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

Junying Wang, Yu Li, Fuping Lu

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. Ca2+ and EDTA had no effects on the activities of α-amylases except AmyF and AmyH, indicating that the six α-amylases were Ca2+ 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, Ca2+-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.

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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 Luxia-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 an enzymatic reaction was cleaned using a Qiagen QIAquick 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 subsequently cloned into the SmaI and AvrII 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 Luxia-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 PstI to confirm their correctness.

2.4. Transformation into P. pastoris

Correct recombinant plasmids were linearized with SalI/SacI. 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 (OD₆₀₀ = 2.0–6.0). The cells were harvested by centrifugation and resuspended in 50 ml of buffered methanol-complex medium (BMMM) 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 Cu²⁺, Mg²⁺, NH₄⁺, Ca²⁺, Fe³⁺, Mn²⁺, 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 As 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, amyE, 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 XbaI and then cloned into the plasmid pPIC5K, 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 acid 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 PstI 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, 19.0, 19.4 and 43.1 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).

Table 2

<table>
<thead>
<tr>
<th>α-Amylase name</th>
<th>Amino acid sequence identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmyD</td>
<td>100.0</td>
</tr>
<tr>
<td>AmyF</td>
<td>55.6</td>
</tr>
<tr>
<td>AmyA</td>
<td>21.3</td>
</tr>
<tr>
<td>AmyC</td>
<td>19.0</td>
</tr>
<tr>
<td>AmyM</td>
<td>18.2</td>
</tr>
<tr>
<td>AmyG</td>
<td>19.1</td>
</tr>
<tr>
<td>AmyE</td>
<td>20.7</td>
</tr>
<tr>
<td>AmyH</td>
<td>21.3</td>
</tr>
<tr>
<td>AmyA</td>
<td>100.0</td>
</tr>
<tr>
<td>AmyD</td>
<td>55.6</td>
</tr>
<tr>
<td>AmyC</td>
<td>21.3</td>
</tr>
<tr>
<td>AmyM</td>
<td>19.0</td>
</tr>
<tr>
<td>AmyG</td>
<td>19.1</td>
</tr>
<tr>
<td>AmyE</td>
<td>20.7</td>
</tr>
<tr>
<td>AmyH</td>
<td>21.3</td>
</tr>
</tbody>
</table>

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.

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%, 90.40% and 89.40% identities with Ap-AmyA, Al-AmyD, Ak-AmyC, Ak-AmyF, Al-AmyA, Ao-Taka AmyA and Al-AmyA, respectively (Fig. 1).

The amino acid sequence alignment revealed that the eight α-amylases from A. niger CBS513.88 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 α-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 catalytic 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: 2gyv.1) [13] and Bacillus halomycalis 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, −2.12, 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 (α/β)8 TIM-barrel domain, domain B, which protrudes out of the barrel as a longer loop between the strand β5 and helix α3 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 α-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

Table 1

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene ID</th>
<th>Protein ID</th>
<th>Number of amino acids</th>
<th>Signal peptide composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>amyA</td>
<td>An09g310100</td>
<td>XP_001393626.1</td>
<td>555</td>
<td>1–25</td>
</tr>
<tr>
<td>amyC</td>
<td>An12g024600</td>
<td>XP_001395328.2</td>
<td>550</td>
<td>1–29</td>
</tr>
<tr>
<td>amyD</td>
<td>An01g136100</td>
<td>XP_001389762.2</td>
<td>557</td>
<td>NO</td>
</tr>
<tr>
<td>amyE</td>
<td>An04g069300</td>
<td>XP_001402054.2</td>
<td>493</td>
<td>1–16</td>
</tr>
<tr>
<td>amyF</td>
<td>An09g031100</td>
<td>XP_001393627.2</td>
<td>567</td>
<td>NO</td>
</tr>
<tr>
<td>amyG</td>
<td>An11g033400</td>
<td>XP_001394335.1</td>
<td>505</td>
<td>1–24</td>
</tr>
<tr>
<td>amyH</td>
<td>An12g069300</td>
<td>XP_001395749.2</td>
<td>499</td>
<td>1–21</td>
</tr>
<tr>
<td>amyM</td>
<td>An15g078000</td>
<td>XP_001397320.1</td>
<td>567</td>
<td>1–22</td>
</tr>
</tbody>
</table>

* Renamed in this study.
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 α-amylases were enhanced by Mn²⁺. In the case of AmyE and AmyG, up to 25% and 20% improvements, respectively, in enzyme activity were observed with the addition of 1 mM Mn²⁺. Ca²⁺ and EDTA had no effects on the activities of amylases except AmyF and AmyH, indicating that the six α-amylases of A. niger were Ca²⁺ independent. Cu²⁺, Fe³⁺ 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⁺. Mg²⁺, NH₄⁺ 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 AmyG, glucose, maltose and maltotriose were observed in the hydrolysates, with maltose and maltotriose accounting for 36.06 and 36.23 (%) 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. AmyF shows acid-stabilization, Ca²⁺-independent and high maltotriose-forming properties, which could potentially...
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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ and EDTA in terms of activity levels except AmyF and AmyH.

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.
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. 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.](image)

![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.](image)
**Table 3**

Effects of metal ions and chemicals on amylase activities.

<table>
<thead>
<tr>
<th>Metal ions &amp; chemicals</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AmyA</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>103 ± 1.2</td>
</tr>
<tr>
<td>Cu^{2+}</td>
<td>86 ± 1.4</td>
</tr>
<tr>
<td>Na^{+}</td>
<td>100 ± 0.8</td>
</tr>
<tr>
<td>Fe^{3+}</td>
<td>83 ± 1.2</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>94 ± 0.2</td>
</tr>
<tr>
<td>Mn^{2+}</td>
<td>100 ± 0.4</td>
</tr>
<tr>
<td>NH(_4)(^+)</td>
<td>98 ± 1.6</td>
</tr>
<tr>
<td>K^{+}</td>
<td>97 ± 2.3</td>
</tr>
<tr>
<td>SDS</td>
<td>19 ± 1.7</td>
</tr>
<tr>
<td>EDTA</td>
<td>97 ± 0.6</td>
</tr>
</tbody>
</table>


**Table 4**

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

<table>
<thead>
<tr>
<th>Amylases</th>
<th>Constituents of end products (% (w/w))(^a)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>G1(^b)</td>
</tr>
<tr>
<td>AmyA</td>
<td>7.84 ± 1.5</td>
</tr>
<tr>
<td>AmyC</td>
<td>8.43 ± 2.4</td>
</tr>
<tr>
<td>AmyD</td>
<td>4 ± 1.8</td>
</tr>
<tr>
<td>AmyE</td>
<td>11.5 ± 1.3</td>
</tr>
<tr>
<td>AmyF</td>
<td>49.64 ± 2.6</td>
</tr>
<tr>
<td>AmyG</td>
<td>2.97 ± 0.4</td>
</tr>
<tr>
<td>AmyH</td>
<td>82.72 ± 3.2</td>
</tr>
<tr>
<td>AmyM</td>
<td>53.14 ± 2.4</td>
</tr>
</tbody>
</table>

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.

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