The Protein Journal, Vol. 24, Nos. 7–8, November 2005 (© 2005)
DOI: 10.1007/s10930-005-7641-z

Characterization of Active *Lentinula edodes* Glucoamylase Expressed and Secreted by *Saccharomyces cerevisiae*

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Received May 9, 2005

The gene encoding *Lentinula edodes* glucoamylase (GLA) was cloned into *Saccharomyces cerevisiae*, expressed constitutively and secreted in an active form. The enzyme was purified to homogeneity by $(NH_4)_2SO_4$ fractionation, anion exchange and affinity chromatography. The protein had a correct N-terminal sequence of WAQSSVIDAYVAS, indicating that the signal peptide was efficiently cleaved. The recombinant enzyme was glycosylated with a 2.4% carbohydrate content. It had a pH optimum of 4.6 and a pH 3.4–6.4 stability range. The temperature optimum was 50°C with stability ≤50°C. The enzyme showed considerable loss of activity when incubated with glucose (44%), glucosamine (68%), galactose (22%), and xylose (64%). The addition of Mn^{++} activated the enzyme by 45%, while Li^{+}, Zn^{++}, Mg^{++}, Cu^{+}, Ca^{++}, and EDTA had no effect. The enzyme hydrolyzed amylopectin at rates 1.5 and 8.0 times that of soluble starch and amylose, respectively. Soluble starch was hydrolyzed 16 and 29 times faster than wheat and corn starch granules, respectively, with the hydrolysis of starch granules using 10× the amount of GLA. Apparent K_m and V_{max} for soluble starch were estimated to be 3.0 mg/ml and 0.13 mg/ml/min (40°C, pH 5.3), with an apparent k_{cat} of 2.9×10^5 min^{-1}.

KEY WORDS: Cloning and expression; glucoamylase; *Lentinula edodes*; *Saccharomyces cerevisiae*.

1. INTRODUCTION

Glucoamylase (1,4-α-D-glucan glucohydrolase; EC 3.2.1.3) is an exoglucosidase that catalyzes the hydrolysis of α-1,4-bonds releasing glucose units successively from the non-reducing end of the starch substrate. The enzyme also acts on α-D-1,6-bonds at the branch point, although hydrolysis occurs at a slower rate (Wong, 1995).

Glucoamylase (GLA) is a microbial enzyme found mainly in fungi. The enzyme is commercially produced from *Aspergillus niger*, and is extensively used for saccharification of soluble starch in the industrial production of sweeteners and bioethanol where thermal stability for high temperature use has been a continuing goal (Ward and Moo-Young, 1988). GLAs from filamentous fungi have a highly glycosylated linker connecting the catalytic domain and the starch-binding domain (Janecek and Sevcik 1999). The hydrolysis reaction proceeds via a single-displacement mechanism involving general acid base catalysis (Koshland, 1959). The rate of hydrolysis increases with the chain length of the substrate, and the end product is exclusively glucose in the β-conformation. GLAs from microbial sources have been identified with raw starch digesting capability (Reilly 2003).

The genes encoding GLAs have been cloned from *Rhizopus oryzae* (Ashikari *et al.*, 1986),

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[3] Abbreviations: CHA, β-cyclodextrin; GLA, glucoamylase; YEP, 2% yeast extract, 1% bactopeptone; YEPG, 2% yeast extract, 1% bactopeptone, 2% glycerol.

A. niger (Boel *et al*., 1984), *A. awamori* (Nunberg *et al*., 1984), *A. oryzae* (Hata *et al*., 1991), *A. shirousami* (Shibuya *et al*., 1990), *Saccharomyces diastaticus* (Yamashita *et al*., 1985), *S. fibuligera* (Itoh, 1987), *L. edodes* (Zhao *et al*., 2000), and *Neurospora crassa* (Stone *et al*., 1993). El-Zalaki and Hamza (1979) compared the amylolytic activities of five basidiomycetous fungi (edible mushrooms)—*Absidia blakesleeana*, *Agaricus bisporus, L. edodes, Peziza auburouniv and Polyporus sulphureus*, and found that *L. edodes* was the most promising in the ability to break down starch. The *L. edodes* GLA hydrolyzed soluble starch, amylose, amylopectin, and glycogen, converting them almost completely into glucose (Yamashi and Suzuki, 1978). More recently, the gene was identified, and the levels of activity and expression of the *L. edodes* strain cultured on a variety of substrates in various development stages were reported (Zhao *et al*., 2000). In the present study, the *L. edodes* GLA gene was cloned into *S. cerevisiae* and constitutively expressed. The physiochemical properties of the recombinant enzyme were characterized.

2. MATERIALS AND METHODS

2.1. Materials

Cloning vector pYEX-S1 was obtained from Clontech (Palo Alto, CA). DEAE-Sepharose and epoxy-activated Sepharose 6B were purchased from Pharmacia (Piscataway, NJ). *E. coli* strains and competent cells were obtained from Invitrogen (San Diego, CA). Restriction enzymes were from New England Biolab (Beverly, MA). Synthetic primers and oligos were synthesized by MWG Biotech (Charlotte, NC). β-Cyclodextrin, soluble starch (S-9765), unmodified corn starch (S-4126), and wheat starch (S-5127) were purchased from Sigma (St. Louis, MO). Amylose (NAPOL L) and amylopectin (NAPOL B) were commercial grades from A. E. Staley (Decatur, IL). Precast gels, staining kits, and protein standards were purchased from Novex (San Diego, CA) or BioRad (Hercules, CA). Membrane concentrators were from Millipore (Bedford, MA) or Vivascience (Carlsbad, CA). Glycoprotein carbohydrate estimation kit and deglycosylation kit were obtained from Pierce (Rockford, IL) and Calbiochem (San Diego, CA). All chemicals and reagents were of analytical grade.

2.2. Cloning and Construction of the GLA Gene

The GLA gene was isolated from the cDNA library of *L. edodes* mycelium as described previously (Lee *et al*., 2001), using PCR with primers: 5′-AGCGGTACCATGTTGCTCTCTGCTGTATTCCTCG and 5′-ACTGCGGCCGCCTACCTCCAAGT GTCATTCGTAGCG. Primer sequences were designed to add a *Kpn*I site and a *Not*I site at the 5′ and 3′ end of the gene, respectively, based on the known sequence reported by Zhao *et al*. (2000). The gene was inserted downstream of the yeast phosphoglycerate kinase (PGK) gene in the pYEX-S1 vector by blunt-end ligation, with the original vector signal sequence eliminated (Wong *et al*. 2002a). The GLA gene sequence in the construct was confirmed using pYEX primers: 5′-CGTAGTTTTTCAAGTTCTTAG and 5′-TCCTTACCTTCCAATAA TTC.

2.3. DNA Manipulation

Plasmid DNA was prepared by alkali lysis using Qiagen mini-prep spin columns. Purification of DNA from agarose gels was performed using a Qiagen MinElute gel extraction kit. Plasmid DNA, oligonucleotides, and PCR products were cleaned using a Qiagen MinElute reaction cleanup kit. Hybridization was performed according to Wallace *et al*. (1981) and Sambrook *et al*. (1992). The DNA probe was radiolabeled by nick translation using [α-^{32}P]dCTP, or by phosphorylation with [γ-^{32}P]ATP.

2.4. Purification of Glucoamylase

Yeast clones secreting recombinant glucoamylase were grown in 2 l YEPG (2% yeast extract, 1% bactopeptone, 2% glycerol) for 72 h at 30°C. The culture was centrifuged at 9000 × *g* for 20 min. The supernatant was subjected to ammonium sulfate fractionation, and the proteins precipitated at 30–70% saturation were combined and dialyzed in 10 m*M* Na acetate buffer, pH 5.3, containing 0.5% glycerol. After buffer exchange, the sample was loaded onto a DEAE-Sepharose column (20×2.5 cm) equilibrated with 25 m*M* Tris, pH 7.4. The column was washed with 350 ml of 10 m*M* Na acetate buffer, pH 5.3, 0.5% glycerol, followed by elution with a gradient from 0 to 0.5 *M* NaCl in the same buffer (Bhella and Altosaar, 1984). The active fractions were combined, exchanged in 0.05 *M* Na acetate,

pH 5.3, containing 5 m*M* NaCl, and applied onto a cyclohepta-amylose-Sepharose 6B column (Silvanovich and Hill, 1976). The column (15×1.5 cm) was washed with 150 ml of the acetate buffer, and 50 ml of the buffer containing 0.3 *M* NaCl. The adsorbed enzyme was eluted with 300 ml of the buffer containing 8 mg/ml of β-cyclodextrin. Active fractions were combined and exchanged into 0.02 *M* Na acetate, pH 5.3, with 0.5% glycerol.

2.5. Electrophoresis

The purified and concentrated enzyme was run on a Tris–glycine gel using 25 m*M* Tris, 192 m*M* Glycine and 0.1% SDS, at 125 V constant for 1.5 h, and stained with Coomassie Blue. For molecular weight determination, the bands were analyzed by image analysis software (Alpha Innotech, CA)

2.6. Amino Acid Sequencing

Proteins were separated on Tris–glycine gels, and blotted onto a PVDF membrane using the recommended transfer buffer (12 m*M* Tris base, 96 m*M* glycine in 20% methanol). The membrane was stained with 0.1% Coomassie Blue R, 40% EtOH, and 10% acetic acid, and the enzyme band was excised and inserted into the sequencing cartridge on a glass fiber filter. Sequencing was performed with an Applied System Model 477A Pulsed Liquid Phase amino acid sequencer equipped with online PTH-amino acid analyzer (Tao and Kasarda, 1989).

2.7. Glucoamylase Activity Assay

Enzyme activity of the clones was monitored by the use of YEP-starch plates (1% yeast extract, 2% bactopeptone, 2% starch, 2% agar). Colonies were incubated for 3–5 days at 30°C, followed by 2-day storage at 4°C to observe halo formation. For liquid assay, the enzyme activity was measured by the DNSA method which reports as reducing ends formed as glucose (Wong *et al.*, 2003). Enzyme activity unit was defined as the amount of enzyme producing 1 μmol of glucose per 1 min at pH 5.3 and 40°C.

2.8. Determination of pH and Temperature Optima and Stability

For pH optimum, the reaction mixture was incubated for 30 min at 37°C, using soluble starch as the substrate at variable pH using a universal buffer. For temperature optimum, the reaction was incubated for 30 min at variable incubation temperature using Na acetate buffer, pH 5.3. For pH stability, the enzyme was incubated at various pH's in a 30°C water bath for 22 h, reconstituted to pH 5.3 with Na acetate buffer, and assayed for residual enzyme activity. For temperature stability, the enzyme was incubated in Na acetate buffer, pH 5.3, at specific temperatures for 20 min, and the residual activity was assayed at 37°C as described above.

2.9. Hydrolysis of Soluble and Raw Starch

Enzyme activity was measured at time intervals using soluble starch, amylose, or amylopectin as the substrate as described in Section 2.7. Before use, the soluble starch solution was heated at 80°C for 10 min. For determining the rate of hydrolyzing raw starch, an approach based on solid granule hydrolysis was performed. A preparation of 1% raw starch in water was centrifuged, and the liquid was removed to leave a wet raw starch paste. Enzyme solution was added to cover the starch, mixed, and incubated at 40°C. Before use, the insoluble starch granule was washed repeatedly to remove the fines that could lead to over-estimation of the extent of hydrolysis. The starch granule was not heated. The reducing sugar equivalent in the reaction was measured using the DNSA method.

2.10. Activity Inhibition by Monosaccharides

Enzyme solution was incubated at 30°C for 24 h, with either 50 m*M* D(+)-glucose, D(+)-xylose, L(+)-arabinose, D(+)-galactose, 2-deoxy-D-glucose, D(+)-glucosamine, or sorbitol. The monosaccharides in the reaction mixtures were removed by a 10,000 MWCO membrane concentrator, and the samples were exchanged into 25 m*M* Na acetate buffer, pH 5.3. After ultrafiltration, the sample was assayed for enzyme activity as described in Section 2.7.

2.11. Effect of Metal Ions on GLA Activity

Enzyme solution was incubated at 30°C for 24 h with 50 m*M* LiCl, $ZnSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$, $CaCl_2 \cdot 2H_2O$, or

EDTA in Na acetate buffer, pH 5.3. The metal concentration was diluted to 1 m*M* by the addition of Na acetate buffer immediately before enzyme activity assay.

2.12. Deglycosylation of GLA

GLA was denatured by heating at 100°C for 5 min in SDS/β-mercaptoethanol sodium phosphate buffer, pH 7.0, followed by addition of *N*-glycosidase F as recommended by the supplier of the deglycosylation kit. For carbohydrate estimation, GLA was oxidized by sodium meta-periodate to aldehydes that reacted with glycoprotein detection reagent to form a purple product with maximum absorbance at 550 nm.

3. RESULTS AND DISCUSSION

Yeast cells (DY150) harboring the recombinant vector (designated as D150[pYEX-GLA]) secreted active GLA, resulting in halo formation on YEP-starch plates (Fig. 1). The clearing of the substrate around the colonies indicates hydrolysis by the secreted enzyme. When cultured in YEPG at 30°C, the GLA in the medium increased with time and leveled off after 144 h incubation (Fig. 2). The amount of activity in the cell extracts was negligible, suggesting that the enzyme was efficiently expressed and secreted. A similar high level of secretion was observed for barley α-amylase in our previous study (Wong *et al*., 2002a). The present result also confirms that the use of glycerol as a carbon source in the yeast medium enhances the synthesis and secretion of recombinant proteins (Wong *et al*., 2002b).

The purified GLA showed a single band of 93.7 kD on SDS-PAGE (lane A, Fig. 3), a sizable variation from the predicted 61,167 Da. The protein, after deglycosylation by *N*-glycosidase-F, showed a band shift in electrophoretics migration to 61.5 kDa (lane B, Fig. 3). The nature and extent of glycosylation was further confirmed by periodate oxidation with an estimated 2.4% carbohydrate content. GLAs from filamentous fungi are known to be highly glycosylated, particularly at the linker region between the catalytic domain and the starch-binding domain (Reilly, 2003). It is not certain in this case that *S. cerevisiae* expressed the *L. edodes* GLA with a glycosylation pattern similar to that of the native enzyme, although the recombinant GLA was active.

Fig. 1. Detection of secreted GLA by halo formation on a YEP-starch plate. The plate was cultured with D150[pYEX-GLA] on the right. The control on the left was *S. cerevisiae* strain D150 transformed with the expression vector pYEX without the GLA gene sequence. Plates were incubated at 30°C for 4 days, followed by storage at 4°C to visualize the halos.

Amino acid sequencing indicated that the N-terminal sequence contained Trp–Ala–Gln–Ser–Ser–Val–Ile–Asp–Tyr–Val–Ala–Ser, identical to the deduced sequence of *L. edodes* GLA (Zhao *et al*. 2000). This result confirms that the GLA was expressed and secreted by the *S. cerevisiae* clone, and that the signal peptide was cleaved at the Gly–Trp position to yield the mature protein.

The recombinant enzyme had a pH optimum of 4.6 and a stability range of pH 3.4 to 6.3 (Fig. 4a and b). The temperature optimum was 50°C, and heat stability $\leq$50°C (Fig. 5a and b). The enzyme retained ~50% of the activity after incubation at 55°C for 20 min, and was completely inactivated at the 60°C incubation. The pH and temperature effect resembles those reported for the native enzyme as well as several *Aspergillus* enzymes. A pH optimum of 4.5, and a broader pH minimum/maximum similar to that of the *L. edodes* GLA has been reported for *A. fumigatus* (Brandani da Silva and Peralta, 1998), *A. saitoi* (Takahashi *et al*., 1981), *A. niger* (Fogarty and Benson, 1983), and *A. awamori* (Yamasaki *et al*., 1977). In contrast, the *Neurospora crassa* enzyme has a pH optimum at 5.4 and stability of pH 2–8 (Baracchini *et al*., 1996).

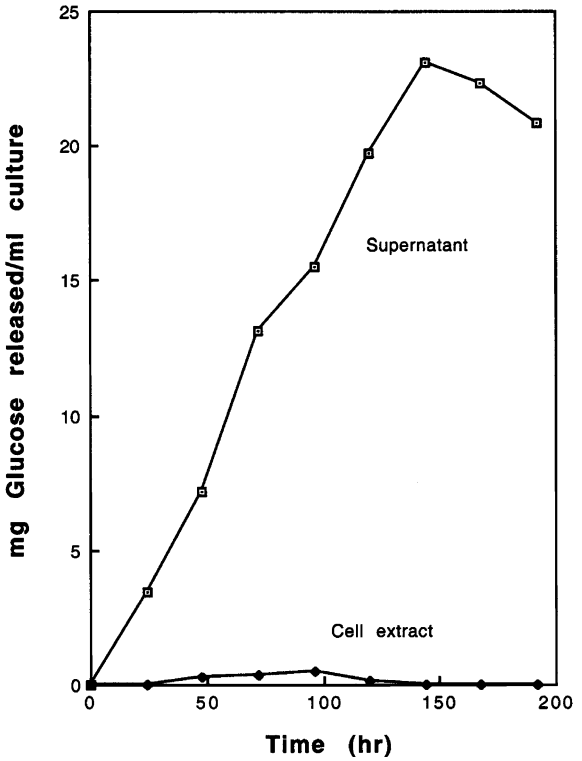

Fig. 2. The time course production of *L. edodes* GLA in *S. cerevisiae*, monitored by activity measurement in the culture medium and in the cell extract. Cultures were incubated in an incubator shaker at 30°C with 250 rpm. Enzyme activity was measured by the DNSA method as described in "Methods".

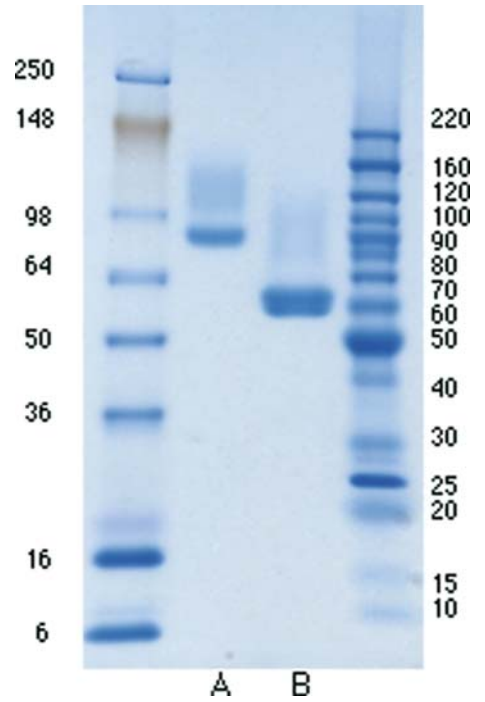

Fig. 3. SDS-PAGE of *L. edodes* GLA (lane A) and deglycosylated GLA (lane B). Precast Tris–glycine gels were used with a running buffer consisting of 25 m*M* Tris, 192 m*M* Glycine, and 0.1% SDS, at 125 V constant for 90 min.

Amylolytic enzymes, like many other enzymes, exhibit end-product inhibition. GLA showed considerable loss of activity when incubated with glucose (44.4%), glucosamine (67.6%), galactose (22.0%), and xylose (63.5%). Sorbitol and arabinose caused mild effects with 6.7% and 5.2% decrease in activity, respectively (Fig. 6). 2-Deoxyglucose did not affect the enzyme activity, suggesting that the C2-OH is essential for inhibition. It is interesting to note that the pentose sugar, xylose, was the strongest inhibitor among all the sugars tested. xylose has been shown to inhibit starch hydrolysis by the extracellular glucoamylase from the yeast *Trichosporon adeninovorans* (Buettner *et al.*, 1987).

Incubation at 30°C for 24 h with 50 m*M* Li^{+}, Zn^{++}, Mg^{++}, Cu^{+}, Ca^{++}, and EDTA had no effect on GLA activity. Under the same incubation conditions, Mn^{++} increased the GLA activity by ~45%. Significant stimulation of the *N. crassa* enzyme by 50–60% at 10 m*M* Mn^{++} has been observed (Baracchini *et al.*, 1996). It has also been shown that Mn^{++} inhibited 12% and 29% of the activity of glucoamylase from *Rhizopus* sp. and *A. saitoi*, respectively (Takahashi *et al.*, 1978, 1981). The activation effect of Mg^{++} and Ca^{++} on the activity of the *A. terreus* GLA has been reported to be 15% and 7% respectively (Ali and Hossain, 1991).

The expressed *L. edodes* GLA hydrolyzed amylopectin at rates 1.5 and 8.2 times that of soluble starch and amylose, respectively (Fig. 7). This result suggests that hydrolysis rate depends on the available points of attack, which is directly a function of the number of non-reducing chain ends or branching of the substrate molecule. Compared to the amount of soluble starch converted to glucose using 1× amount of enzyme (0.7 μg), the use of 10× the amount (7 μg) of enzyme converted 6.3% and 3.5% wheat and corn starch granules to glucose, respectively (Figs. 7 and 8). This means soluble starch was hydrolyzed at rates 16 and 29 times faster than wheat and corn starch granules, respectively, even though 10 × the amount of enzyme was used for solid granule hydrolysis. Kimura and Robyt (1995) reported on the need for greater amounts of *R. niveus* GLA to effect hydrolysis of various starch

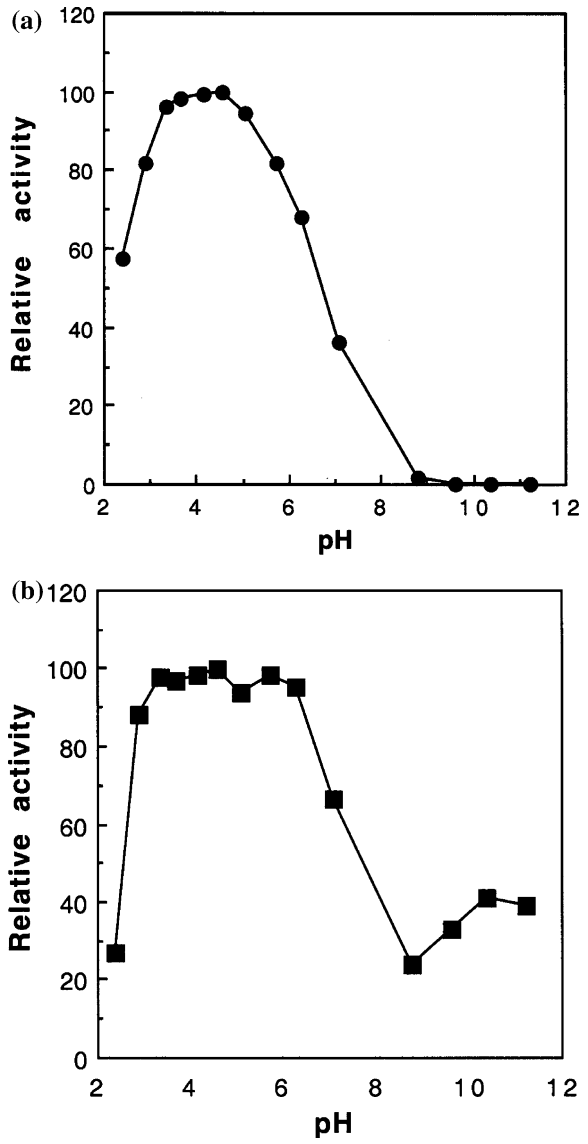

Fig. 4. Effect of pH on (a) optimum activity, and (b) stability of *L. edodes* GLA. Reactions were conducted for 30 min at 37°C, using soluble starch as the substrate at variable pH using a universal buffer. For pH stability measurements, the enzyme (0.7 μg) was preincubated at various pH's in a 30°C water bath for 22 h, reconstituted to pH 5.3 with Na acetate buffer, and assayed for residual enzyme activity.

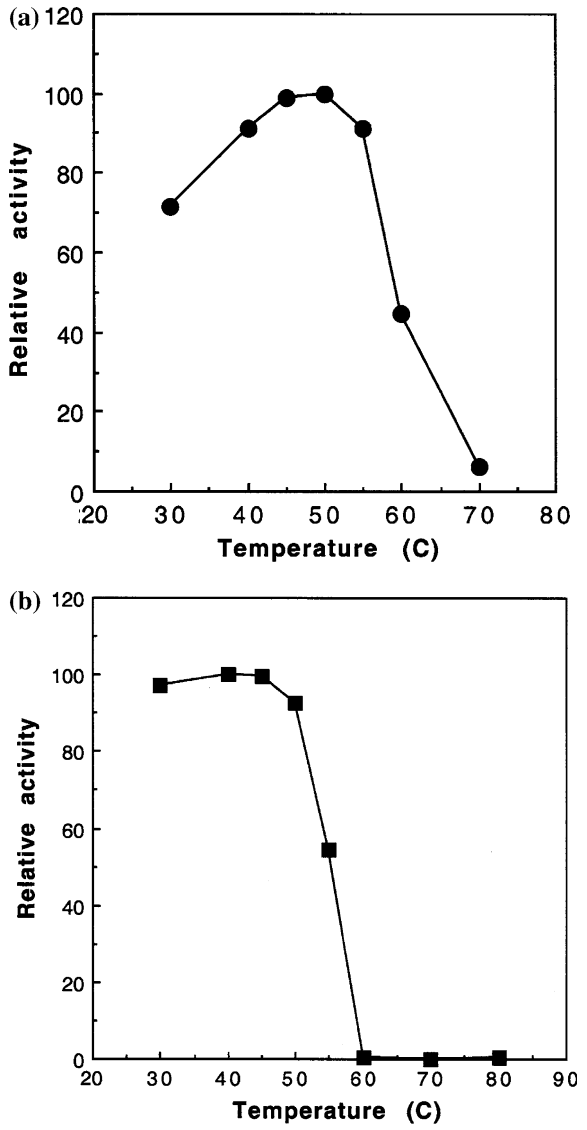

Fig. 5. Effect of temperature on (a) optimum activity, and (b) stability of *L. edodes* GLA. For temperature optimum, the reaction was incubated for 30 min at variable incubation temperature using Na acetate buffer, pH 5.3. For temperature stability, the enzyme (0.7 μg) was incubated in Na acetate buffer, pH 5.3, at specific temperatures for 20 min, before assay for residual activity.

granules. They identified three starch groups with varying susceptibility: waxy corn starch; barley, corn, and tapioca; and the least susceptible group, amylomaize-7, shoti, and potato starch. Smith and Lineback (1976) followed the action of the same enzyme on wheat and corn starch granules with scanning electron microscopy, showing that wheat starch was attacked along the equatorial grove and that corn starch produced a Swiss-cheese appearance with deep holes into the granule. The apparent K_m and V_{max} values of *L. edodes* GLA were calculated from a Lineweaver–Burk plot to be 3.0 mg/ml and 0.13 mg/ml/min, respectively (40°C, pH 5.3) (Fig. 9). The apparent k_{cat} was $2.9\times10^5 min^{-1}$. For *A. niger* GLA, a K_m of 2.98 mg/ml and a V_{max} of 0.24 mg/ml/min have been reported for the

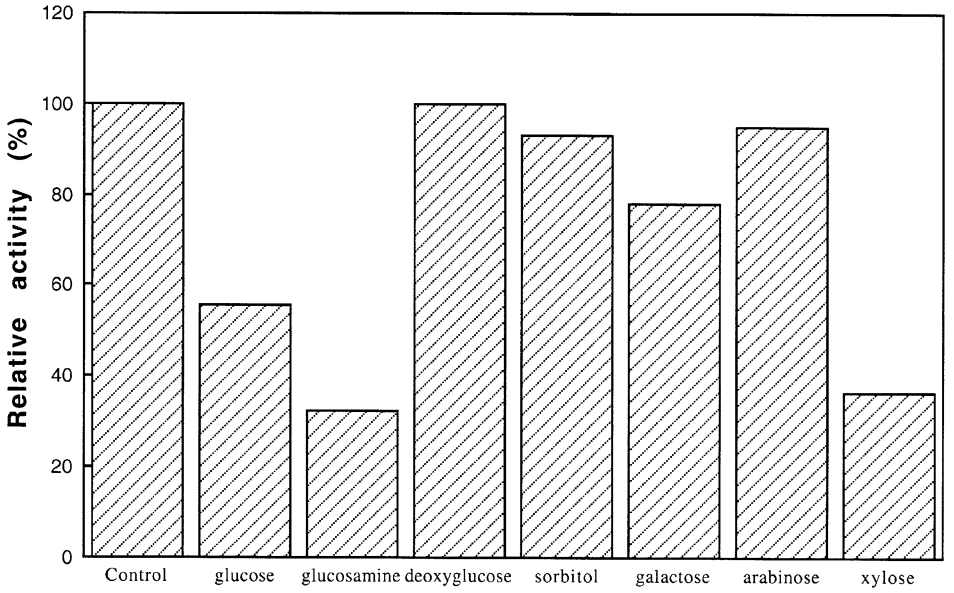

Fig. 6. Effect of monosaccharides on the activity of *L. edodes* GLA. The enzyme (0.7 μg) was incubated with 50 m*M* sugar at 30°C for 24 h, desalted, and analyzed for activity.

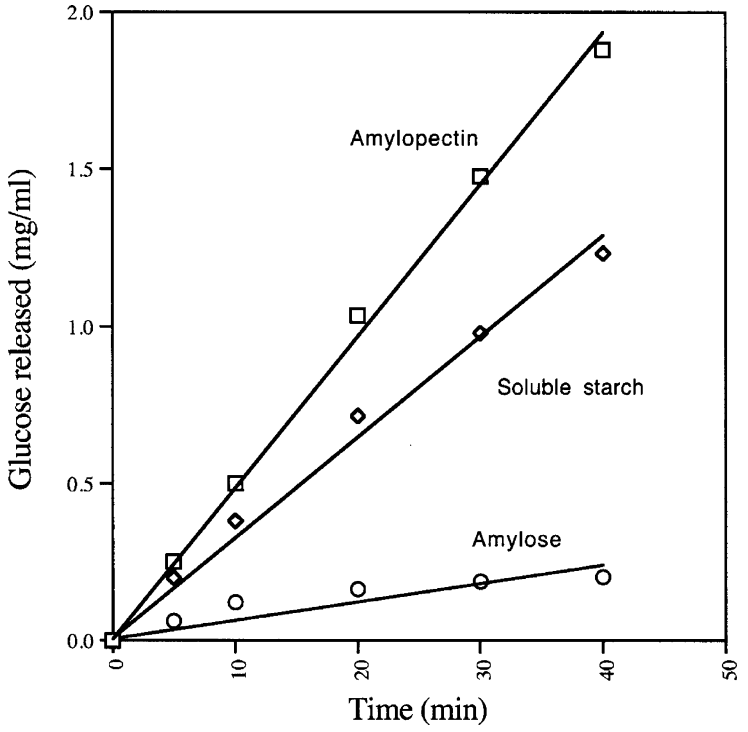

Fig. 7. The time course for the formation of D-glucose in the reaction of *L. edodes* GLA with amylopectin, soluble starch, and amylose. The reaction mixture contained 0.7 μg (2 μl) GLA, 100 μl 1% starch solution, with 25 m*M* Na acetate buffer, pH 5.3, added to a final volume of 200 μl. After incubation at 40°C, 200 μl DNSA was added, and the mixture was heated at 80°C for 30 min. The reaction was cooled in ice water, and the absorbance at 562 nm was measured.

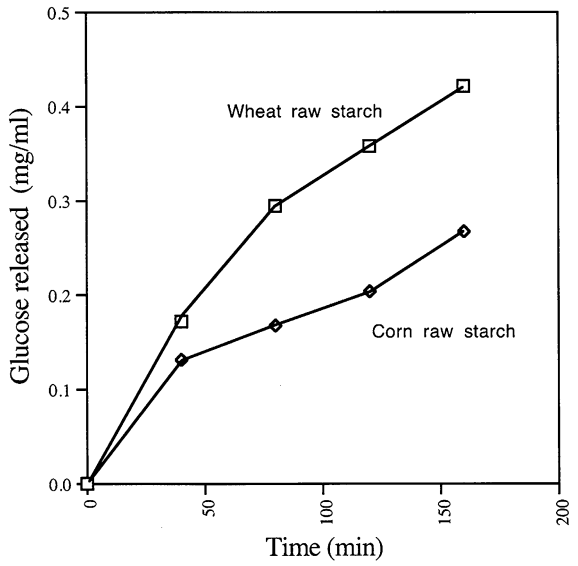

Fig. 8. The time course for the formation of D-glucose in the reaction of *L. edodes* GLA with wheat and corn starch granules. The reaction mixture contained 7 μg, (20 μl) of GLA, and a wet slurry of starch granule prepared from 100 μl of 2% starch. After incubation at 40°C, Na acetate buffer (25 m*M*, pH 5.3) was added to a final volume of 200 μl, followed by DNSA detection as described for Fig. 7.

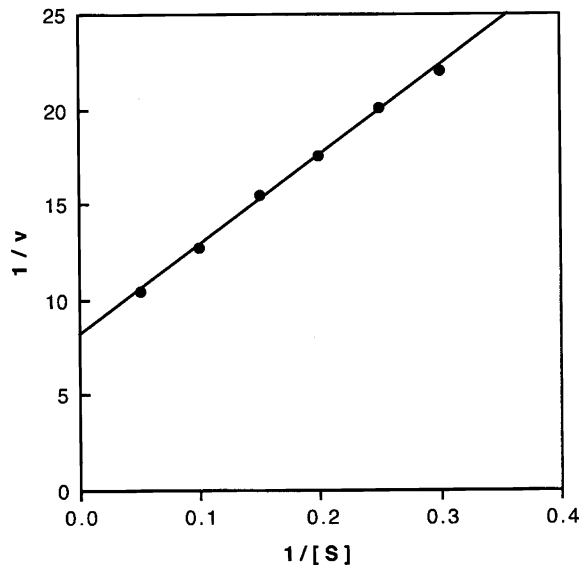

Fig. 9. A Lineweaver–Burk double-reciprocal plot of 1/V as a function of 1/[S]. The reaction mixture contained 100 μl of GLA (0.87 μg) in Na acetate buffer, pH 5.3, and 100 μl of 0.33% to 2% soluble starch, incubated at 40°C for various time intervals. The formation of glucose was measured by the DNSA method as described for Fig. 7.

hydrolysis of soluble starch (40°C, pH 4.4). The same enzyme had K_m and V_{max} values of 6.22 mg/ml and 0.47 mg/ml/min when a different type of soluble starch was used (Sanroman *et al.*, 1996). Kinetics values are widely scattered in large part because the starch substrate is not well characterized, hence, the use of mass concentration is almost universally employed. The values can also vary when GLA from other sources or different conditions are used. The structural properties, such as size, shape, and branching of the starch substrate may also affect the kinetic behavior of the hydrolysis.

4. CONCLUSIONS

L. edodes glucoamylase was constitutively expressed and secreted from *S. cerevisiae* in an active form. The physiochemical properties of the recombinant enzyme were characterized. The apparent kinetic behavior of the enzyme-catalyzed hydrolysis of starch substrates was comparable to those of glucoamylases from fungal sources.

ACKNOWLEDGMENT

The authors would like to thank Dr. William Vensel for N-terminal sequencing. Reference to a company and/or products is only for purposes of information and does not imply approval of recommendation of the product to the exclusion of others that may also be suitable. All programs and services of the U.S. Department of Agriculture are Offered on a nondiscriminatory basis without regard to race, color, national origin, religion, sex, age, marital status, or handicap.

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