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Heterologous Expression and Biochemical Characterization of α-Glucosidase from *Aspergillus niger by Pichia pastroris*

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The *aglu* of *Aspergillus niger* encodes the pro-protein of α-glucosidase, and the mature form of wild-type enzyme is a heterosubunit protein. In the present study, the cDNA of α-glucosidase was cloned and expressed in *Pichia pastoris* strain KM71. The activity of recombinant enzyme in a 3 L fermentor reached 2.07 U/mL after 96 h of induction. The recombinant α-glucosidase was able to produce oligoisomaltose. The molecular weight of the recombinant enzyme was estimated to be about 145 kDa by SDS−PAGE, and it reduced to 106 kDa after deglycosylation. The enzymatic activity of recombinant α-glucosidase was not significantly affected by a range of metal ions. The optimum temperature of the enzyme was 60 °C, and it was stable below 50 °C. The enzyme was active over the range of pH 3.0−7.0 with maximal activity at pH 4.5. Using *p*NPG as substrate, the K_m and V_{max} values were 0.446 mM and 43.48 U/mg, respectively. These studies provided the basis for the application of recombinant α-glucosidase in the industry of functional oligosaccharides.

KEYWORDS: α-Glucosidase; *Aspergillus niger*; *Picha pastoris*; expression; characterization

INTRODUCTION

α-Glucosidases (EC3.2.1.20) are a group of typical exotype carbohydrases, which catalyze the liberation of α-glucose from non-reducing terminals of substrates (*1*). Various types of α-glucosidases are widespread in nature, distributed in microorganisms, insects, plants and mammals (*2*). Some α-glucosidases, e.g. those from *Aspergillus niger* (*3*), *Bacillus stearothermophilus* (*4*), *Saccharomyces cerevisiae* (*5*) etc., not only hydrolyze glycosides but also can catalyze transglucosidation reaction. For example, *A. niger* α-glucosidase can transfer a glucosyl residue to the 6-OH of the accepting glucose unit and yield isomaltose, panose, isomaltotriose and tetrasaccharides from maltose (*6*, *7*). Isomaltose, panose, isomaltotriose and tetrasaccharides are defined as isomaltooligosaccharides (IMOs) (*8*). Since IMOs are stimulating materials to *Bifidobacteria* and total lactic acid bacteria of human intestines (*7*), they are of special interest to the food industry. In addition, IMOs are also utilized in animal feed to increase dry matter and calcium digestibility (*9*).

It has been reported that, among all the α-glucosidases, *A. niger* α-glucosidase gives a notable yield of total transglycosylating products (*10*), therefore, the preparation of α-glucosidase from *A. niger* has been long investigated by many researchers. However, low yield of α-glucosidase from wild-type *A. niger* makes it uneconomical for large-scale industrial application, thus genetic engineering approach is highly necessary for the expression of this enzyme in a new host of efficiency. Previously, *A. niger* α-glucosidase gene *aglu* was identified by Kimura (*1*). Biochemical studies showed that aglu was a pro-protein (*11*). It was suggested that the pro-protein was subjected to limited proteolysis after secretion from the *A. niger* cells and 14 residues inside the polypeptide were cleaved, which resulted in a heterosubunit protein of the mature form of α-glucosidase (*11*). Probably due to this special phenomena, expression of *aglu* has been attempted only in fungal species, such as *Aspergillus nidulans* (*11*) and *Emericella nidulans* (*12*). Increasing copy number of *aglu* by chromosomal integration could also improve the yield of α-glucosidase in *A. niger* (*13*). However, the yield obtained by these efforts was still low and could not reach the requirement of industrial applications.

The yeast *Pichia pastoris* expression system, as a eukaryotic expression system, has been a favorite system for expressing heterologous proteins due to its many advantages, such as high production and high secretion efficiency (*14*). The *Pichia* expression system has been extensively used in the enzyme industry for the production of recombinant proteins (*15*). In our previous work, the cDNA of *A. niger* (No. SG136) α-glucosidase was cloned by overlap-PCR and expressed in *P. pastoris* (*16*). However, it was found that the biological activity of the recombinant enzyme was low. This study described the cloning of the cDNA of α-glucosidase from another strain of *A. niger* (CICIM F0620) by RT-PCR and its expression in *P. pastoris*. In addition, the characterization of the recombinant enzyme from *P. pastoris* was also investigated. These studies provide the basis for the application of recombinant α-glucosidase in the industry of IMOs production.

MATERIALS AND METHODS

Strains, Vectors and Materials. The strain of *A. niger* (No. CICIM F0620) was from China Center for Type Culture Collection (CCTCC).

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P. pastoris KM71 and the plasmid pPIC9K were obtained from Invitrogen. The EZ-10Spin Column Plasmid Mini-Preps kit, agarose gel DNA purification kit, restriction enzymes, and T4 DNA ligase were obtained from TakaRa (Dalian, China). *p*-Nitrophenyl-α-D-glucopyranoside (*p*NPG) was obtained from Seebio Biotech. Inc. (Shanghai, China). Endo H_f was obtained from New England Biolabs (Beijing, China). Other chemicals were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). DNA primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China). DNA sequencing was performed by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd.(Shanghai, China).

Gene Cloning of *A. niger* α-Glucosidase. The cDNA of *A. niger* α-glucosidase exclude the fragment of its signal peptide was cloned by RT-PCR. Total RNA of *A. niger* was extracted and purified as described previously (*17*). cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA). The obtained first-strand cDNA was served as template for PCR. The cloning primer sequences were designed according to GenBank (GeneID: 4991096) as follows: ATTAATGCGGCCGCGTCCACCACTGCCCCT TCC (forward primer) and AGCACTAGCGGCCGCCCATTCCAATACCCAGT TTTCC (reverse primer). The *Not*I restriction site (underlined) was designed into the primers. The amplification was carried out under the following conditions: the first step was at 95 °C for 4 min, followed by 30 cycles of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 4 min, and the final extension was carried out at 72 °C for 10 min. The PCR product was digested with *Not*I, gel-purified and then ligated into pPIC9K which was subjected to a similar treatment. The recombinant plasmid, pPIC9K/*aglu*, was identified by restriction analysis and sequencing.

Expression of α-Glucosidase in *P. pastoris*. The recombinant plasmid pPIC9K/*aglu* was linearized with *Bgl*II and then electroporated into *P. pastoris* KM71. The transformants were selected at 30 °C on the MD agar plates for 2−4 days. The presence of the α-glucosidase gene in the transformants was confirmed by PCR using yeast genomic DNA as template. For expression, the colonies were grown in 10 mL of YPD medium at 30 °C for 24 h, then inoculated into 50 mL of BMGY medium and shaken (200 rpm) at 30 °C until OD_{600} of 5−6 was reached. The cells were collected by centrifugation at 5000 rpm for 5 min at 4 °C, resuspended in 25 mL of BMMY. To maintain induction, methanol was supplemented every 24 h to a final concentration of 1.0% (v/v) throughout the induction phase.

The recombinant *P. pastoris* with the highest α-glucosidase yield was used to scale up fermentation in a 3 L fermentor (BIOFLO 110, America). The fermentation began at batch growth phase in 1.5 L BSM at 30 °C and pH 5.5, and the pH was maintained with ammonium hydroxide. After the level of dissolved oxygen increased, continuous glycerol feeding was carried out until the OD_{600} reached 100. When the dissolved oxygen increased again, a methanol solution was added to the fermentor. The level of dissolved oxygen was maintained above 30% throughout the induction phase. DO-stat methanol feeding strategies were applied.

Hydrolytic Activity of Enzyme. The hydrolytic activity of α-glucosidase was measured as the amount of *p*-nitrophenol (*p*NP) released from *p*NPG (*18*). The reaction mixture contained 1 mL of 100 mM sodium acetate buffer (pH 5.5), 0.05 mL of 10 mM *p*NPG, and 0.05 mL of appropriately diluted enzyme. The reaction was incubated at 50 °C for 15 min and terminated by 1 mL of 1 M sodium carbonate solution. One unit (U) of enzyme activity was defined as the amount of 1 μmol *p*NP produced per min under the above conditions.

Transglycosylation Activity of Enzyme. The reaction mixture (2.5 mL), which consisted of 2 mL of 10% (w/v) maltose solution in 100 mM sodium acetate buffer (pH 5.5) and 500 μL of enzyme (to reach a final activity of 0.4 U/mL), was incubated at 60 °C. At different intervals, 100 μL reaction mixtures were taken and incubated at 100 °C for 5 min to inactivate the enzyme. Samples were centrifuged at 12,000 rpm for 5 min and analyzed by HPLC. The HPLC analysis was performed on an Agilent separation module (model 1200) equipped with quaternary pump, using a Hypersil NH_2 column (4.6 × 250 mm). The mobile phase consisted of 75% acetonitrile and 25% water used at a flow rate of 1 mL/min. A refractive index detector (Agilent model 1200) was used (*5*).

Purification of Recombinant α-Glucosidase. The culture supernatant of engineered *P. pastoris* was obtained by centrifugation at 10000*g* for 20 min and then concentrated by ultrafiltration (30 kDa cutoff membrane, Amicon). α-Glucosidase was precipitated with 70% (v/v) ethanol and collected by centrifugation (10000*g*, 20 min). The precipitate was dissolved in 50 mL of buffer A (20 mM sodium acetate buffer, pH 5.5), and dialyzed against two liters of buffer A at 4 °C overnight. Solid $(NH_4)_2SO_4$ was added to the dialyzed sample to a final concentration of 20% (w/v). The sample was filtered (0.22 μm) and loaded onto a Phenyl HP Sepharose FF column pre-equilibrated with 20% $(NH_4)_2SO_4$ in buffer A. A reverse gradient from 20% to 0% $(NH_4)_2SO_4$ in buffer A was applied at a flow rate of 1.0 mL/mim over 60 min. The fractions containing *p*NPG hydrolase activity were pooled and dialyzed against 1 L of buffer A overnight. The purified enzyme was stored at −80 °C.

Molecular Weight Determinations. The subunit molecular weight of recombinant α-glucosidase was determined by SDS−PAGE. The native molecular weight of recombinant α-glucosidase was determined by gel filtration utilizing a Superdex 200 10/300GL column. The elution volume was determined in triplicate for all samples and standards.

Deglycosylation of Recombinant α-Glucosidase. Ten micrograms of recombinant α-glucosidase was denatured with glycoprotein denaturing buffer at 100 °C for 10 min. After the addition of G5 reaction buffer, Endo H_f was added and the reaction mixture was incubated for 1 h at 37 °C (*19*).

HPLC Analysis of Purified Recombinant Enzyme. The purified enzyme was analyzed by gel filtration HPLC on an Agilent separation module (model 1200) equipped with quaternary pump, using a TSK-gel G3000SWXL column (7.5 × 300 mm). The mobile phase consisted of 10 mM sodium phosphate buffer (pH 6.8) used at a flow rate of 0.6 mL/min. In addition, the enzyme was also subjected to a reverse-phase HPLC with a C_{18} column (3.9 × 150 mm) (Delta-Pak). The reverse-phase HPLC analysis was performed according to the previous report (*2*).

Temperature Optimum and Thermostability. The optimal temperature of the recombinant enzyme was measured at temperatures ranging between 30 and 90 °C at pH 5.5, using *p*NPG as substrate in 100 mM acetate buffer. Since the pH of acetate buffer is temperature-dependent, the pH of the buffers was adjusted to 5.5 at the desired temperatures. At each temperature, the buffer and *p*NPG were preincubated for 5 min. The reaction was initiated by the addition of the enzyme and allowed to proceed for an additional 15 min. The thermostability was determined by incubating the enzyme in 100 mM acetate buffer (pH 5.5) at the various temperatures (50−70 °C). At different intervals, samples were taken and assayed for residual activity. The experiments were carried out in three independent experiments.

pH Optimum and Stability. pH optimum of the recombinant enzyme was measured over a pH range of 3.0−7.0 by using acetate buffer (pH 3.0−5.0), sodium phosphate buffer (pH 5.0−6.0) and Tris-HCl buffer (pH 6.0−7.0), respectively. To determine the pH stability, the enzyme was preincubated in the various buffers described above at 4 °C for 24 h, and then assayed for residual activity at pH 5.5. The experiments were carried out in three independent experiments.

Determination of Kinetic Parameters. Enzyme assays were performed in acetate buffer (pH 5.5) at 50 °C using *p*NPG as substrate. Substrate concentrations were in the range of 0.1−2.0 mM. The Michaelis−Menten parameters, V_{max} and K_m, were calculated from double reciprocal plots of reaction curve (*20*). The experiments were carried out in three independent experiments.

Effect of Ions on Enzyme Activity. To determine the effect of metal ions (Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Zn^{2+}, Fe^{2+}, Ba^{2+}, Ni^{2+}, Cu^{2+}, Al^{3+}, and Pb^{2+}) on the recombinant α-glucosidase activity, the enzyme was preincubated with each metal ion at a 1 mM concentration in 100 mM acetate buffer (pH 5.5) at 50 °C for 1 h. The residual enzyme activity was assayed at 50 °C. The experiments were carried out in three independent experiments.

RESULTS

Cloning and Expression of α-Glucosidase in *P. pastoris*. The cDNA of α-glucosidase excluding the fragment of signal peptide was reverse transcribed from the total RNA of *A. niger* (CICIM F0620) and cloned into *P. pastoris* expression vector pPIC9K. Nucleotide sequence analysis showed that the gene length was 2883 bp encoding a protein consisting of 960 amino acids. The cDNA sequence and amino acid sequence were compared with those of *A. niger* CBS513.88 α-glucosidase. It was shown that the cDNA sequence shared 99.8% homology with that of *A. niger*

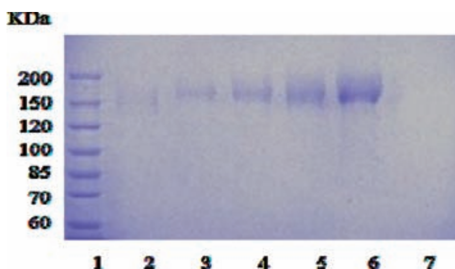

Figure 1. SDS−PAGE analysis of culture supernatant of engineered *P. pastoris*. Lane 1 marker; lanes 2−6 culture supernatant of recombinant *P. pastoris* after methanol induction from 1 to 5 days at the shaker flask level; lane 7 control.

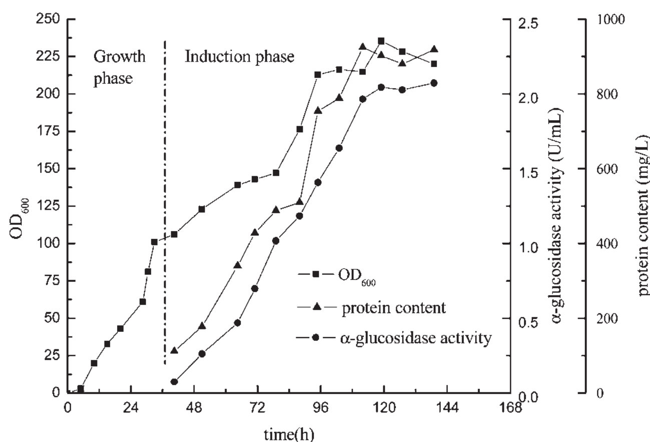

Figure 2. Time profiles for batch cultivations of recombinant *P. pastoris* in 3 L fermentor.

CBS513.88 α-glucosidase (GenBank Accession No. 4991096) and differed by four nucleotides. The amino acid sequence shared 100% identity with that of *A. niger* CBS513.88 α-glucosidase (NCBI Accession No. XP_001402053).

For expression, the recombinants were screened in MD and YPD/G418 plates, and the insert was identified by PCR. The *P. pastoris* KM71 transformed with vector pPIC9K was used as control. After 120 h of induction on methanol in a shake flask, the *p*NPG hydrolase activity in the culture supernatant of recombinant *P. pastoris* KM71/pPIC9K-*aglu* reached 1.15 U/mL, which was 18.2-fold higher than that of native α-glucosidase extracted from *A. niger* (CICIM F0620). The protein concentration in the culture medium was 517 μg/mL. SDS−PAGE analysis showed that there was one major band of protein, approximately 145 kDa, secreted into the culture medium (**Figure 1**). No *p*NPG hydrolase activity was detected in the culture supernatant of the control strain under the same culture conditions.

The expression efficiency of the engineered *P. pastoris* was further explored in a 3 L fermentor. As shown in **Figure 2**, after methanol induction for 5 days, α-glucosidase activity and protein concentration in the culture supernatant were 2.07 U/mL and 918 μg/mL, which were 1.80-fold and 1.78-fold higher than those in shake condition, respectively. The yield of recombinant enzyme in the culture media of engineered *P. pastoris* was 32.8-fold higher than that of native α-glucosidase extracted from *A. niger* (No. CICM F0620).

Transglycosylation Activity of Recombinant α-Glucosidase. To determine whether the expressed α-glucosidase has transglycosylation activity, the culture supernatant of recombinant *P. pastoris* KM71/pPIC9K-*aglu* was incubated with 10% (w/v) maltose solution at 60 °C. The progress of the reaction was monitored by HPLC. To identify the components in the reaction mixture, the retention time of each peak from HPLC was compared with those of IMOs standards. As shown in **Figure 3**, isomaltose, panose, isomaltotriose were able to be detected in the reaction mixture by HPLC. The results showed that recombinant α-glucosidase had transglycosylation activity.

Purification of the Recombinant α-Glucosidase. The recombinant α-glucosidase was purified from culture supernatant by ultrafiltration, ethanol fraction and hydrophobic interaction chromatography. The purified enzyme was homogeneous by SDS−PAGE with a specific activity of 2.52 U/mg.

Physical Properties. The molecular weight of recombinant enzyme as determined by SDS−PAGE was 145 kDa (**Figure 4**). The molecular weight of the native enzyme determined by gel filtration chromatography was 277 kDa (**Figure 5**), 1.9 times the molecular weight determined by SDS−PAGE. Therefore, recombinant α-glucosidase is predicted to have a dimeric structure in solution.

In addition, the calculated molecular weight of the mature α-glucosidase was 106 kDa (http://www.expasy.ch/tools/pi_tool.html), which is different from that estimated from SDS−PAGE. This difference is probably caused by glycosylation. Sequence analysis showed that there were 18 potential N-glycosylation sites in *A. niger* α-glucosidase (http://www.cbs.dtu.dk/services/). In order to confirm whether the recombinant enzyme is glycosylated, the purified enzyme was subjected to be treated by deglycosylase (Endo H_f). As shown in **Figure 4**, the molecular weight of the expressed enzyme was reduced after the treatment. These results indicated that the recombinant protein was glycosylated in the host of *P. pastoris*.

Furthermore, the purified enzyme showed one single peak by HPLC with a TSK-gel column, while it showed two peaks by HPLC with a reverse phase column (**Figure 6**).

Temperature Optimum and Thermostability. The optimum temperature curve of the purified α-glucosidase showed that the enzyme activity increased with increasing temperature from 30 to 60 °C and decreased from 60 to 90 °C (**Figure 7 A**). At the optimum temperature of 60 °C, the activity was 5-fold higher than that at 30 °C.

The thermostable experiment showed that the enzyme retained 50% of activity after 72 h at 50 °C or 3 h at 60 °C, but was rapidly inactivated above 70 °C (**Figure 7 B**). Since the stability of the recombinant α-glucosidase is similar to the wild-type enzyme (*2*), which was stated to be stable, the recombinant enzyme from the yeast expression system in the present study is also considered to be stable against heat.

pH Optimum and Stability. The optimum pH of recombinant α-glucosidase was 4.5 (**Figure 8 A**). The enzyme exhibited the highest enzymatic activity (> 90% of maximum) between pH 3.5 and 5.5. It retained more than 50% of its maximal activity between pH 3.0 and 8.0 after incubation at 4 °C for 24 h (**Figure 8 B**).

Kinetic Studies. The kinetics of the recombinant enzyme was analyzed using *p*NPG as substrate. At 50 °C, the K_m and V_{max} of the recombinant enzyme were 0.446 mM and 43.48 U/mg, respectively. The K_m value was similar to that of the native enzyme, which was 0.620 mM (*12*).

Metal Requirement. Previously, it was reported that the activity of α-glucosidases from *Escherichia coli* (*21*), *Mucor racemosus* (*22*) and *Thermotoqa maritima* (*23*) was able to be stimulated in the presence of Al^{3+}, Ca^{2+}, K^+, Mg^{2+} or Mn^{2+} (1−10 mM), while there is no metal requirement information for α-glucosidase of *A. niger*. In the present study, the metal requirement of the recombinant α-glucosidase was analyzed by using 1 mM metal ions (Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Zn^{2+}, Fe^{2+}, Ba^{2+}, Ni^{2+}, Cu^{2+}, Al^{3+}, and Pb^{2+}). The enzyme was preincubated with each

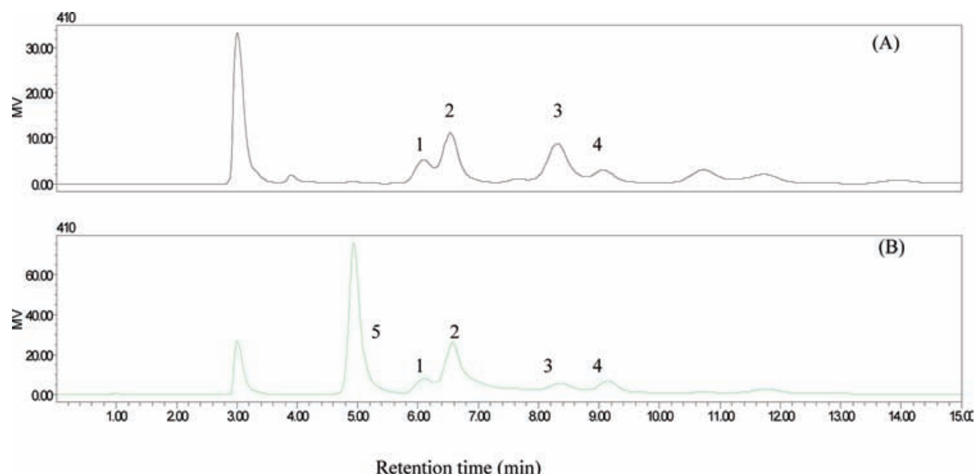

Figure 3. Analysis of transglucosidation products by HPLC. (**A**) Standard (commercial IMOs). Peak 1, maltose; peak 2, isomaltose; peak 3, panose; peak 4, isomaltotriose. (**B**) Transglucosidation products by recombinant α-glucosidase. Peak 5, glucose.

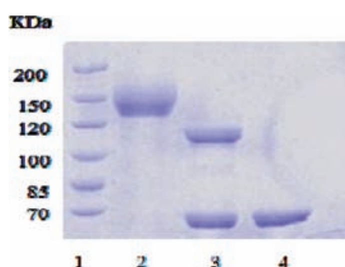

Figure 4. SDS−PAGE analysis for deglycosylation of recombinant α-glucosidase. Lane 1, marker; lane 2, recombinant α-glucosidase; lane 3, deglycosylated recombinant α-glucosidase; lane 4, endoglycosidase H_f (Endo H_f).

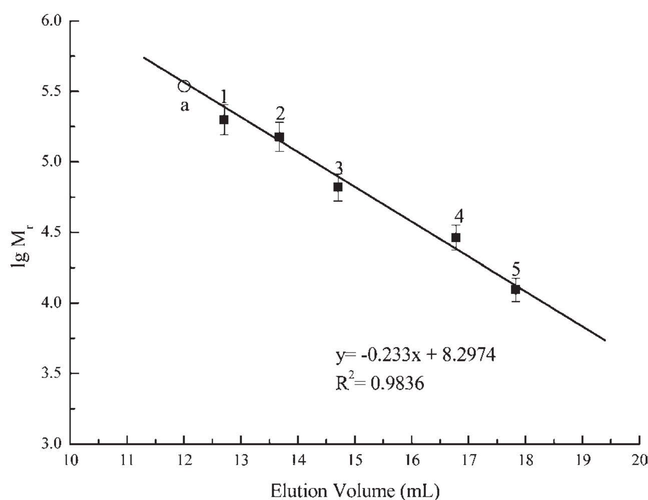

Figure 5. Molecular weight determination of native recombinant α-glucosidase by Superdex 200 10/300 gel filtration chromatography. 1, standard; 2, β-amylase (M_r 200,000); 3, alcohol dehydrogenase (M_r 150,000); albumin bovine serum (M_r 66,000); carbonic anhydrase (M_r 29,000); cytochrome C (M_r 12,400); a, recombinant α-glucosidase. Error bars correspond to the standard deviation of three determinations.

metal ion and then assayed for the activity. The results showed that none of these metals significantly affected the activity of recombinant enzyme, which suggested that the structure and the catalysis of the enzyme are not sensitive to the metal ions in the experimental condition.

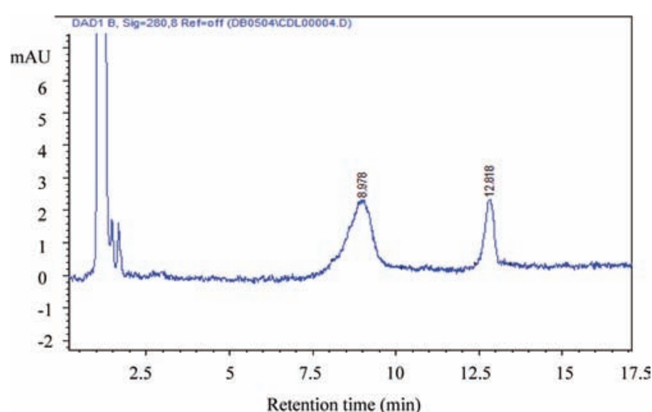

Figure 6. Reverse-phase HPLC of recombinant α-glucosidase.

DISCUSSION

In the industry of functional oligosaccharides, the transglycosylation activity of *A. niger* α-glucosidase has been applied to produce IMOs (*7*). In order to improve the yield of enzyme production, in the present study, cDNA of *A. niger* α-glucosidase was cloned and expressed in *P. pastoris* KM71. Previously, *A. niger* α-glucosidase was expressed in *A. nidulans* (*11*) and *E. nidulans* (*12*), and the expression level was reported to be 0.04 U/mg and 0.96 U/mg, respectively. In addition, the yield of recombinant α-glucosidase obtained from *E. nidulans* was reported to be 0.65 U/mL (*11*). In the present study, the enzyme activity in the culture supernatant of a 3 L fermentor could reach 2.07 U/mL and 2.21 U/mg protein. The high expression level obtained in the present study probably due to that, compared with filamentous fungal expression systems, the *P. pastoris* expression system has several advantages for expression of heterologous proteins, including use of the strong and regulated *AOX1* promoter, the effectively selectable markers, secretion signals and methods for coping with proteases (*24*).

Previously, cDNA of *A. niger* (No.SG136) α-glucosidase was cloned and expressed in *P. pastoris* in our laboratory; however, the yield of recombinant α-glucosidase was only 0.08 U/mL. The low expression level compared to that in the present study of *A. niger* (CICIM F0620) might be caused by nonidentical gene sequences in both cases as well as the low copy number of the target gene in the host cell. Thus, to the best of our knowledge, the production of recombinant α-glucosidase obtained in the present study represents the highest yield reported so far.

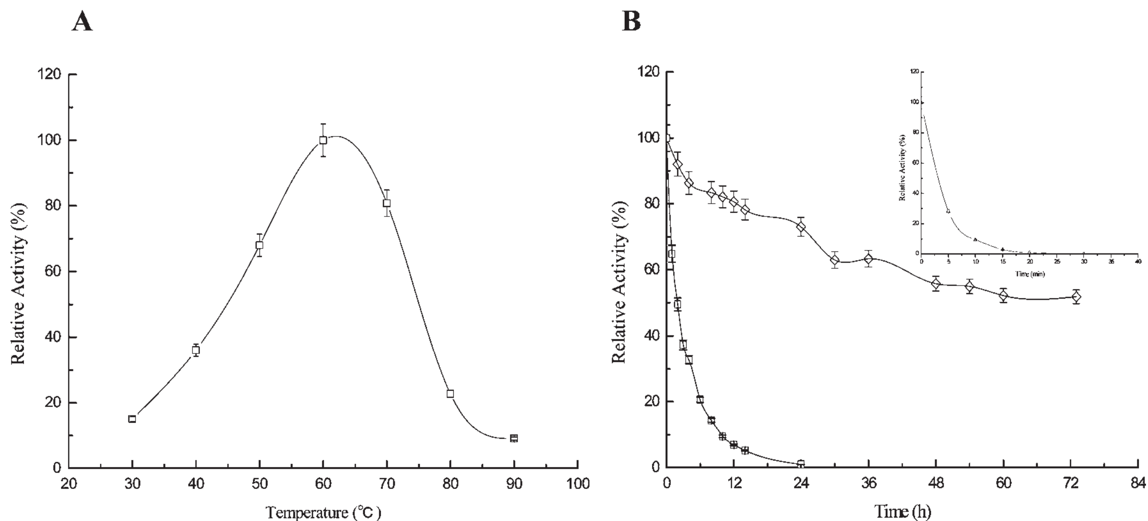

Figure 7. Effects of temperature on activity and stability of recombinant α-glucosidase. (**A**) Temperature optimum. The activity of recombinant α-glucosidase at 60 °C was defined as 100%. (**B**) Thermostability of the enzyme, 50 °C (◇), 60 °C (□) and 70 °C (△). The activity of recombinant α-glucosidase without heat treated was defined as 100%. Error bars correspond to the standard deviation of three independent determinations.

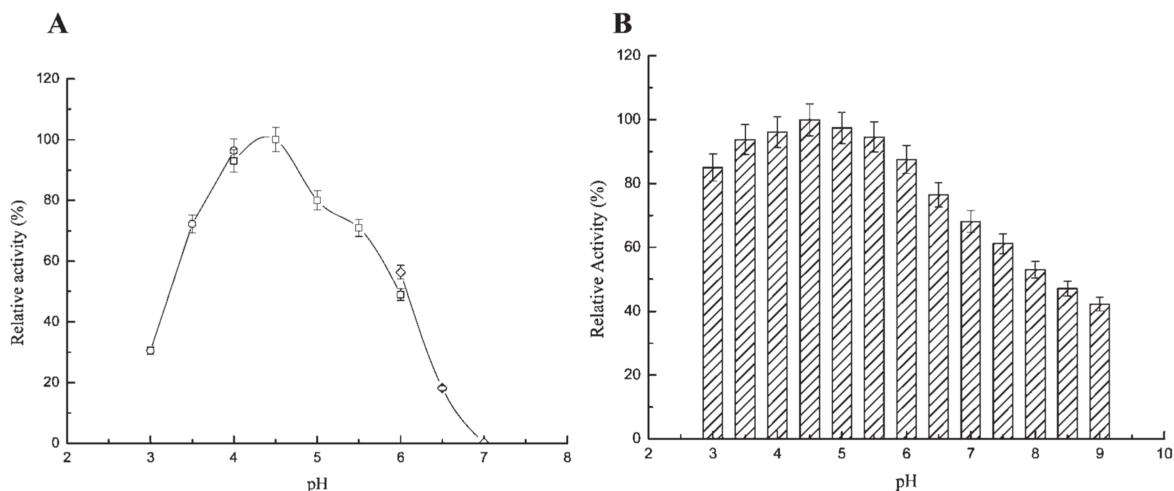

Figure 8. Effects of pH on activity and stability of recombinant α-glucosidase. (**A**) pH optimum. The activity of recombinant α-glucosidase at pH 4.5 was defined as 100%. (**B**) pH stability. The activity of recombinant α-glucosidase at pH 4.5 was defined as 100%. Error bars correspond to the standard deviation of three independent detrminations.

Chemical characterization of recombinant *A. niger* α-glucosidase has been performed previously when the gene of *A. niger* α-glucosidase (including introns and extrons) was cloned and expressed using fungal species as host cell (*11*−*13*). Although in our previous study, the cDNA of *A. niger* α-glucosidase was cloned and expressed in yeast, only the condition of transglycosylation reaction of the recombinant enzyme was investigated (*16*). In order to demonstrate if the α-glucosidase from the fungal source *A. niger* has been successfully post-translational modified in the yeast expression system, in the present study, the biochemical properties of the recombinant enzyme were characterized in detail.

Comparative biochemical characterization of native α-glucosidase from *A. niger* and recombinant enzyme from *P. pastoris* indicated that they had similar optimal pH and temperature. The recombinant α-glucosidase remained active at a wide pH range and was stable against thermal denaturation. These results showed that the recombinant α-glucosidase was potentially to be effectively useful in the preparation of IMOs.

Wild-type α-glucosidase from *A. niger* is a glycoprotein containing 25.5−27.6% carbohydrate (*25*). Deglycosylation analysis of the recombinant enzyme indicated that it was glycosylated in *P. pastoris*. Considering the similar catalytic properties between the wild-type and recombinant enzyme, it seems like the recombinant enzyme has the correct glycosylation patterns to ensure its biological activity.

Previously, it was reported that the gene length of *aglu* is 3124 bp, containing three introns and four extrons. It encodes 985 amino acids, and the N-terminal sequence from Met-1 to Leu-25 is predicted to be a signal peptide which is similar to the typical eukaryotic signal sequence (*11*). In addition, it was found that the mature wild-type α-glucosidase was actually a heterosubunit protein, in which the two heterosubunits were composed of residues 26−252 and 267−985 of aglu respectively. Even though the mechanism of this mature protein formation process has not been demonstrated, it was suggested that the pro-protein of aglu was subjected to a limited proteolysis after secretion from the cells (*11*). Interestingly, it was found that the two heterosubunits had a very tight interaction and could not be separated by SDS−PAGE, while they were able to be separated by reverse-phase HPLC (*2*). In the present study, a similar phenomenon was also observed. The recombinant enzyme exhibited one

band on SDS−PAGE, one peak by gel filtration HPLC, and two peaks by reverse-phase HPLC. Attempting to N-terminal sequence the recombinant enzyme failed probably due to glycosylation issues. Considering that the biochemical properties of recombinant enzyme in the present study are similar to those of the wild-type, it is assumed that the recombinant enzyme was also proteolyzed after synthesis and formed a heterosubunit protein. If this is correct, it may be presumed that the protease which hydrolyzes the pro-α-glucosidase has no strict substrate specificity. It not only exists in *A. niger* but also exists in *P. pastoris*. Further information is needed to support this hypothesis.

In summary, cDNA of *A. niger* α-glucosidase was cloned, expressed and characterized in detail. The expression level of 2.07 U/mL and 2.21 U/mg protein obtained in the culture media in the present study represents the highest yield of *A. niger* α-glucosidase reported so far. Detailed biochemical characterization demonstrated that the recombinant enzyme from *P. pastoris* was similar to that of native α-glucosidase from *A. niger*, suggesting that the recombinant enzyme has been successfully post-translationally modified in the *P. pastoris* expression system. Further enhancement of the yield of the recombinant α-glucosidase by fermentation technology is currently underway in our laboratory, and these studies will provide the basis for the application of recombinant α-glucosidase in the industry of IMOs production.

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