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Full Length Research Paper

Cloning, recombinant expression and characterization of a new glucoamylase gene from *Aureobasidium pullulans* NRRL 12974 and its potential application in raw potato starch degradation

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A new amylase gene APGA1 was cloned from *Aureobasidium pullulans* NRRL 12974 and expressed in *Pichia pastoris*. This is the first report on cloning and expression of amylolytic gene from the industrially important microorganism *A. pullulans*. The purified recombinant protein with MW of 66 kDa and specific activity of 298.02 Umg^{-1} protein was verified as a glucoamylase by its hydrolytic mode. This recombinant glucoamylase with optimal pH of 4.5, and temperature of 60°C, showed good hydrolytic activity against raw potato starch. At 60°C, 83.1% of raw potato starch slurry (150 gl^{-1}) was hydrolysed into glucose by 0.1 U mg^{-1} starch purified recombinant glucoamylase in less than 2.5 h. This is the highest raw starch hydrolysis efficiency report about recombinant fungal glucoamylase. This useful property indicated that this glucoamylase may find important applications in the starch saccharification industry and in bioethanol production.

Key words: Glucoamylase*, Aureobasidium pullulans*, expression, raw starch degradation.

INTRODUCTION

Starch is an important reproducible industrial substrate for the production of biofuel, protein and chemicals (Connor and Liao, 2009; Giordano et al., 2008; Li et al., 2007).In starch, industrial utilization, liquefaction and saccharification at high temperature are necessary processing steps. However, they are time-wasting and energy intensive. Some glucoamylase can hydrolyse raw starch granules into glucose in one step at low temperature (Nagasaka et al., 1998). This raw starch direct degradation will be popular and valuable due to saving on power costs and simplifying the process. Many new glucoamylases with this useful property have been investigated and reported (Asoodeh et al., 2010; Chen et al., 2007; Da Silva et al., 2009; Gill and Kaur, 2004) but few of them have been used in direct enzymatic conversion of raw starch by industry scale, such as potato starch; is a main industrial starch source. But the large granule size of raw potato starch (up to 100 µm) makes the glucoamylase direct degradation less efficient. It is a key to the utilisation of raw potato starch that obtains a glucoamylase with high direct raw potato starch degrading ability. So many glucoamylases with excellent properties have been reported in literature (Kumar and Satyanarayana, 2003; Liu et al., 2008; Yang et al., 2010; Kumar et al., 2007).

Aureobasidium pullulans is an industrially important microorganism, as it can produce extracellular

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Abbreviations: **MW**, Molecular weight; **LB**, luria-bertani; **NaCl**, sodium chloride; **PCR**, polymerase chain reaction; **BMGY**, buffered glycerol-complex; **BMMY**, buffered methanol-complex; **SDS-PAGE**, **sodium dodecyl sulfate polyacrylamide gel electrophoresis;** **DNS**, 3,5-dinitrosalicylic acid; **MALDITOF/TOF,** matrix-assisted laser desorption/ionization time-of-flight/time-of-flight; **HPLC**, high-performance liquid chromatography.

polysaccharide pullulan and highly active hydrolytic enzymes such as fructofuranosidase, xylanase, pectinase, tannase, cellulase and glucoamylase (Chi et al., 2009; Lodato et al., 1997; Kudanga and Mwenje, 2005). Glucoamylase from this organism has great potential for use in the raw starch degradation because of its optimal acidic pH, thermostability and ability of degrading high concentration raw potato starch slurry. Because of the low yield and high production cost of wild type strains, recombinant expression of this glucoamylase in engineering microorganism may be a better production strategy but the glucoamylase gene from this fungus remains unknown so far. In this work, we report the cloning, expression and characterisation of the glucoamylase gene, APGA1.The recombinant protein was purified to homogeneity and characterized with respect to substrate, pH and temperature profiles. Additionally, the hydrolysis profiles of raw potato starch were characterized. Our research result would contribute to this new glucoamylase with great activity found in the potential application of it to the direct hydrolysis of raw starch for industrial application.

MATERIALS AND METHODS

Strains, plasmids and media

The fungal strain *A. pullulans* NRRL 12974 in this study was a gift from Dr. James Swezey, collection manager of ARS culture collection (NRRL). This strain was maintained and grown in YPD medium containing 2.0% (w/v) glucose, 1.0% (w/v) yeast extract and 2.0% (w/v) polypeptone at 4°C. *Escherichia coli* DH5α [supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-l relA1] was used as the host cell to amplify the plasmids carrying cloned genes and was grown at 37°C in LB medium containing 1.0% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl overnight. pMD19T-Simple vector for cloning of PCR products was purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The expression vector and host yeast strain used in this study were pPIC9k and *P. pastoris* KM71. The transformation and induction carried out refer to previous *P. pastoris* heterologus expression reports (Yang et al., 2010). The electroporation yeast transformants were grown and selected at 30°C in MD medium [1.34% YNB, 4×10^{-4}% biotin, 2% glucose (pH 7.0)] by PCR. The host yeast strain and the yeast transformants were cultivated at 28°C in BMGY[1.34% YNB, 4×10^{-4}% biotin, 1.0% glycerol in 100 mM phosphate buffer (pH 7.0)] and induced in BMMY [1.34% YNB, 4×10^{-4}% biotin and 0.5% methanol in 100 mM phosphate buffer (pH 7.0)], respectively.

Cloning of the full-length gene encoding glucoamylase

Gene cloning was carried out by using the methods as described in previous reports (Sambrook et al., 1989). The partial fragment of glucoamylase cDNA was amplified using two degenerate primers: JBF5'-YTNGCNAAYCAYAAR-3' and JBR5'-GCRTANSWCCAN-GTNAR-3' which were designed based on the conserved motifs LANHK and LTWSYA. The partial fragment PCR products were cloned into pMD19-T Simple vector and sequenced by Invitrogen Co. Ltd (Shanghai, China). The amino acid sequence of the cloned partial cDNA fragment was deduced and aligned using the BlastX online programs of NCBI. After the alignment, it was found that the amino acid sequence deduced from the cloned partial cDNA (438 bp) was 68% identical to glucoamylase gene of *Talaromyces emersonii.* So, we supposed that the fragment is part of a novel glucoamylase gene from *A. pullulans* strain NRRL 12974. In order to get the full-length gene, SMART RACE cDNA amplification kit (Cat 634914) from Clontech Co. Ltd. was used to carry out 5′ and 3′RACE. The RACE primers were designed based on the partial fragment sequence.

Recombinant expression in *P. pastoris* KM71

The expression primers were APGA1-F (5'-**GAATTC**ATGGA TCACAGCACGATTCATT-3') and APGA1-R (5'-**GCGGCCGC**CCT CCAACTATCGTTCTC-3'); the restriction enzyme cleavage sites *EcoRI* and *NotI* were underlined and in bold. The PCR product was digested and ligated into digested pPIC9k. This recombinant plasmid linearized with *SacI* was the transformed *P. pastoris* KM71 by electroporation. Strains transformed with the parent plasmid without the APGA1 gene were used as the control. The expression manipulations were performed according to the protocols in the pPIC9k manual from Invitrogen. After culturing in BMGY medium for 2 days, the yeast cells were collected by low speed centrifugation and transferred to BMMY medium in the sterile operation. During the next five days, some filter sterilized (0.22 μm) methanol was added into the medium at 24 h intervals to induce the extracellular protein expression. After seven days, the supernatant of the medium was collected by centrifugation of 12000 *g* for 10 min for the next step purification.

Recombinant glucoamylase purification and characterisation

The recombinant protein was purified to homogeneity from the cell-free culture broth of KM71 stepwise by ultra-filtration with Cogent μScale system (Millipore), gel filtration chromatography with Sephacryl S-100 HR (GE Healthcare) and anion-exchange chromatography with Toyopearl DEAE-650M (Sigma-Aldrich). Finally, the purified protein was verified for purity by SDS-PAGE.

In the purification of the recombinant glucoamylase, the standard activity assay protocol was 0.5 ml and the reaction mixture contained 10 gl^{-1} soluble starch in 0.2 M acetate buffer (pH 4.5) mixed with 0.2 U enzymes at 60°C for 30 min. The reducing sugar was determined using the DNS method (Rick and Stegbauer, 1974). One unit of amylase activity was defined as the amount of enzyme causing the release of reducing sugars equivalent to 1 μM of glucose from starch per minute under the assay conditions. Protein concentration was measured by the Bradford method (Bradford, 1976). The relative activity is determined as the enzyme units composed per mg protein.

In order to confirm the purified protein as recombinant APGA1 indeed, mass spectrometry was performed using an Applied Biosystems MALDITOF/TOF mass spectrometer (Framingham, MA, USA). The protocol was carried out as previous reports (Peng et al., 2010). The tryptic digests were cocrystallized with a matrix of CHCA and spotted on the target wells. The signals of peptide mass fingerprinting (PMF) were acquired in the reflectron mode in the m/z range from 700 to 4,000. Calibration was performed externally using standard peptide mixtures and internally using the peptide fragments of trypsin autolysis products. On the basis of the PMF signals, the one strongest peptide with higher accuracy and higher abundance was further analyzed in the tandem mass spectroscopy (MS/MS) mode. The MS/MS data were exported in a suitable format and submitted to the database for the search for proteins with MASCOT software (Matrix Science, London, UK). The latest version of the fungi database in the NCBI nr databases was used in

the protein search.

In characterisation of recombinant glucoamylase in order to identify the hydrolysis mode of the APGA1, the hydrolysis product of soluble starch was determined by HPLC. The protocols were referred to as in previous reports (Balkan and Ertan, 2005). The Waters Breeze HPLC system composed of 152 Binary HPLC Pump, 2487 dual λ absorbance detector and waters symmetry C18 HPLC column (4.6×150 mm) were used. The mobile phase was made of 75% acetonitrile dissolved in dd H_2O and pumped at a flow rate of 1 $mlmin^{-1}$ after an injection of 20 µl sample.

Substrate profile of recombinant APGA1 was tested using six common starch substrates. Optimal pH was determined by conducting assays in buffer systems with different pH between pH 3 and 9. Optimal temperature and thermostability were determined by conducting assays at 30 to 80°C.

Hydrolysis of raw potato starch

Direct raw starch hydrolysis capacity is a very important property for glucoamylase. However, it is difficult for most glucoamylase to direct the high concentration hydrolysis of starch slurry. Raw potato starch is more difficult to be degraded because of larger granules. So, we selected common raw potato starch slurry with industrial concentration as test substrate for the recombinant APGA1. Hydrolysis test was carried out as protocols in previous reports (Nagasaka et al., 1998; Kumar et al., 2007). The reaction mixture containing 1 ml acetate buffer (200 mM, pH 4.5), some raw potato starch and purified glucoamylase was shaken at 180 rpm and 60°C for 2 to 3 h. Based on the amounts of reduced sugars released, the substrate hydrolysis ratio was calculated. Next, temperature effect on raw potato starch (100 gl^{-1}) hydrolysis ratio was tested at 55, 60 and 65°C, respectively; enzyme doses effect on hydrolysis ratio was tested at five enzyme doses ranging from 0.02 to 0.2 Umg^{-1} starch.

RESULTS

Molecular cloning and sequence analysis

A 438 bp cDNA fragment sequence was obtained using degenerate primers. This sequence was confirmed as internal sequence of a new glucoamylase by BlastX and its flanking sequences obtained by 5′ and 3′RACE were 955 and 715 bp. This full-length cDNA was amplified by APGA1-F and APGA1-R primers and named APGA1 whose sequence was deposited in the GenBank database under the accession no. HM246718.

The CDS translated from the APGA1 gene consisted of 626 amino acids with a calculation of 66.7 kDa MW, which is a relatively small molecular weight among fungal glucoamylases. The glucoamylases from *Aspergillus niveus*, *Fomitopsis palustris* and *Paecilomyces variotii* have the estimated molecular weight of 76, 72 and 86kDa, respectively (Da Silva et al., 2009; Yoon et al., 2006; Michelin et al., 2008). On the contrast, the molecular weight of glucoamylase from *Thermomucor indicae-seudaticaehad* is merely 42 kDa (Kumar and Satyanarayana, 2003). The specific activity of recombinant glucoamylase reached 298.02 Umg^{-1} protein which is similar to other fungi derived counterparts (Nagasaka et al., 1998; Chen et al., 2007; Yang et al., 2010; Yoon et al., 2006; Michelin et al., 2008).

Alignment results with other glucoamylase CDS are shown in Figure 1. A forecasted signal peptide of 25 amino acids (underline marked) was predicted in http://cbs.dtu.dk/services/SignalP/.The five most probable N-linked glycosylation sites of APGA1 predicted in http://cbs.dtu.dk/services/NetNGlyc are marked with stars. Two conserved Glu residues (arrow marked) of the glycosyl hydrolase family 15, were thought to participate in the hydrolysis mechanism by removing or adding a proton. These analysis results indicated that this glucoamylase is a typical extracellular fungal glucoamylase, a member of glycoside hydrolase family 15. It was more homologous to enzymes from filamentous fungi other than yeast, although *A. pullulans* is sometimes called “black yeast”.

Recombinant expression, purification and characterisation

Figure 2 shows that the extracellular supernatant displayed one specific band with about 66 kDa MW (Lanes 2, 3, 4 and 5), which was nearly consistent with the deduced 66.7 kDa MW of APGA1 gene. No similar band was observed from the control sample (Lane 6). After ultra-filtration and gel filtration, the purified protein band which was presumed to be the recombinant APGA1 had 66 kDa MW by SDS-PAGE analysis (Lane 7). MALDI-TOF/TOF result of the purified APGA1 indicated that 12 peptide sequences could match the CDS of APGA1 completely. The 12 peptide sequences were 1: 779.41-DSALVFK; 2: 823.42-VVTDSFR; 3: 983.48-ATAMIAYSR; 4: 1141.63-ALVDQLIAGNK;5: 1427.75-ALVEGNNLATQ LGK; 6: 1449.62-SNPDYFYTWTR; 7: 1561.66-AQDG SVTWESDPNR; 8: 1688.90-SLEPLIQQYISAQAK; 9: 1876.96- SIYSINQGIA- QGSGVAVGR; 10: 1976.98-FEVDLTPFTGAWGRPQR; 11: 2089.86-SYTVTGNCAGT ATEND SWR; 12: 2948.49-ATGSLSSWLSSENTVAL QGVLNNIGASGSK.

The one order mass spectrum of protein and second order mass spectrum of peptide (ALVDQLIAGNK) were shown in Figure 3. The mentioned results confirm that this protein band with about 66 kDa MW was a recombinant APGA1 indeed.

One of the primary differences between glucoamylase and other amylase is the mode of hydrolysis against starch substrates. Glucoamylase is an exonclease which hydrolyzes and releases glucose from non-reducing ends of the starch molecule. One glucose molecule is released in one hydrolysis reaction and at the end of the hydrolysis, almost all the product is glucose except occasionally a small amount of maltose. Hydrolysis mode determined the classification of APGA1 in terms of the HPLC detection of hydrolysis product. Figure 4 demonstrates that the hydrolysis product was composed of

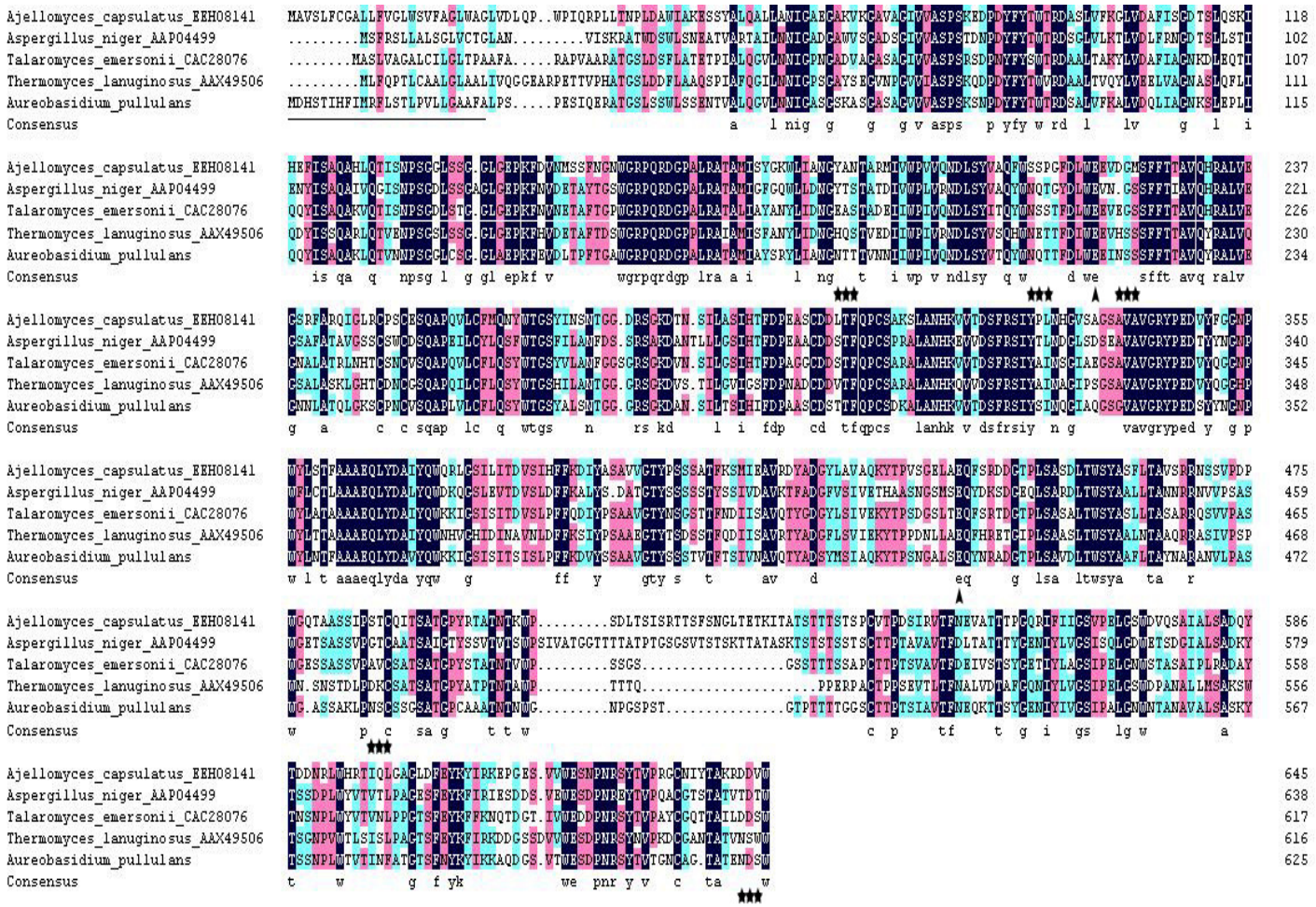

Figure 1. Amino acid alignment and analysis result of APGA1 with other fungal glucoamylases.

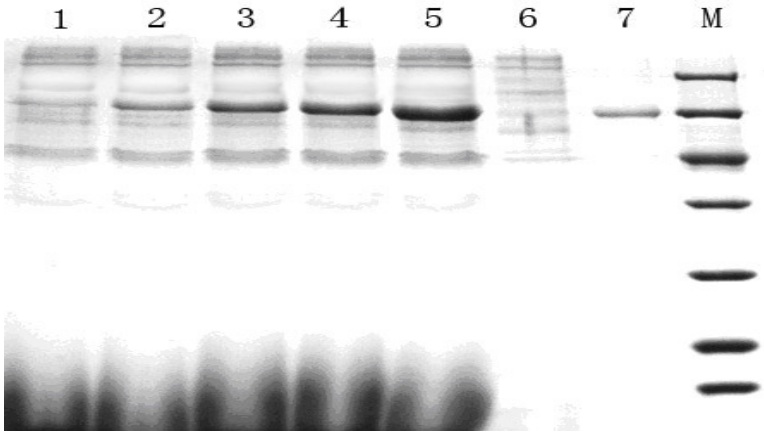

Figure 2. SDS-PAGE analysis of APGA1 expression and purification. Proteins were visualised by Coomassie-brilliant blue staining. Lane 1, 24 h; lane 2, 48 h; lane 3, 72 h; lane 4, 96 h; lane 5, 120 h; lane 6, supernatant from *P. pastoris* KM71 without the APGA1 gene; lane 7, purified recombinant glucoamylase; lane M, protein molecular weight marker (116, 66.4, 45, 35, 25, 18.4 and 14.4 kDa from the top).

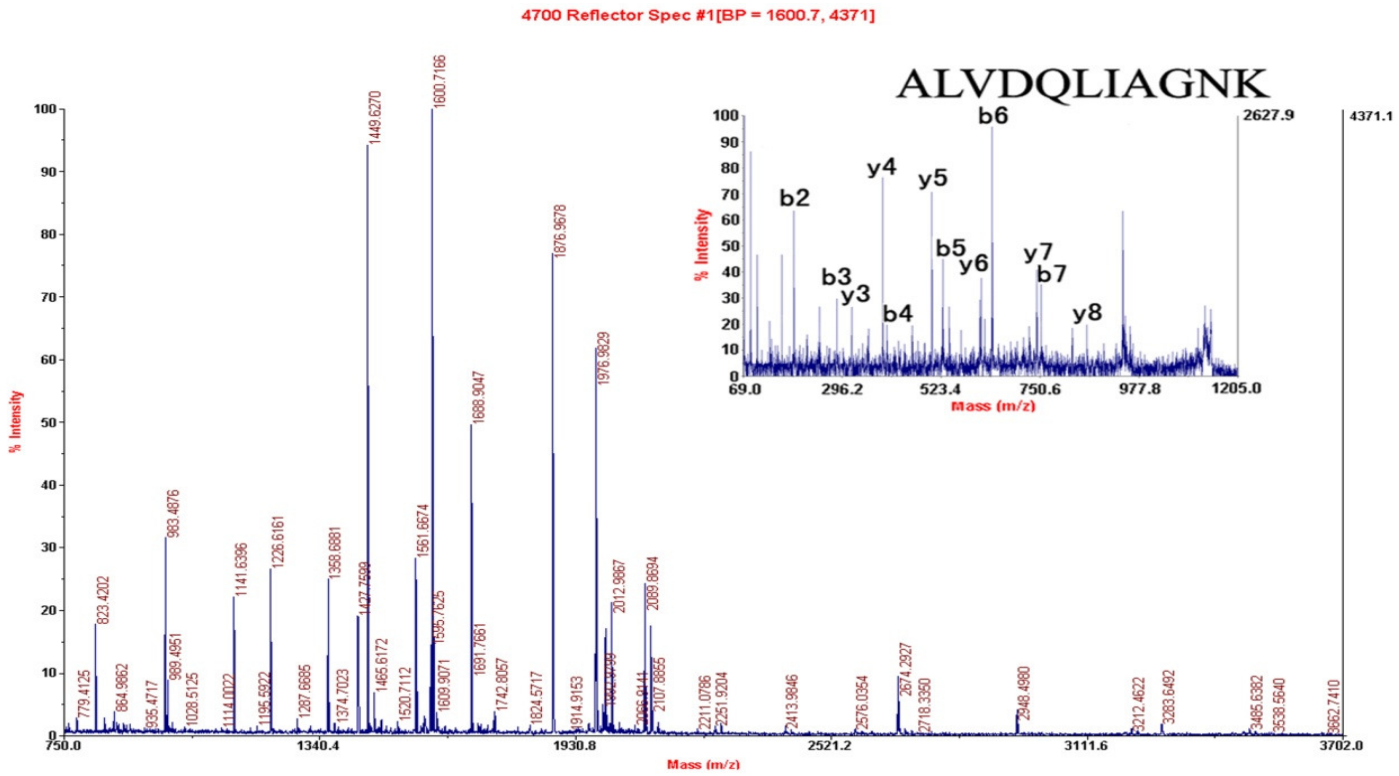

Figure 3. MALDI-TOF/TOF mass spectra analysis of the purified recombinant glucoamylase. A total of 12 parent ions' amino acid sequences were identified by matching the CDS of glucoamylase gene. MS/MS spectra and the sequence of one parent ion with MW of 1141.636 are shown.

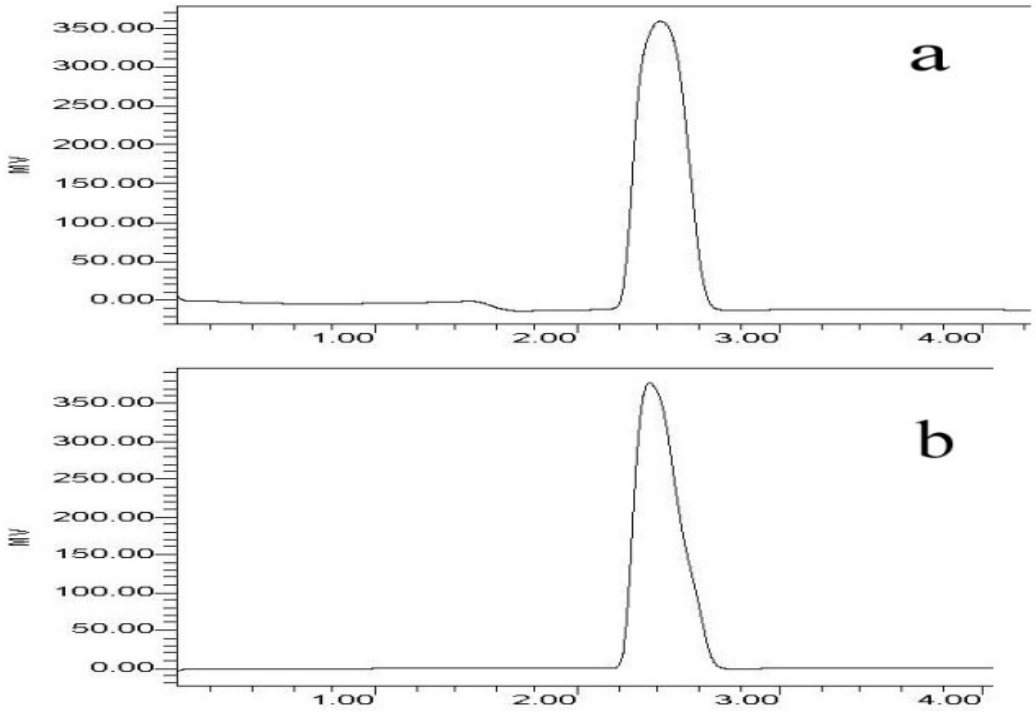

Figure 4. HPLC result of the hydrolysis products of the soluble starch generated by the purified recombinant APGA1. (a) Glucose standard; (b) the hydrolysis product.

Table 1. Hydrolysis activity of the purified glucoamylase against different substrates.

Substrate	Relative activity (%)*
Soluble starch	100
Amylopectin	124
Amylose	45.4
Glycogen	79.8
Pullulan	5.5
Laminarin	9.7

*The reaction conditions were 0.5 ml reaction mixture containing 10gl^{-1} substrate in 0.2 M acetate buffer (pH 4.5) mixed with 0.2 U enzyme at 60°C for 30 min. The reducing sugar in the diluted sample was determined using the DNS (3,5-dinitrosalicylic acid) method. The relative activity was calculated based on relative content of the reducing sugar. Absolute (100%) quantitation was the sugar concentration of soluble starch hydrolysate. The starch substrates were purchased from Sigma.

almost entire glucose, supporting the conclusion that APGA1 is a glucoamylase.

Substrate profile of amylase is mainly determined by the ability of breaking down different glycosidic linkages. However, for glucoamylase, non-reducing end amount of substrates is also important sometimes. Table 1 shows that amylopectin was the best substrate for APGA1 (relative activity 124%) because it has more non-reducing ends and a large number of α-1, 4-glycosidic linkages. On the contrary, although glycogen contains many more non-reducing ends than amylopectin, the relative activity against it was lower than the latter (relative activity 45.7%). The reason is possibly because there is too much α-1, 6-glycosidic linkage in glycogen, which are more difficult than α-1, 4-glycosidic linkages for APGA1 to break down. Pullulan has both α-1, 4 and α-1, 6-glycosidic linkages. Thus, the lowest relative activity (5.5%) against pullulan was caused by the extremely rare non-reducing ends in pullulan which is almost a complete linear molecule. The low activity against laminarin (relative activity 9.7%) indicated that APGA1 hardly hydrolyze β-1, 3 and β-1, 6-glycosidic linkage.

The recombinant glucoamylase activity measured as a function of temperature from 30 to 80°C showed that the activity was highest at 60°C (Figure 5). This is a useful property for industrial glucoamylase. The thermostability test result is shown in Figure 5. The glucoamylase activity still remained 98% over the control after the treatment at 50°C for 1 h. However, the enzyme was inactivated rapidly at temperatures higher than 60°C and was inactivated totally at 65°C within 20 min. The half-life was 15 min under incubation at 60°C.The recombinant glucoamylase was less thermostable than the native glucoamylase from this kind of microorganism which was named glucoamylase A and investigated in previous reports (Badal et al., 1993). The possible reason is that the glycosylation status of the native protein is more beneficial to its thermostability. Optimal temperature of glucoamylase from *Chaetomium thermophilum* is 65°C (Chen et al., 2007), however, the glucoamylase MpuGA-I from *Monascus purpureus* RY3410 has an optimal temperature of 50°C (Tachibana and Yasuda, 2007). So, among other fungal glucoamylase, the thermostabilty of APGA1 is relatively intermediate.

Purified recombinant glucoamylase activity and stability was measured at various pHs in buffers of the same ionic concentrations. Figure 6 shows that most fungal glucoamylases APGA1 were acidophilic with optimal activity at pH 4.5 and stability between pH 3 and 6. The catalytic activity of the enzyme was reduced quickly at pH higher than 5 and was totally lost at pH higher than 7. This result confirms that glucoamylase from *A. pullulans* is acidophilic, which is one of the universal properties of fungal glucoamylase. The glucoamylase from *C. thermophilum* exhibited optimum catalytic activity at pH 4.5 and 5 (Chen et al., 2007). *Paecilomyces variotii* produce a glucoamylase which has optima pH of 5 (Michelin et al., 2008), while the glucoamylase produced by *Aspergillus niveus* has the optimum pH of 5 to 5.5 (Da Silva et al., 2009). On the other hand, the glucoamylase from thermophilic mold *Thermomucor indicae-seudaticaehad* acted optimally at pH 7 (Kumar and Satyanarayana, 2003).

Hydrolysis of raw starch by purified recombinant glucoamylase

Figure 7 indicates that 60°C was the optimal reaction temperature of the raw potato starch hydrolysis. This temperature happens to be the starch saccharification temperature in industry. Figure 8 shows that the hydrolysis ratio of the raw potato starch slurry (150 gl^{-1}) was 83.1% using 0.1 U mg^{-1} substrate enzyme doses. Many raw starch-degrading amylases have been investigated, but few have been applied in the starch industry largely because of low activity against high concentration raw starch. In our study, the recombinant APGA1, demonstrated an excellent ability to degrade raw potato starch directly and when the enzyme dose was increased to 0.1 U mg^{-1}starch, the hydrolysis ratio of raw potato starch slurry (150 gl^{-1}) reached 83.1% in 3 h (Figure 8). This result is attractive for industrial production due to the starch slurry (150 gl^{-1}) which is usually used in the production practice of the starch industry.

DISCUSSION

Along with the petroleum source exhaustion, new power source such as biofuel has attracted people's attentions (Connor and Liao, 2009). Bioethanol, the most successful biofuel, has been produced by enzymatic transforming starch and cellulose substrates in industrial scale

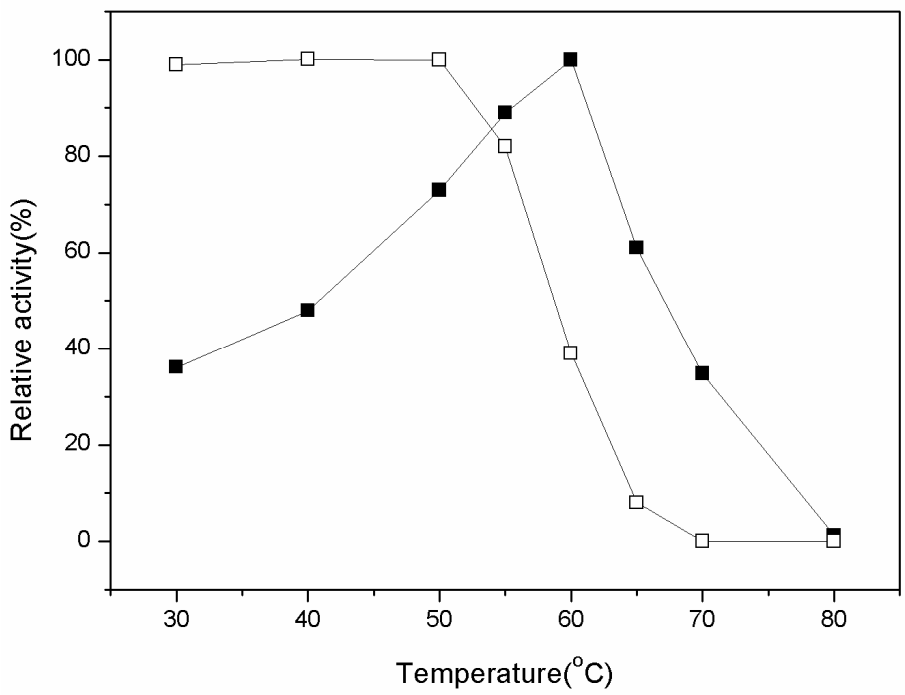

Figure 5. Effect of temperature on activity (closed square) and stability (open square). Activity was determined between 30 and 80°C in 100 mM acetate buffer (pH 4.5). Thermostability was determined by measuring residual activity of samples pre-incubated at various temperatures for 0.5 h at pH 4. Relative activity was in percentage (%). Absolute activity = 298.02 U/mg protein.

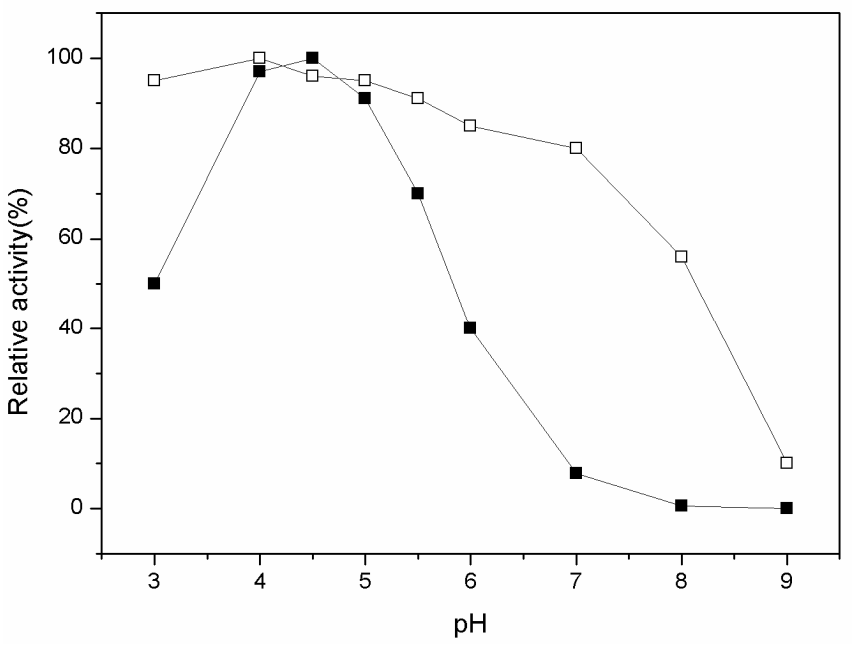

Figure 6. Effect of pH on activity (closed square) and stability (open square). Buffers used were 100 mM acetate for pH 3 to 6, 100 mM Tris-HCl for pH 6 to 8 and 100 mM $Na_2B_4O_7 \cdot 10H_2O$-H_3BO_3 for pH 7 to 10. Activity was determined by measuring activity assays between pH 3 and 9. Stability was determined by assaying residual activity of samples pre-incubated at various pHs for 10 h at 4°C. Relative activity was in percentage (%). Absolute activity = 298.02U/ mg protein.

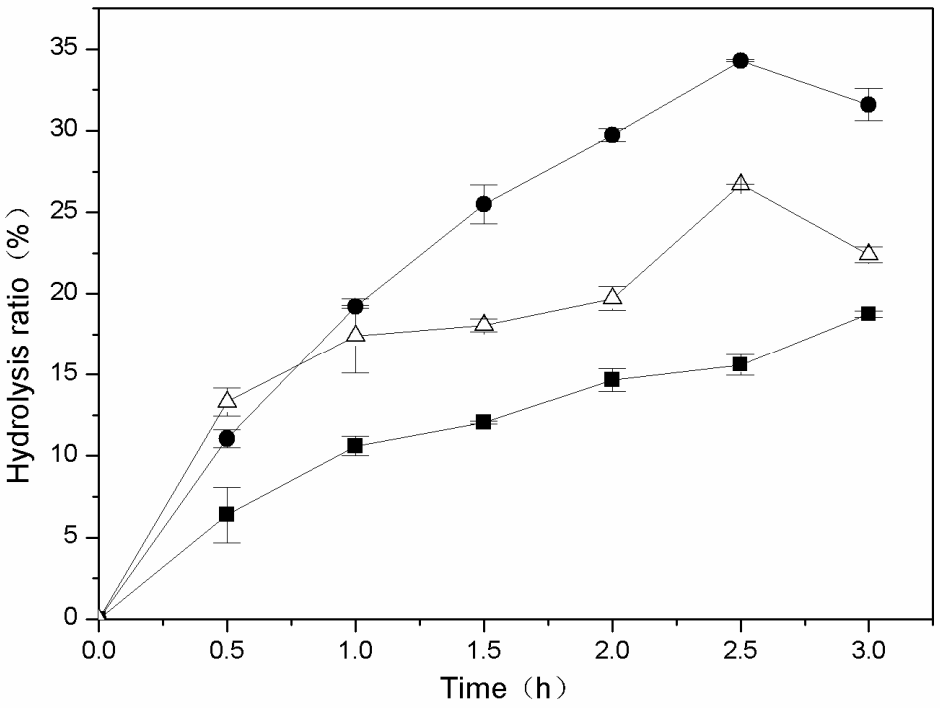

Figure 7. Effect of temperatures on hydrolysis ratio of raw potato starch. Data shown were averages of three independent experiments. Error bars represent standard deviation. Buffer, 100 mM acetate (pH4.5); substrate concentration, 150 gl^{-1}; enzyme dose, 0.02 U/mg starch; temperature, 55℃ (closed square), 60℃ (closed circle) and 65℃ (open triangle).

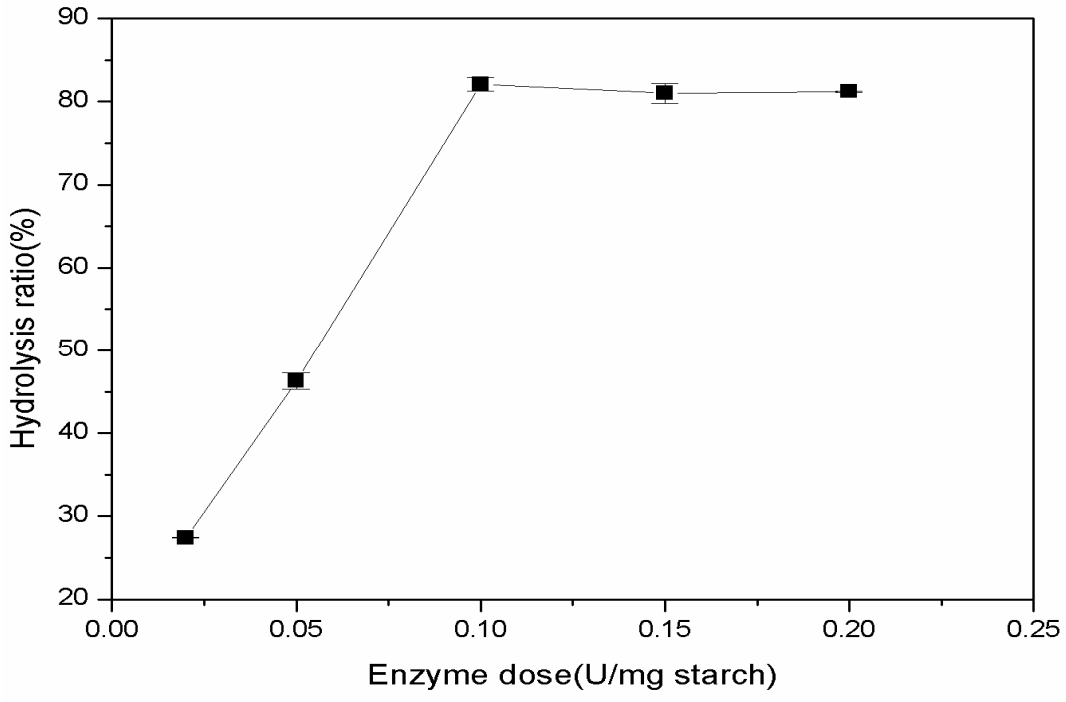

Figure 8. Effect of enzyme doses on hydrolysis ratio of raw potato starch. Data shown were averages of the three independent experiments. Error bars represent standard deviation. Buffer, 100 mM acetate (pH 4.5); substrate concentration, 150 gl^{-1}; temperature, 60℃; time, 2.5 h.

(Giordano et al., 2008). Among starch transforming enzymes, raw starch degrading enzymes (RSDE) is most potential and attractive (Li et al., 2007). Besides, RSDE is useful in the production of organic acids, amino acids, cyclodextrin and SCP. Yang et al. (2010) reported that *Sacharomyces cerevisiae* expressing *Rhizopus* glucoamylase could utilize starch as energy source to produce ethanol up to a final concentration as 5% (Yang et al., 2010). Tateno et al. (2007) reported the production of L-lysine by *Corynebacterium glutamicum* secreting alpha-amylase; raw corn starch was used as the sole carbon resource. RSDE can be divided into two class groups; glucoamylase and α-amylase. Glucoamylase can directly degrade raw starch into glucose, the good bioethanol production substrate. However, α-amylase only degrades raw starch into oligosaccharide which is not a convenient fermentation substrate. So, exploring good RSDE glucoamylase will contribute more to bioethanol industry.

As past reports, glucoamylase belonging to RSDE can be produced by *Acremonium, Aspergillus, Cladosporium gossypiicola, Corticium rolfsii, Gibberella pulicaris, Nodilusporium, Penicillium, Rhizopus, Thermomucor indicae-seudaticae*, *Saccharomycopsis fibuligera* and *Aureobasidium pullulans* (Sun et al., 2010). However, all these enzyme producers are fungi. The reaction temperature optima for them were mostly between 40 and 70°C and pH optima were mostly between 4.5 and 6.5. These properties are very suitable for raw starch hydrolysis. However, few of them possess high degrading activity with exceeding 10% (w/v) raw starch slurry, which is the lowest inventory of stuff in the industry. So, presently, it is difficult to direct hydrolyze raw starch using glucoamylase in industry. Discovering and screening more novel fungal glucoamylase and genes is a better solution.

A. pullulans NRRL 12974 has powerful starch degrading capacity as previously reported. Its native α-amylase and glucoamylase have been studied (Badal et al., 1993) but its amylolytic enzyme gene has remained unknown. In our research, glucoamylase gene APGA1 of NRRL 12974 was cloned and recombinant was expressed for the first time in *P. pastoris*. APGA1 was expressed in *E. coli* in our early research. The recombinant enzyme was soluble but had no activity (data not shown). Lack of glycosylation is a possible reason why *Pichia* yeast expression system was chosen in this research. The characterization of purified active recombinant enzyme from yeast indicated that APGA1 has excellent performance in degrading high concentration of raw potato starch slurry. The hydrolysis ratio of the raw potato starch slurry (150 gl^{-1}) was 83.1% using 0.1 U mg^{-1} substrate enzyme doses. By contrast, the purified glucoamylase from thermophilic *Bacillus* sp. strain WN11 can convert 82% of raw potato starch into glucose; however, the substrate concentration is only 10 gl^{-1} (Gill and Kaur, 2004). Another example is that the glucoamylase produced by *Penicillium* sp. X-1 can convert 79.5% of raw corn starch (150 gl^{-1}) into glucose with an enzyme dose of 0.06 U mg^{-1} starch; however, the process time is 6 h (Lin et al., 2011). The efficiency of raw starch hydrolysis of the APGA1 was the best of all the reported glucoamylases.

At the same time, this enzyme has good thermostability. So, valuable applications should be found in the raw potato starch saccharification process and other related processes. However, we must now face the problem of the yield of the recombinant APGA1 which was not high enough for large-scale industrial application. Thus, high-density fermentation research, targeting the improvement of the enzyme yield, is currently been carried out in our lab.

ACKNOWLEDGEMENTS

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