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Research article

Molecular cloning and biochemical characterization of an α-amylase family from *Aspergillus niger*

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ABSTRACT

Background: α-Amylase is widely used in the starch processing, food and paper industries, hydrolyzing starch, glycogen and other polysaccharides into glucose, maltose and oligosaccharides. An α-amylase gene family from *Aspergillus niger* CBS513.88 encode eight putative α-amylases. The differences and similarities, biochemical properties and functional diversity among these eight α-amylases remain unknown.
Results: The eight genes were cloned and expressed in *Pichia pastoris* GS115 by shaking-flask fermentation under the induction of methanol. The sequence alignment, biochemical characterizations and product analysis of starch hydrolysis by these α-amylases were investigated. It is found that the eight α-amylases belonged to three different groups with the typical structure of fungal α-amylase. They exhibited maximal activities at 30–40°C except AmyG and were all stable at acidic pH. Ca^{2+} and EDTA had no effects on the activities of α-amylases except AmyF and AmyH, indicating that the six amylases were Ca^{2+} independent. Two novel α-amylases of AmyE and AmyF were found. AmyE hydrolyzed starch into maltose, maltotriose and a small amount of glucose, while AmyF hydrolyzed starch into mainly glucose. The excellent physical and chemical properties including high acidic stability, Ca^{2+}-independent and high maltotriose-forming capacity make AmyE suitable in food and sugar syrup industries.
Conclusions: This study illustrates that a gene family can encode multiple enzymes members having remarkable differences in biochemical properties. It provides not only new insights into evolution and functional divergence among different members of an α-amylase family, but the development of new enzymes for industrial application.

1. Introduction

α-Amylase (EC 3.2.1.1) belonging to glycosyl hydrolase family 13 is a digestive enzyme that catalyzes the hydrolysis of starch, glycogen and related polysaccharides and therefore has wide applications in many industries, including starch processing, food and paper [1,2,3]. α-Amylase can be produced by plants, animals and microorganisms. However, from a bio-industrial viewpoint, microorganisms are the most important sources [4]. To meet the growing demands of the sugar industry, many of the microbial α-amylase genes were investigated, including molecular cloning, and expression and regulatory analyses [5,6,7,8]. Compared with the bacterial α-amylases, fungal α-amylases are preferred for starch hydrolysis applications in the baking, brewing and sweeteners industries owing to their generally recognized as safe status [9].

Aspergillus niger is an important industrial fermentation microorganism that is widely used for the production of industrial enzymes and organic acids [10]. α-Amylases of *A. niger* have been identified and characterized in recent decades. For example, the three-dimensional structure of an acid α-amylase (An11g03340) from *A. niger* was first reported in 1990 [11]. The functions of two α-amylase genes, An05g02100 and An12g06930, from *A. niger* var. *awamori* were determined in the genome [12], and one α-amylase was purified, crystallized and structurally defined [13]. However, no further details of the enzymological properties or characteristics of the hydrolysis on starch were discussed. Three putative cell wall-associated and GPI-anchored α-amylases (An09g03100, An12g02460 and An15g07800) of *A. niger* showed transglycosylation activities, therefore, belonged to the 4-α-glucanotransferases (EC 11 2.4.1.25) [14]. An intracellular fungal

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α-amylase (An01g13610) from *A. niger* was heterologously expressed, purified and characterized in 2007 [15].

Genome sequencing of *A. niger* CBS513.88 was completed and published in 2007 [16], and this made it possible to identify additional α-amylases-like enzymes. By analyzing the genome sequence information, at least eight genes were found to encode α-amylases. Among these enzymes, two α-amylases (An04g06930 and An09g03110, designated as AmyE and AmyF in this work) had not been reported previously. Additionally, the differences and similarities, biochemical properties and functional diversity among these eight α-amylases remain unknown. In this study, eight α-amylase genes from *A. niger* were successfully cloned and expressed in *Pichia pastoris*. Their biochemical and enzymatic characteristics were also investigated. To the best of our knowledge, this is the first comprehensive study on all of the α-amylases from *A. niger*.

2. Experimental

2.1. Bacterial strains and plasmids

Restriction enzymes and the DNA ligation kit were purchased from TaKaRa (Dalian, China). DNA gel extraction and plasmid DNA isolation kits, T4 DNA ligase and a 1-kb DNA ladder were obtained from Fermentas (MD, USA). *Escherichia coli* JM109 was used to propagate plasmid DNA. *P. pastoris* strain GS115, and vector pPIC9K were obtained from Invitrogen (Carlsbad, CA, USA). *E. coli* JM109 cells with plasmids were grown aerobically in Luria-Bertani medium supplemented with 100 μg/ml ampicillin. The media and culture conditions for the expression of recombinant α-amylases were performed according to the Pichia Expression Kit manual and the Pichia Fermentation Process Guidelines (Invitrogen).

2.2. Phylogenetic analysis of α-amylases

Signal peptide prediction was made using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). Multiple amino acid sequence alignments were constructed using the Clustal X2 software package. Phylogenetic relationships among α-amylases from different microorganisms were constructed using the program MEGA 4.0 with the Neighbor-joining method.

Three-dimensional (3D) models of *A. niger* α-amylases were performed with SWISS-MODEL (http://swissmodel.expasy.org/) which utilizes the MODELLER program. The 3D structure was analyzed by PYMOL software.

2.3. DNA manipulation

Total RNA was isolated from *A. niger* CBS513.88 using the RNA isolation reagent (Ambion, Inc. Austin, TX, USA) and treated with DNase-free water (Ambion) to remove contaminating DNA from the RNA samples. The total RNA was reverse-transcribed with heat denaturation of the RNA using the RETROscript kit (Invitrogen, Carlsbad, CA, USA). The enzymatic reaction was cleaned using a PCR Purification kit (Qiagen Inc.) followed by PCR amplification. Using cDNA sequences of α-amylases from *A. niger* CBS513.88 (NW_014013630.1) as templates, primers were designed using the software Primer Premier 5.0. PCR products were visually inspected on agarose gels, cleaned with a Qiagen QIAquick PCR purification kit and sent for sequencing at Huada Gene, Inc. (Beijing, China).

The amplified PCR products were subsequently cloned into the *Sna*BI and *Avr*II sites of plasmid pPIC9K. The recombinant plasmids were transformed into *E. coli* JM109 competent cells. Ten colonies of each transformant were picked, inoculated into 3 ml of Luria-Bertani medium supplemented with ampicillin and grown overnight in a shaking incubator at 37°C. Recombinant plasmids were purified from the liquid culture using the Miniprep kit (Qiagen) and digested with *Pst*I to confirm their correctness.

2.4. Transformation into P. pastoris

Correct recombinant plasmids were linearized with *Sal*I/*Sac*I. Then, the digestion mixture was purified and transformed into *P. pastoris* GS115 by electroporation according to the Pichia Expression Kit manual (Invitrogen). His^+ Mut^+ transformants were initially selected by MD medium (1.34% yeast nitrogen base, 4×10^{-5}% biotin, 2% dextrose) plates. After incubation at 30°C for 3 d, 50 larger colonies from MD plates were selected, pointed onto YPD plates containing 0.5 mg/ml G418 and grown at 30°C for 2–3 d. The grown clones were moved to YPD plates supplemented with 2 mg/ml G418.

2.5. α-Amylase gene expression

The clones from the YPD plates containing 2 mg/ml G418 were selected and cultivated in a shaking flask. The seeds were inoculated into 25 ml of buffered glycerol-complex medium (BMGY) at 30°C on a rotary shaker at 220 rpm until the culture reached the logarithmic phase (OD_{600} = 2.0–6.0). The cells were harvested by centrifugation and resuspended in 50 ml of buffered methanol-complex medium (BMMY) and incubated at 30°C and 200 rpm for 5 d with 0.5% (*v*/*v*) methanol added daily. At the end of fermentation, the culture solution was centrifuged at 10,000 × g for 10 min. The supernatant was collected and dialyzed against 0.5 mM NaAc-HAc buffer (pH 5.0) overnight and lyophilized for further tests.

2.6. Enzyme assay

A modified 3,5-dinitrosalicylic acid method was performed to assess the amylase activity [17]. Briefly, 0.25 ml of 1% (*w*/*v*) soluble starch was used as a substrate and mixed with 0.25 ml 0.2 M NaAc-HAc buffer (pH 5). The mixture was incubated at 37°C for 5 min followed by the addition of 0.5 ml of enzyme solution and further incubation for 1 h. Upon the addition of 1.5 ml of dinitrosalicylic acid, the sample was boiled for 7 min and 10 ml of water was then added. The amylase activity was subsequently evaluated by measuring the reducing sugars released following starch hydrolysis at 550 nm. One unit of amylase activity was defined as the amount of enzyme needed to liberate 1.0 μg of reducing sugar per min using glucose as a standard under the assay conditions.

2.7. Biochemical characterization of recombinant α-amylase

The optimal temperatures of recombinant α-amylases were determined in the 20–70°C range. The amounts of released reducing sugar were afterward measured under the standard assay conditions. Temperature stability was studied by measuring the residual activity of the enzyme was after being pre-incubated at each temperature for 1 h. The α-amylase activities were determined in triplicate experiments.

Different buffer systems, including 20 mM NaAc-HAc buffer (pH 3–5) and citric acid-sodium citrate buffer (pH 6–7) were prepared to study the optimum pH values of recombinant α-amylases. To study the stabilities of α-amylases, these enzymes were incubated in buffers with pH values ranging from 3 to 7 at 20°C for 1 h. Residual activity was assayed as mentioned above.

The α-amylase activities were investigated in the presence of a number of different metal ions or chemicals (1 mM), including Cu^{2+}, Mg^{2+}, NH_4^+, Ca^{2+}, Fe^{3+}, Mn^{2+}, Na^+, K^+, ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl sulfate (SDS), by pre-incubating the enzyme separately with each reagent for 30 min at 0°C, followed by measuring the residual activity under optimum conditions. The remaining activity was expressed in percentage activity and assuming

the activity of the control sample (in the absence of any additives) as 100%. All determinations were performed in triplicate.

2.8. Product analysis of starch hydrolysis

A reaction mixture containing 5 U of α-amylases and soluble starch (1%) in NaAc-HAc buffer (0.2 M, pH 5) was incubated at 37°C for 12 h. Sugars released were analyzed by high-performance liquid chromatography using an evaporative light-scattering detector (Alltech Elsd detector 2000s, Grace, USA) and a Tskgel Amide 80 (Amide, 4.6 × 250 mm, 5 μm). The mobile phase was 65% acetonitrile and 35% water, and the flow rate was 1 ml/min.

3. Results

3.1. Sequence analysis and heterologous expression of α-amylase in P. pastoris

Based on the genome information of *A.s niger* CBS513.88, nine possible genes encoding α-amylases were identified using the web site of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/guide/) and renamed as *amyA*, *amyB*, *amyC*, *amyD*, *amy*E, *amyF*, *amyG*, *amyH* and *amyM* (Table 1). Among them, the *amyB* and the *amyH* sequences were exactly the same. According to the gene information for the eight α-amylases from *A. niger*, the corresponding nucleotide primers were designed. Using cDNA as a template, eight α-amylase genes were amplified. The amplified PCR products were subsequently purified and digested with *Xba*I and then cloned into the plasmid pPIC9K, yielding recombinant plasmids pPIC-*amyA*, pPIC-*amyC*, pPIC-*amyD*, pPIC-*amyE*, pPIC-*amyF*, pPIC-*amyG*, pPIC-*amyH* and pPIC-*amyM*. The genes encoding eight α-amylases were sequenced and analyzed. The amino acids sequences were deduced based on the nucleotide sequences. The eight cloned genes from *A. niger* CBS513.88 possessed complete open reading frames (Table 1). After the correctness of these recombinant plasmids was confirmed by *Pst*I digestion, they were linearized and electrotransformed into *P. pastoris* GS115. The transformants GS115 (pPIC-*amyA*), GS115 (pPIC-*amyC*), GS115 (pPIC-*amyD*), GS115 (pPIC-*amyE*), GS115 (pPIC-*amyF*), GS115 (pPIC-*amyG*), GS115 (pPIC-*amyH*) and GS115 (pPIC-*amyM*) were obtained. As a result, in 50-ml shaking flasks, the eight transformants showed activities of 6.25, 5.08, 8.83, 31.33, 7.41, 27.33, 24.25 and 6.17 U/ml, respectively.

3.2. Amino acid sequence comparison and structure modeling of A. niger α-amylases

Based on comparing these α-amylases two by two (Table 2) and analysis of genome sequence [16], the members of α-amylase family belonged to three different groups: α-amylase (AmyE, AmyG and AmyH), GPI-anchored α-amylase-like (AmyA, AmyC and AmyM) and intracellular α-amylase-like (AmyF and AmyD). The amino acid sequence of members from one group displays very high similarity with each other, but different groups showed low similarity (Table 2). For example, the amino acid sequence of AmyE had 73.4% similarity with AmyH, while AmyA showed 45.8% similarity with AmyH, and AmyF revealed 19.4% similarity with AmyH.

Table 1
Basic characteristics of α-amylases from *A. niger* CBS513.88.

Genes[a]	Gene ID	Protein ID	Number of amino acids	Signal peptide composition
amyA	An09g03100	XP_001393626.1	555	1–25
amyC	An12g02460	XP_001395328.2	550	1–29
amyD	An01g13610	XP_001389762.2	557	NO
amyE	An04g06930	XP_001402054.2	493	1–16
amyF	An09g03110	XP_001393627.2	567	NO
amyG	An11g03340	XP_001394335.1	505	1–24
amyH	An12g06930	XP_001395749.2	499	1–21
amyM	An15g07800	XP_001397301.1	567	1–22

[a] Renamed in this study.

Table 2
Pairwise analysis of the eight α-amylases.

α-Amylase name	Amino acid sequence identity (%)							
	AmyD	AmyF	AmyA	AmyC	AmyM	AmyG	AmyE	AmyH
AmyD	100.0	55.6	21.3	19.0	18.2	19.1	19.0	20.7
AmyF	55.6	100	20.2	19.5	18.1	19.4	19.0	19.4
AmyA	21.3	20.2	100.0	57.5	39.7	45.0	48.1	45.8
AmyC	19.0	19.5	57.5	100.0	40.3	45.6	46.4	43.1
AmyM	18.2	18.1	39.7	40.3	100.0	45.4	47.9	45.9
AmyG	19.1	19.4	45.0	45.6	45.4	100.0	64.6	65.5
AmyE	19.0	19.0	48.1	46.4	47.9	64.7	100.0	73.4
AmyH	20.7	19.4	45.8	43.1	45.9	65.5	73.4	100.0

According to the amino acid sequences of α-amylases from different microorganisms, a phylogenetic tree was constructed using Clustal X2 and MEGA 4.0 programs (Fig. 1). The eight α-amylases from *A. niger* CBS513.88 were different from each other and belonged to three different groups. Among them, the An-AmyB and the An-AmyH sequences were exactly the same. The evolutionary tree reflected the genetic distance of α-amylases from different microorganisms. An-AmyA, An-AmyC, An-AmyD, An-AmyE, An-AmyF, An-AmyG, An-AmyH and An-AmyM shared 78.14%, 96.17%, 93.51%, 94.93%, 90.94%, 95.84%, 99.40% and 89.40% identities with Ap-AmyA, Ak-AmyD, Ak-AmyC, Ak-AmyA, Al-amyB, Ak-amyB, Ao-Taka AmyA and Al-AmyA, respectively (Fig. 1).

The amino acid sequence alignment revealed that the eight α-amylases from *A. niger* CBS 513.88 had the three same putative active sites (Asp, Glu and Asp; red font in Fig. 2), as deduced from a comparison with the *A. oryzae* α-amylase. All known α-amylases of the GH13 family share 4 to 7 conserved sequence regions (CSRs) [18,19]. In this study, the eight amylases of *A. niger* have seven CSRs (marked in yellow in Fig. 2). The region containing CSR I, II, III and IV may be associated with the catalyst and the substrate-binding sites of α-amylase [2].

To further improve the protein sequence alignment, the 3D structure modeling of AmyA, AmyE and AmyF (choosing one representative for each group) were constructed with Swiss-Model using the crystal structure of α-amylase from *Aspergillus niger* (PDB ID: 2gvy.1) [13] and *Bacillus halmapalus* α-amylase (PDB ID: 2gjp.1) [20] as the template, respectively (Fig. 3). The QMEAN Z-Score of the 3D structure constructed were −2.03, −1.22, and −2.05, respectively.

Despite the low sequence similarities, the predicted three-dimensional structures of AmyA, AmyE and AmyF contained three conserved domains: domain A, which is the catalytic central $(\alpha/\beta)_8$ TIM-barrel domain, domain B, which protrudes out of the barrel as a longer loop between the strand β_3 and helix α_3 of domain A, and domain C, which is folded into antiparallel β-sandwich containing a Greek key motif at the C-terminal end of the enzyme [21,22]. The structure of domain A and domain C about the three α-amylases are similar to each other, but domain B varies substantially in size and structure among them (Fig. 3).

3.3. Biochemical characterization of recombinant α-amylase

The optimal temperature was determined by incubating the amylases in the 20 to 70°C range (Fig. 4a). AmyA, AmyD and AmyF exhibited maximal activity levels at 30°C; the optimum temperatures of AmyC, AmyE, AmyH and AmyM were 40°C, whereas AmyG showed optimum temperature at 60°C. The thermostability of the recombinant α-amylases were determined by measuring their residual activity levels after enzyme incubations at different temperatures (20–70°C) for 1 h (Fig. 4b). AmyA, AmyD, AmyF and AmyM were stable at

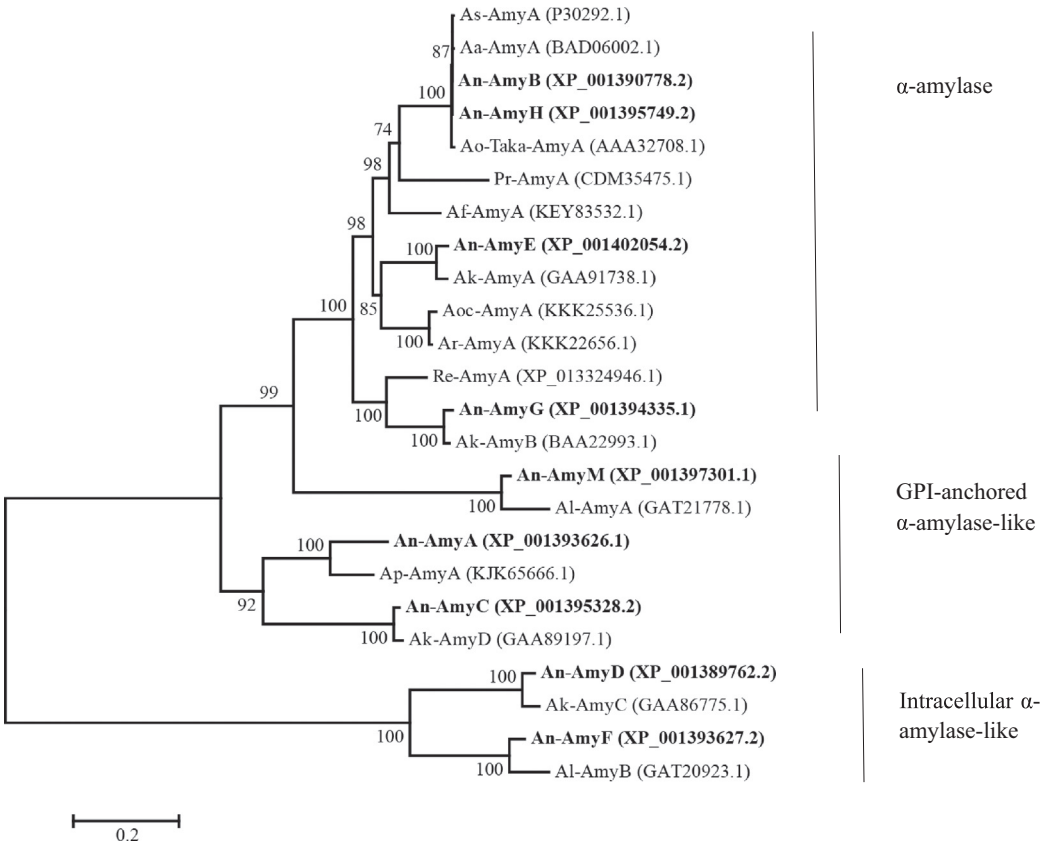

Fig. 1. Phylogenetic relationships of α-amylases from different microorganisms. α-Amylases from *A. niger* CBS 513.88 are in bold. The lengths of the line segments represent the distances calculated by MEGA 4.0. The numbers on the branch node represent the bootstrap percentages. As-amyA, Aa-amyA, Ao-Taka-amyA, Pr-amyA, Af-amyA, Ak-amyA, Aoc-amyA, Ar-amyA, Re-amyA, Ak-amyB, Al-amyA, Ap-amyA, Ak-amyD, Ak-amyC and Al-amyB represents amylases from *A. shirousamii*, *A. awamori*, *A. oryzae*, *Penicillium roqueforti* FM164, *A. fumigatus* var. RP-2014, *A. kawachii* IFO 4308, *A. ochraceoroseus*, *A. rambellii*, *Rasamsonia emersonii* CBS 393.64, *A. kawachii*, *A. luchuensis*, *A. parasiticus* SU-1, *A. kawachii* IFO 4308, *A. kawachii* IFO 4308 and *A. luchuensis*, respectively.

temperatures below 40°C. At temperatures between 40 and 50°C, AmyC and AmyE maintained more than 60% activity. Interestingly, AmyG and AmyH were more thermostable, with 43 and 65% residual activity at 70°C, respectively.

The optimum pH levels of recombinant α-amylases were studied in different buffers having a pH range of 3–7 (Fig. 4c). AmyA, AmyC, AmyE and AmyF displayed maximal activities at pH 4.5, whereas AmyG and AmyM exhibited maximal activities at pH 5. The optimum pH levels of AmyD and AmyH were pH 6. The α-amylases were all stable at acidic pH levels (Fig. 4d). AmyA, AmyC, AmyF and AmyH were stable at pH 3–5, while AmyD, AmyE, AmyG and AmyM were stable at pH 4–6.

The effects of metal ions and chemicals on α-amylase activities were shown in Table 3. Most amylases were enhanced by Mn^{2+}. In the case of AmyE and AmyG, up to 25% and 20% improvements, respectively, in enzyme activities were observed with the addition of 1 mM Mn^{2+}. Ca^{2+} and EDTA had no effects on the activities of amylases except AmyF and AmyH, indicating that the six α-amylases of *A. niger* were Ca^{2+} independent. Cu^{2+}, Fe^{3+} and SDS inhibited the activities of these eight amylases to different degrees, especially exhibiting a strong inhibitory effect on AmyG. Na^{+} had no apparent influence on the activities of most amylases, whereas the activity of AmyG was increased 10% by Na^{+}. Mg^{2+}, NH_4^{+} and K^{+} exhibited slight inhibitory effects on the activities of most amylases.

3.4. Hydrolysis of starch by A. niger α-amylases

All eight of the recombinant α-amylases can hydrolyze starch, but the starch conversion rates and hydrolysates were very obviously different among them (Table 4). After hydrolysis of 1% starch substrates by AmyF, AmyH and AmyM at 37°C for 12 h, the only detectable end-product was glucose. AmyD had a relatively low hydrolyzing activity on starch, producing mainly maltotriose. Hydrolysis of starch by AmyA and AmyC resulted in the formation of maltotetraose and a small amount of glucose. When the starch was hydrolyzed by AmyE, glucose, maltose and maltotriose were observed in the hydrolysates, with maltose and maltotriose accounting for 36.06 and 36.23 (*w*/*w*) of the total, respectively. The main end-products formed by AmyG from starch were maltose and maltotriose. The maltose and maltotriose contents in the hydrolysates were 34.34 and 43.72% (*w*/*w*), respectively.

4. Discussion

α-Amylase is a starch-degrading enzyme that has received much attention owing to its economic benefits and technological significance. It accounts for the whole of the enzyme preparation and ~25% of the market share. In recent years, many different sources of α-amylases have been investigated [23,24,25]. With the implementation of the human genome project, it was revealed that a microbial genome often contains several open reading frames encoding α-amylases. The *A. niger* genome contains nine possible α-amylase open reading frames (the *amyB* and the *amyH* sequence were exactly the same). The aim of this study was to investigate the functions of the open reading frames encoding α-amylases from *Aspergillus niger* using molecular cloning and expression techniques. We analyzed the enzymatic properties and starch hydrolysis properties of six α-amylases on the basis of previous studies [11,12,13]. And, we focused on two new α-amylases (AmyE and AmyF) in details. AmyE shows acid-stabilization, Ca^{2+}-independent and high maltotriose-forming properties, which could potentially

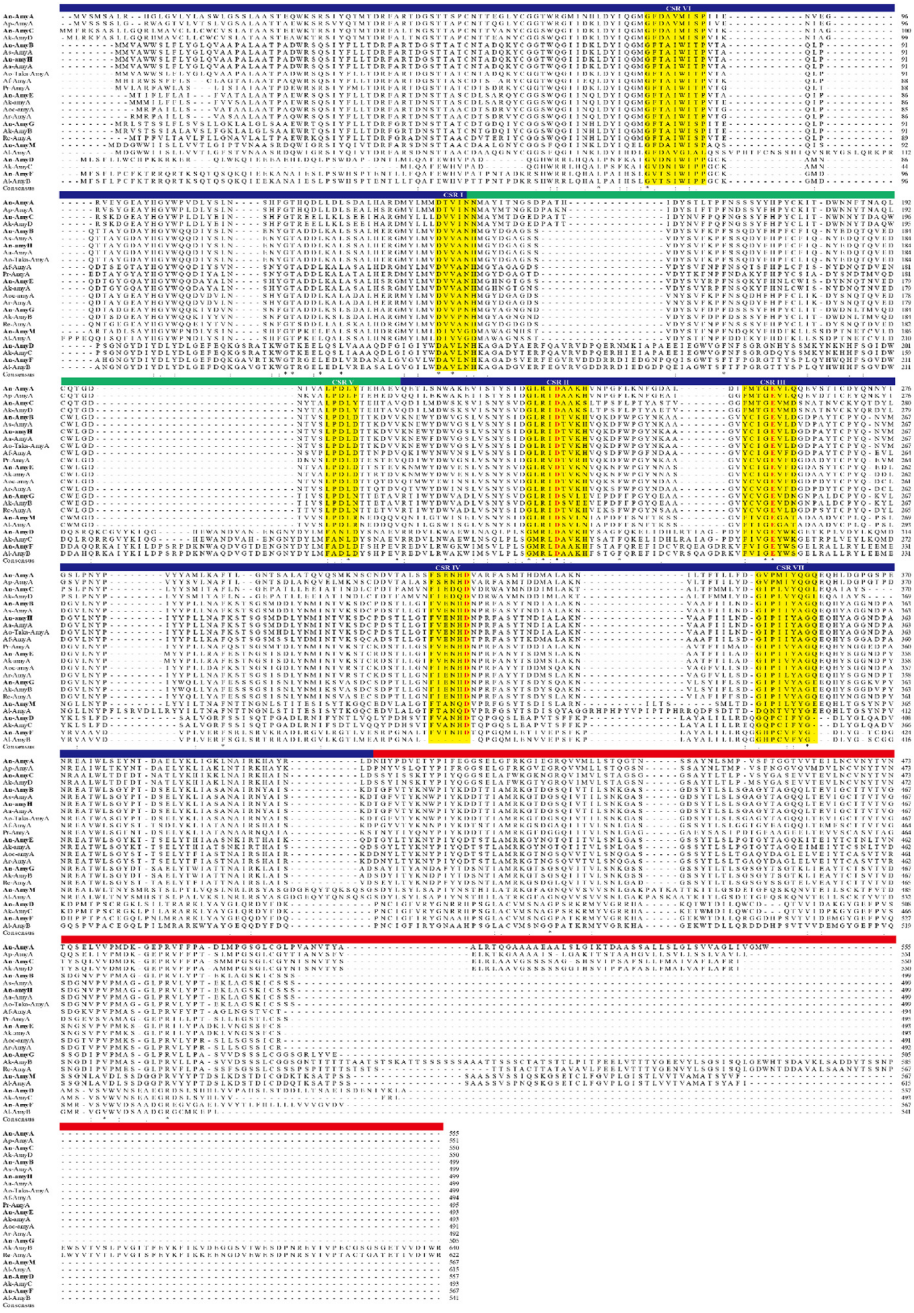

Fig. 2. Multiple sequence alignment of amylases from different sources. (*), conserved amino acids; (:), conservative replacement; (.), half conservative replacement. The seven conserved sequence regions (CSRs) are marked in yellow, and putative catalytic triplet are highlighted in red font. Structures of catalytic domain A, domain B and C-terminal domain C are highlighted by blue, green and red bars, respectively, above the sequences.

benefit α-amylase industrial applications. It also gives new insights into evolution and functional divergence among different members of an α-amylase family.

The optimum temperatures of the seven α-amylases (AmyA, AmyC, AmyD, AmyE, AmyF, AmyH and AmyM) ranged from 30 to 40°C, which are lower than those of α-amylases from *Rhizopus oryzae* (60°C) [26], *Neosartorya fischeri* (NFamy-2, 50°C) [27] and *Aspergillus foetidus* ATCC 10254 (45°C) [28]. However, the range was the same as that of Taka-amylase from *A. Oryzae* ATCC 9376 (30–40°C) [29], which is currently a widely researched and used fungal amylase. These α-amylases with lower optimum temperatures can reduce energy consumption, thus having industrial potential. AmyG showed optimum temperature at 60°C and had high thermal stability. This is in agreement with the reported by Minoda et al. [30].

According to the current literature [31,32], an amylase with an optimum pH of 2.5 to 4.5 is an acidic amylase, while a neutral amylase usually has an optimum pH of 5.0 to 6.5. AmyA, AmyC, AmyE and AmyF displayed maximal activities at pH 4.5, whereas AmyG and AmyM exhibited maximal activities at pH 5. The optimum pH levels of AmyD and AmyH were pH 6. Thus, the eight α-amylases from *A. niger* CBS513.88 were acidic or neutral amylases, similar to those from *R. oryzae* (pH 4–6) [26].

Generally, α-amylases need Ca^{2+} to maintain their activity and stability levels [33]. Van der Kaaij [15] studies on the intracellular fungal α-amylase (AmyD) from *A. niger* showed that 1 mM Ca^{2+} or EDTA did not have a significant effect on the starch hydrolytic activity. In this study, the α-amylases of *A. niger* showed no response to added Ca^{2+} and EDTA in terms of activity levels except AmyF and AmyH,

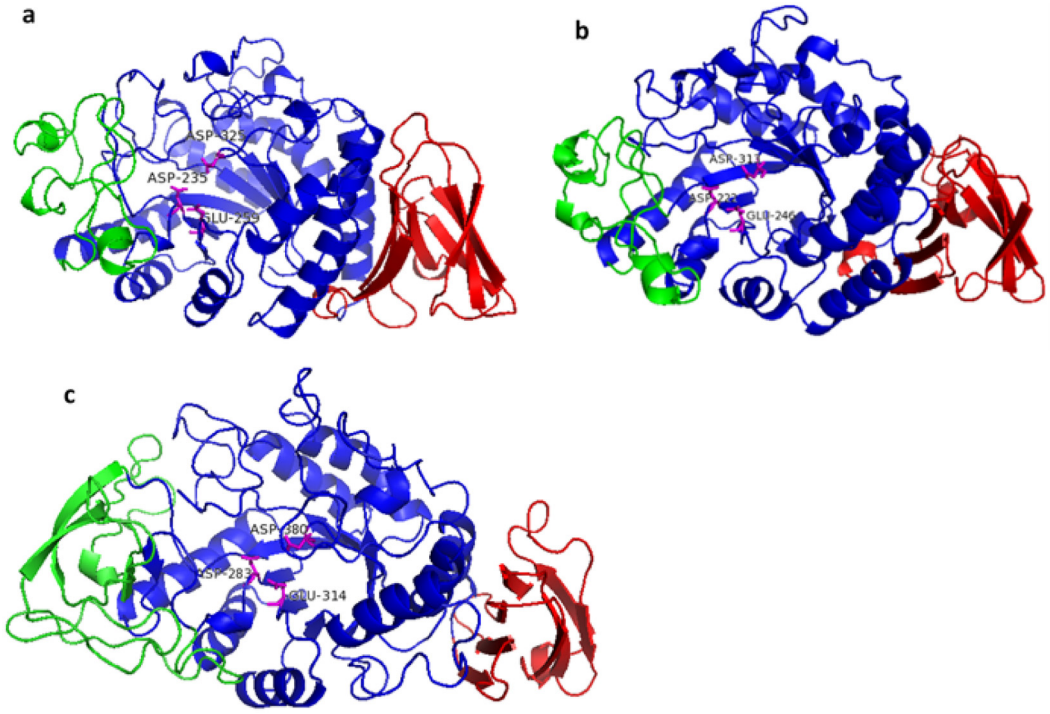

Fig. 3. The 3D structure modeling of *A. niger* α-amylases. (a) AmyA; (b) AmyE; (c) AmyF. Domain A, domain B and domain C are shown in blue, green and red, respectively. The catalytic triad (ASP, Glu and ASP) are shown as sticks.

indicating that most α-amylases of *A. niger* may be Ca^{2+} independent. This feature resembles the α-amylase from *R. oryzae* [26]. Ca^{2+} enhanced AmyG activity, apart from conferring thermal stability. This is consistent with the results of Ramasesh et al. studies [34]. This author inferred that Ca^{2+} helps in the formation of intramolecular cross linkages by disulfide bridges, which help stable conformation of the enzyme and thus helps enzyme action.

There are big differences in the hydrolysate and enzymatic properties among different biological sources of α-amylases [1]. Bacterial α-amylases (such as those from *Bacillus licheniformis* and

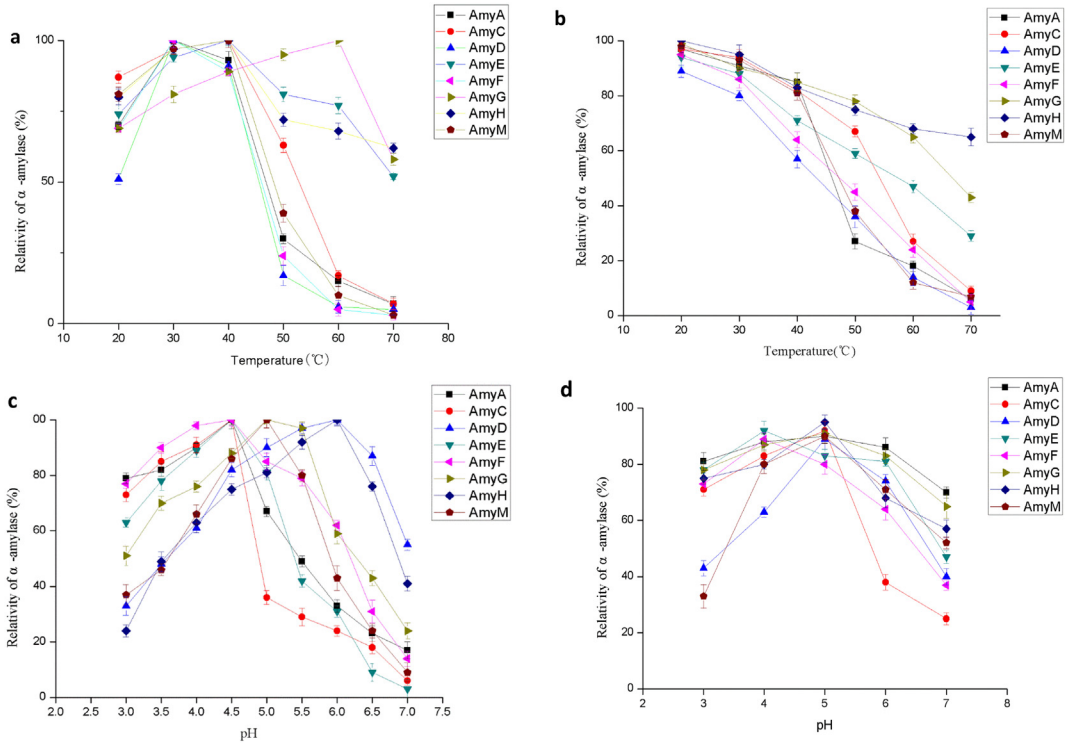

Fig. 4. Biochemical characterization of recombinant α-amylase. (a) The optimal temperature of recombinant α-amylases. (b) The thermostability of recombinant α-amylases. (c) The optimal pH of recombinant α-amylases. (d) The pH stability of recombinant α-amylases.

Table 3
Effects of metal ions and chemicals on amylase activities.

Metal ions & chemicals	Relative activity (%)							
	AmyA	AmyC	AmyD	AmyE	AmyF	AmyG	AmyH	AmyM
Control	100	100	100	100	100	100	100	100
Ca^{2+}	103 ± 1.2	100 ± 2.0	98 ± 1.1	101 ± 1.7	115 ± 0.4	100 ± 2.1	112 ± 0.6	97 ± 0.9
Cu^{2+}	86 ± 1.4	90 ± 1.4	88 ± 3.1	92 ± 2.3	85 ± 1.1	60 ± 0.8	87 ± 0.2	73 ± 0.3
Na^{+}	100 ± 0.8	94 ± 0.5	97 ± 2.1	98 ± 1.1	95 ± 0.7	110 ± 1.3	96 ± 1.8	95 ± 1.5
Fe^{3+}	83 ± 1.2	91 ± 0.6	80 ± 0.7	90 ± 0.9	50 ± 1.8	70 ± 0.5	85 ± 2.4	82 ± 1.3
Mg^{2+}	94 ± 0.2	93 ± 2.3	97 ± 0.6	99 ± 3.1	92 ± 2.5	98 ± 1.4	93 ± 0.9	96 ± 2.4
Mn^{2+}	100 ± 0.4	103 ± 1.0	114 ± 1.2	125 ± 2.4	90 ± 1.3	120 ± 2.6	121 ± 0.6	99 ± 2.0
NH_4^+	98 ± 1.6	94 ± 0.9	95 ± 0.7	95 ± 1.6	80 ± 2.0	96 ± 0.7	98 ± 0.8	98 ± 1.9
K^{+}	97 ± 2.3	97 ± 1.6	99 ± 0.9	100 ± 0.4	93 ± 1.2	101 ± 2.2	94 ± 1.5	100 ± 0.7
SDS	19 ± 1.7	68 ± 2.4	55 ± 2.2	75 ± 3.0	15 ± 0.9	40 ± 0.6	70 ± 0.7	33 ± 3.6
EDTA	97 ± 0.6	95 ± 3.1	95 ± 0.4	102 ± 1.2	70 ± 0.5	90 ± 2.4	66 ± 1.3	94 ± 1.7

Bacillus amyloliquefaciens) hydrolyze starch into mainly short chain malt dextrin and oligosaccharides. Different sources of fungal α-amylases, such as Taka-amylase, as well as *R. oryzae* and the *Penicillium expansum* amylases, have different biochemical properties but they usually degrade starch and dextrin into mainly maltose [4,26,35]. Thus, they are mainly used for the production of high maltose syrup. In this research, two recombinant α-amylases, AmyG and AmyE, hydrolyzed starch into maltose, maltotriose and a small amount of glucose (Table 4). This feature differed from most of the reported amylases that hydrolyze starch mainly into glucose and maltose. Thus, AmyG and AmyE may be used to prepare maltooligosaccharides with maltotriose as the main ingredient. Additionally, the products formed during the hydrolysis of starch by AmyG and AmyE did not contain maltotetraose and maltopentaose, making it beneficial to isolate maltotriose from maltooligosaccharides. Thus, the recombinant amylases AmyG and AmyE have application potential in the preparation of maltotriose.

Normally, α-amylases classified in family GH13 have three-domains, A, B and C. In this paper, Domain B varies substantially in size and structure (Fig. 3) among the three different α-amylases from *A. niger*, AmyA, AmyE and AmyF (one representative for each group). Domain B forms a large part of the substrate binding clef and is presumed to be important for the substrate specificity differences observed between α-amylases [36]. In addition, domain B may be related to stability of α-amylases, the pH stability and thermal stability of α-amylases were significantly influenced by changing amino acids of this area [33]. This may be one reason of differences in biochemical properties of these eight α-amylases from *A. niger*.

Brown CA studied the evolution and functional divergence of subtelomeric gene families in the yeast lineage [37]. According to the sequence alignment, comparison of similarities and differences about biochemical properties and 3D structure modeling, we can deduce that the eight α-amylases of *A. niger* may derived from a common ancestor, but evolved into three groups of enzymes (α-amylase, GPI-anchored α-amylase and intracellular α-amylase) with different physiological function: the members of α-amylase group show high activity for starch hydrolysis. The biochemical characterization of two homologous GPI-anchored α-amylases from *A. niger* has revealed that they had a very low hydrolyzing activity on starch, but clearly showed α-glucanotransferase activity on maltooligosaccharides and starch [14]. The representative of intracellular α-amylase group, AmyD has a relatively low hydrolyzing activity on starch, a possible function of these enzymes in relation to cell wall α-glucansynthesis [15].

Currently, acid-stable and maltooligosaccharides producing α-amylases are required in the food industry, especially in baking, because adding these α-amylases could improve shelf-life, loaf volume and crumb color [38,39] of bread. Furthermore, in the starch industry, exploring acid-stable and Ca^{2+}-independent α-amylase can simplify liquefaction and saccharification processes and thus reduces the cost of the products [40]. Due to the novel α-amylase, AmyE of *Aspergillus niger* is acid-stable, Ca^{2+}-independent and has high maltotriose-forming ability, the application experiments of AmyE in the baking and starch processing industry deserve further investigation.

Table 4
Analysis of end-products formed during the hydrolysis of 1% (*w*/*v*) starches by α-amylases.

Amylases	Constituents of end products (% (*w*/*w*))[a]			
	G1[b]	G2	G3	G4
AmyA	7.84 ± 1.5	ND	ND	35.21 ± 2.6
AmyC	8.43 ± 2.4	ND	ND	36.47 ± 3.5
AmyD	4 ± 1.8	10.14 ± 2.8	32.14 ± 2.8	ND
AmyE	11.5 ± 1.3	36.06 ± 2.9	36.23 ± 1.5	ND
AmyF	49.64 ± 2.6	ND	ND	ND
AmyG	2.97 ± 0.4	34.34 ± 1.7	43.72 ± 2.2	ND
AmyH	82.7 ± 3.2	ND	ND	ND
AmyM	53.14 ± 2.4	ND	ND	ND

ND: not detected or the content is negligible.
[a] Constituents of end products (% (*w*/*w*)) are defined as (concentration of end-product/original starch concentration) × 100%.
[b] G1, G2, G3 and G4 represent glucose, maltose, maltotriose and maltotetraose, respectively.

5. Conclusions

Eight putative genes encoding α-amylases from *A.s niger* CBS513.88 were successfully expressed in *P. pastoris* GS115, and some biochemical characterizations of the recombinant α-amylases were investigated, including the optimality and stability of pH and temperature, the effects of metal ions and chemicals on the activity of α-amylase, and product analysis of starch hydrolysis by α-amylases. The recombinant α-amylases AmyG and AmyE exhibit acid-stabilization and high levels of maltotriose-forming ability, which gives them application potential in the food and starch processing industry. This study illustrates that a gene family can encode multiple enzymes members having remarkable differences in biochemical properties.

Conflict of interest statement

There are no conflicts to declare.

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