maximum activity at pH 4.8, and was stable at pH 4–5.8 (Fig. 4). In general, the reported pH optima of fungal α -glucosidases have been in the region of 3.5–5.0, whereas pH optima of yeast α -glucosidases tend to be in the range 6.5–7.0 (Kelly & Fogarty, 1983). The enzyme showed maximal activity at 60 °C. The half life of the α -glucosidase was 6 h at 55 °C, 125 min at 60 °C and 45 min at 65 °C (Fig. 5). The temperature optima (60 °C) resembles that of *Lipomyces starkeyi* α -glucosidase (Kelly *et al.*, 1985). In various other fungal α -glucosidases, the temperature optimum has been 50–55 °C (Kelly & Fogarty, 1983). Costantino *et al.* (1990) recorded a broad temperature optimum of

Fig. 4. Effect of pH on activity and stability of the α -glucosidase. Enzyme activity was determined at 60 °C using the following buffer systems: citrate at pH 3.0–5.0, phosphate at pH 5.5 to 7.5, and borate at pH 8.0. To determine pH stability, the enzyme preparations were kept at 4 °C for 24 h, with buffers pH 3.0–8.0 and enzyme activity was measured thereafter at pH 4.8 and 60 °C. $\circ$, Activity; $\bullet$, stability. Error bars are SEM of four observations.

Fig. 5. Effect of temperature on α -glucosidase stability. The enzyme was incubated in citrate buffer (pH 4.8) at various temperatures, and residual activity was measured at various time intervals described in the text. Error bars are SEM of four observations.

about 105–115 °C for *Pyrococcus furiosus* α -glucosidase, which is the highest temperature optimum recorded for a purified enzyme.

Catalytic properties

Table 2 shows relative rates of hydrolysis of various substrate by *M. sulfurea* α -glucosidase. The α -glucosidase hydrolysed pNPG at the highest rate; sucrose, maltose and maltotriose were hydrolysed at 31%, 12% and 7.2% of this rate, respectively. Isomaltose, methyl- α -D-glucoside and starch were not hydrolysed by the α -glucosidase. In its substrate specificity, *M. sulfurea* α -glucosidase showed most similarity to the yeast α -glucosidase, which hydrolyses sucrose more rapidly than maltose and pNPG more rapidly than either. Sucrose-hydrolysing activity was found to be associated with electrophoretically homogenous *M. sulfurea* α -

Table 2. Substrate specificity of the purified α -glucosidase from *M. sulfurea*

Results are expressed as the arithmetic mean $\pm$ SEM of four observations. Activities are expressed relative to the pNPG α -glucosidase activity of 0.35 ± 0.02 U under standard assay conditions. Substrates tested without significant activity (less than 5%) were: α -methyl-D-glucoside, isomaltose and starch. Experimental methods are described in the text.

Substrate	Hydrolysis (%)
<i>p</i> -Nitrophenyl- α -D-glucopyranoside	100 ± 2.5
Sucrose	31 ± 1.1
Maltose	12 ± 0.76
Maltotriose	7.2 ± 0.66

Table 3. Inhibitory effects of various chemicals on the activity of *M. sulfurea* α -glucosidase

Results are expressed as the arithmetic mean $\pm$ SEM of four observations. Chloride salts (10 mM) of Cu^{2+} , Hg^{2+} , Mg^{2+} , Zn^{2+} , Ca^{2+} and Ag^+ were supplemented separately in the enzyme assay mixtures. The α -glucosidase assay is described in the text. The 100% activity corresponds to 0.35 ± 0.014 U α -glucosidase activity under the standard assay conditions.

Substance	Residual activity (%)
Cu^{2+} (10 mM)	84.36 ± 5.3
Mg^{2+} (10 mM)	100 ± 7.2
Zn^{2+} (10 mM)	100 ± 4.6
Hg^{2+} (10 mM)	64 ± 7.4
Ca^{2+} (10 mM)	111 ± 12.12
Ag^+ (10 mM)	58.61 ± 6.22
EDTA (25 mM)	86.23 ± 7.3
Histidine (10 mM)	100 ± 8.3
2-Mercaptoethanol (2 mM)	130 ± 15.4
<i>p</i> -Chloromercuribenzoate	66.86 ± 7.8

glucosidase. It indicates that hydrolysis of α -1,2 linkages by an α -1,4 linkage-specific α -glucosidase is not unusual. Most yeast α -glucosidases hydrolyse sucrose more slowly than pNPG (Chiba *et al.*, 1973). Highly purified maltase and α -glucosidase preparations from *Bacillus amyloliquefaciens* and *Bacillus licheniformis*, respectively, hydrolyse α -1,2 linkages in sucrose (Fogarty *et al.*, 1985; Kelly *et al.*, 1986). The *M. sulfurea* α -glucosidase resembles *Bacillus amyloliquefaciens* α -glucosidase (Fogarty *et al.*, 1985) in its inability to hydrolyse isomaltose. It supports the conclusion of Fogarty *et al.* (1985) that for a single enzyme to hydrolyse α -1,4 and α -1,6 glucosidic linkages is not as common as was previously supposed (Wang & Hartman, 1973).

The effects of various chemicals on the α -glucosidase activity were tested. Hg^{2+} , Cu^{2+} , Ag^+ and *p*-chloromercuribenzoate exerted inhibitory effects, whereas 2-mercaptoethanol enhanced enzyme activity (Table 3). Inactivation by Cu^{2+} and Hg^{2+} is indicative of the presence of thiol groups, carboxyl groups, or histidine

residues in the enzyme molecule (Kundu & Das, 1970). Inhibition by *p*-chloromercuribenzoate indicates that cysteine is involved at the active site. Addition of 2-mercaptoethanol increased enzyme activity, indicating the presence of oxidation-sensitive thiol groups on the enzyme (Plant *et al.*, 1988). EDTA (25 mM) had no significant effect on the enzyme activity, suggesting that metal ions are not important to the enzyme activity.

The extracellular α -glucosidase from the thermophilic fungus *M. sulfurea* is distinct from other fungal α -glucosidases in its substrate specificity. The purified α -glucosidase showed hydrolysis of α -1,2 linkages as an inherent property.

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