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Gene Cloning, Heterologous Expression, and Characterization of a High Maltose-Producing α-Amylase of *Rhizopus oryzae*

Song Li · Zhirui Zuo · Dandan Niu · Suren Singh · Kugenthiren Permaul · Bernard A. Prior · Guiyang Shi · Zhengxiang Wang

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Abstract A putative α-amylase gene, designated as *RoAmy*, was cloned from *Rhizopus oryzae*. The deduced amino acid sequence showed the highest (42.8%) similarity to the α-amylase from *Trichoderma viride*. The *RoAmy* gene was successfully expressed in *Pichia pastoris* GS115 under the induction of methanol. The molecular weight of the purified RoAmy determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis was approximately 48 kDa. The optimal pH and temperature were 4–6 and 60 °C, respectively. The enzyme was stable at pH ranges of 4.5–6.5 and temperatures below 50 °C. Purified RoAmy had a K_m and V_{max} of 0.27 mg/ml and 0.068 mg/min, respectively, with a specific activity of 1,123 U/mg on soluble starch. Amylase activity was strongly inhibited by 5 mM Cu^{2+} and 5 mM Fe^{2+}, whereas 5 mM Ca^{2+} showed no significant effect. The RoAmy hydrolytic activity was the highest on wheat starch but showed only 55% activity on amylopectin relative to soluble corn starch, while the pullulanase activity was negligible. The main end products of the polysaccharides tested were glucose and maltose. Maltose reached a concentration of 74% (*w*/*w*) with potato starch as the substrate. The enzyme had an extremely high affinity (K_m=0.22 mM) to maltotriose. A high ratio of glucose/maltose of 1:4 was obtained when maltotriose was used at an initial concentration of 40 mM.

Keywords α-Amylase · Maltose · *Rhizopus oryzae* · Heterologous expression · Enzyme characterization

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S. Li · Z. Zuo · G. Shi · Z. Wang (✉)
Research Center of Bioresource & Bioenergy, School of Biotechnology, Jiangnan University, Wuxi 214122, China
e-mail: zxwang@jiangnan.edu.cn

D. Niu · B. A. Prior
Department of Microbiology, University of Stellenbosch, Matieland 7602, South Africa

S. Singh · K. Permaul
Department of Biotechnology, Durban University of Technology, Durban 4000, South Africa

Introduction

α-Amylase (1, 4-α-D-glucan-glucanhydrolase, EC.3.2.1.1) is classified as a member of family 13 of the glycosyl hydrolases and catalyzes the hydrolysis of α-1, 4 glycosidic linkages of glycogen, starch, and related polysaccharides to produce glucose, oligosaccharides, and dextrins [1, 2]. α-Amylases are widely used in the starch processing, food, fermentation, detergent, textile, and paper industries [3]. Although there are many sources (e.g., plants, animals, and microorganisms), microorganisms are the most important sources of α-amylases for industrial purposes.

Compared to the bacterial α-amylases, α-amylases of fungal origin are preferred for starch hydrolysis applications in the baking, brewing, and sweeteners industries [4] due to their more accepted generally recognized as safe status. Two main types of fungal α-amylases are typically used in industrial applications: the common neutral types (such as Taka-amylase from *Aspergillus oryzae* [5] or the Taka-amylase-like enzyme (amyA/amyB) from *Aspergillus niger* [6] and the acid-stable types from *A. niger* [7], *Aspergillus kawachi* [8] and *Rhizomucor pusillus* [9]).

Recently, an extensive study of enzyme production by the *A. niger* N402 strain revealed the presence of 27 α-glucan acting enzymes. Four of these were α-amylases related to starch degradation, and two were putative α-amylases which are potentially involved in cell wall α-glucan synthesis [6]. Several other filamentous fungi, such as *Trichoderma* spp., *Aspergillus flavus*, *Aspergillus usamii* [10], *Phanerochaete chrysosporium* [11], and *Rhizopus* spp. [12], also produce α-amylases. Many *Rhizopus* species are able to produce α-amylases and glucoamylases and are important in the industrial production of glucoamylase [13], as well as in the production of various alcoholic beverages [12, 14].

In the present work, a putative α-amylase gene from *Rhizopus oryzae*, designated as *RoAmy*, was cloned based on the *R. oryzae* genome sequence (www.broad.mit.edu) and successfully expressed in *Pichia pastoris* GS115. The methylotrophic yeast *P. pastoris* was chosen as the expression system for *RoAmy* because of its high expression level of recombinant proteins and its capacity for functional posttranslational modification of recombinant proteins. *Pichia* has been used to express a wide range of heterologous proteins [15], including a number of α-amylases [16–18]. To the best of our knowledge, this is the first report of a purified recombinant *R. oryzae* α-amylase that degrades starch to maltose as the main end product.

Materials and Methods

Strains and Plasmids

Strains and plasmids were obtained from the Culture and Information Center of Industrial Microorganisms of China Universities (http://cicim-cu.jiangnan.edu.cn/), except for the *P. pastoris* GS115 expression kit (Catalog no. K1710-01), which was purchased from Invitrogen Company (Carlsbad, CA, USA). *R. oryzae* (CICIM 0071) was used for the isolation of the *RoAmy* gene. *Escherichia coli* JM109 was used to propagate plasmid DNA.

Media and Culture Conditions

Spores of *R. oryzae* were cultivated on potato dextrose agar at 30 °C, and a fresh mycelium was used for isolation of total RNA. Luria–Bertani medium was used for *E. coli* cultivation. The media and culture conditions for *P. pastoris* GS115 and bioreactor expression of *R.*

oryzae α-amylase were performed according to the *Pichia* Expression Kit manual and the *Pichia* Fermentation Process Guidelines (Invitrogen).

Cloning of *RoAmy* and Construction of an Expression Cassette

Total RNA of *R. oryzae* was isolated with TriPure Isolation Reagent (Roche Applied Science, Sweden), and the cDNA of putative amylase was amplified with a Single Step RT-PCR Kit and specific primers (forward: 5′-ATCATGAAGTCTTTCTTAAGTCTCCTTTGCA-3′, reverse: 5′-CTGCCCGGGTTATTTCTTTTGGAA-3′). A 1.4-kb α-amylase gene fragment was amplified and cloned into plasmid pUC19 to yield a recombinant plasmid pUC19-*RoAmy*. The amylase gene fragment was sequenced (ABI PRISM™ Big Dye™ dideoxy nucleotide termination sequencing, Applied Biosystems, Foster City, CA, USA), and the sequence was deposited in GenBank (accession number: HM234170). The *RoAmy* fragment was amplified from pUC19-*RoAmy* with *Pfu* DNA polymerase and specific primers (forward: 5′-ATTCCCGGGGTGCCTGTCATCAA-3′; reverse: 5′-CTGCCCGGGTTATTTCTTTTG-GAA-3′). The amplified *RoAmy* was subsequently cloned into the *Sna*BI site of plasmid pPIC9K, yielding the expression plasmid pPIC9K-*RoAmy*.

Bioinformatics Assay

The composition and properties of the *RoAmy* nucleotide sequence and its deduced amino acid sequence were analyzed with DNAMAN 6.0, SignalP-3.0 (http://www.cbs.dtu.dk/services/signalP/), and Blast/blastp (http://www.ncbi.nlm.nih.gov/Blast). Distance trees were generated via neighbor-joining methods using Mega version 3.1 [19] after preliminary multiple alignments of the sequences with Clustal W version 1.83 [20].

Transformation of *P. pastoris*

Plasmid pPIC9K-*RoAmy* was linearized with *Stu*I, and 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 isolated on minimal methanol medium plates containing 0.5% methanol, 1.34% yeast nitrogen base (Difco, USA), 0.4 mg/l biotin, and 0.2% soluble starch. After incubation at 30 °C for 3 days, transformants showing strong halo formation with Lugol's iodine were selected.

Expression and Purification of RoAmy

Bioreactor expression of RoAmy was performed according to the *Pichia* Fermentation Process Guidelines (Invitrogen) in a 15-l fermentor. The temperature and pH during fermentation were maintained at 30 °C and 5, respectively. The broth was centrifuged at 10,000×*g* for 10 min, the supernatant was filtered through a 0.45-μm membrane, and the filtrate was then concentrated with a 10-kDa cutoff membrane (Pall Corporation, NY, USA) and dialyzed against distilled water at 4 °C overnight. RoAmy was purified by size exclusion chromatography (Sephacryl S-100) with 50 mM phosphate buffer (pH 6.5) as the elution buffer, and the fraction with the highest amylase activities was collected and concentrated by lyophilization. The concentrated fraction was dissolved in 20 mM phosphate buffer (pH 6.5) and loaded on ion exchange (Mono Q 4.6/100 PE) chromatography column (GE Healthcare Bioscience). Proteins were eluted with a liner NaCl gradient (0 to 1 M) in 20 mM phosphate buffer (pH 6.5). The total protein content of

samples was measured by the Bradford method [21] with bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as a standard. The molecular mass of the purified enzyme was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and protein bands were stained with Coomassie Brilliant Blue G-250 [22] with a protein ladder (SM0061; Fermentas China Co., Ltd, Shenzhen, China) as a standard.

Amylase Activity Assays

To determine RoAmy activity, 1 ml 1% soluble starch was mixed with 0.25 ml citric acid–Na_2HPO_4 buffer (0.2 M, pH 5) incubated at 55 °C for 5 min followed by addition of 0.1 ml enzyme solution and further incubation for 5 min. The reducing sugar formed was determined according to Miller [23], using maltose as a standard. One unit of α-amylase activity was defined as the amount of enzyme that released 1 mg reducing sugar per minute under the above conditions.

The optimal pH of RoAmy was studied between pH 3 and 8 (citric acid–Na_2HPO_4, 0.2 M) at 55 °C for 5 min. To study the stability of RoAmy, the purified enzyme was diluted 10-fold with buffers between pH 3 and 8 and incubated at 55 °C for 10 min. Residual activity was determined by diluting the sample 10-fold with 0.2 M citric acid–Na_2HPO_4 buffer (pH 5) and measuring the activity. The temperature optimum of RoAmy was determined by carrying out assays between 20 °C and 75 °C at pH 5. To determine the temperature stability, enzyme samples were incubated at different temperatures (40–70 °C) in 0.2 M citric acid–Na_2HPO_4 buffer (pH 5) for up to 20 min. Samples were withdrawn and placed on ice before the residual activities were assayed. Activities were determined on soluble corn starch (0.2–5 mg/ml) and maltotriose (4–40 mM) as substrates and kinetic constants were calculated using Lineweaver–Burk plots. The effects of metal ions on enzyme activity were determined by adding metal ions at 1 or 5 mM concentrations to 10-fold diluted purified RoAmy in 0.2 M citric acid–Na_2HPO_4 buffer (pH 5, 55 °C) and comparing the observed activity to that without added metal ions. The effects of Ca^{2+} on the activity and stability of RoAmy was investigated after the enzyme was dialyzed against 5 mM EDTA.

The substrate specificity of RoAmy was determined using 1% solutions of various raw starches (Sigma) and amylopectin from maize (Fluka). Substrates were boiled to increase solubility and then digested with 0.1 U of RoAmy at pH 5 and 55 °C for 5 min.

End Product Analysis

A reaction mixture containing 10 U of RoAmy in citric acid–Na_2HPO_4 buffer (0.2 M, pH 5) and maltotriose (up to 200 mM) and various starches (1%) was incubated at 40 °C. Sugars released were analyzed by high-performance liquid chromatography (HPLC) using an Agilent ZORBAX NH2 column (4.6×250 mm, 5 μm) with 65% acetonitrile as the mobile phase and the flow rate was 1 ml/min.

Results and Discussion

Cloning and Sequence Analysis of *RoAmy*

The nucleotide sequence of the *RoAmy* gene consisted of 1,386 bp encoding a protein of 462 amino acids. The nucleotide sequence and its deduced amino acid sequence alignment

indicated that RoAmy was distinct from other α-amylases reported or deposited in GenBank. The RoAmy shared the highest amino acid similarity (42.8%) to the α-amylase from *Trichoderma viride* but was only 36.8% and 36.4% similar to the acid-stable α-amylase from *A. kawachi* and the Taka-amylase from *R. oryzae*, respectively (Fig. 1). The mature RoAmy had a predicted molecular weight of 48.7 kDa after the removal of a putative 20-amino acid signal peptide. Three potential N-glycosylation sites were found at Asn 57, 382, and 457, and the calculated isoelectric point of the polypeptide is 5.82. A search of the NCBI blastp database revealed that the RoAmy would consist of a conserved α-amylase catalytic domain (residues 39–378) and a C-terminal beta-sheet domain (residues 392–462) and together it covered four common conserved regions of the α-amylase family [24]. Three conserved amino acids—Asp 135, Asn 139, and His 140—that are important for enzyme stability and activity were exhibited in region I (Asp 135–His 140). Three key residues—Asp 216, Glu 240, and Asp 307—that are possibly important for the hydrolyzing activity of α-amylases [25] were found in region II (Asp 211–His 220), region III (Gly 239–Ser 243), and region IV (Phe 302–Asp 307), respectively.

Heterologous Expression and Purification of RoAmy

His^+ Mut^+ transformants of *P. pastoris* were isolated and used for the production of RoAmy in a 15-l fermentor following induction by methanol. We found that proteases in the fermentation medium were particularly active at pH 5, so 1% Casamino acids was added to reduce the degradation of the RoAmy protein. However, at pH 3, no RoAmy protein expression or amylase activity was detected following methanol induction which did not allow enzyme production at this pH value. Production at pH 5 and 30 °C in the presence of 1% Casamino acids resulted in the recovery of 382 mg/l of RoAmy protein at the end of the fermentation. The growth of *P pastoris*, extracellular α-amylase activity levels, and RoAmy protein secretion after methanol induction are illustrated in Fig. 2.

RoAmy recovered from the culture supernatant was purified 1.3-fold with a yield of 86% and specific activity of 1,123±28 U/mg (mean ± standard deviation of triplicate determinations). The purified protein migrated as a single band of 48 kDa on SDS-PAGE

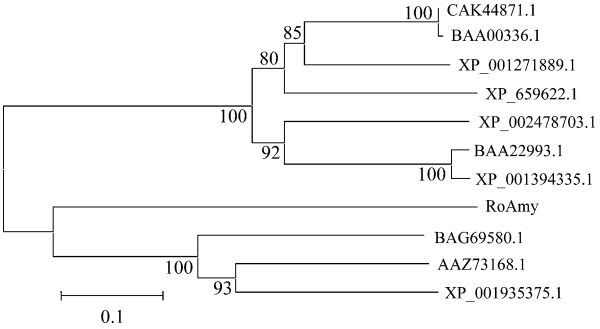

Fig. 1 Tree showing phylogenetic relationships between *R. oryzae* α-amylase (RoAmy) and other fungal homologues. *A. niger* α-amylase amyA/amyB (CAK44871.1); *A. oryzae* α-amylase (BAA00336.1); *Aspergillus clavatus* putative α-amylase (XP_001271889.1); *Aspergillus nidulans* α-amylase (XP_659622.1); *Talaromyces stipitatus* α-amylase (XP_002478703.1); *A. kawakii* acid α-amylase (BAA22993.1); *A. niger* acid α-amylase (XP_001394335.1); *T. viride* α-amylase (BAG69580.1); *Gibberella moniliformis* α-amylase (AAZ73168.1); *Pyrenophora tritici-repentis* α-amylase A type-3 precursor (XP_001935375.1)

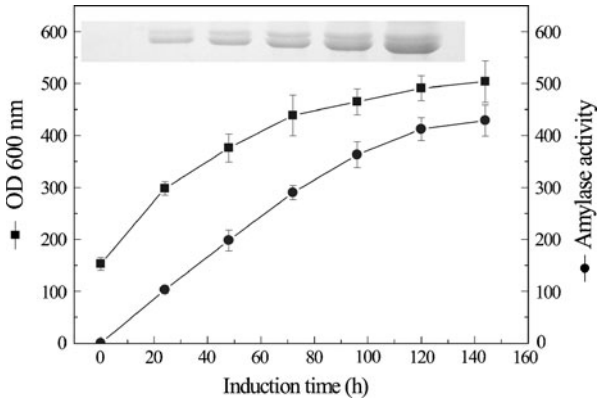

Fig. 2 Time course of amylase activity (units per milliliter) during growth (OD_{600}) in a fed-batch bioreactor at 30 °C and pH 5. Secreted RoAmy protein bands at time intervals as detected by SDS-PAGE are shown at the *top of the figure*. Data points represent the mean ± standard deviation of three independent fermentation experiments

(Fig. 3). The molecular weights of proteins expressed by fungi are usually larger than those predicted from the deduced amino acid sequences of the mature proteins. This discrepancy is probably a result of glycosylation of the expressed proteins during the posttranslational secretion process [26]. However, the apparent molecular weight of the purified RoAmy was 48 kDa (Fig. 3), which was almost equivalent to its predicted mature molecular weight (48.7 kDa). Based on these molecular weight similarities, apparently RoAmy might not be glycosylated but this observation requires further confirmation.

Characterization of the RoAmy Properties

The RoAmy enzyme had a pH optimum between pH 4 and 6 (Fig. 4a) and was most stable between pH 4.5 and 6.5 (Fig. 4b). The optimum temperature was 60 °C (Fig. 5a), and the enzyme was only stable at temperatures below 50 °C (Fig. 5b). At higher temperatures, the enzyme rapidly lost activity over 20 min. The optimum temperature and heat stability of RoAmy was comparable to that of *Penicillium expansum* α-amylase [27], which was 5–10 °C higher than that of *A. niger* and *A. oryzae* α-amylases [1], and this would make RoAmy be better suited to the conditions in industrial starch conversion. The enzyme had K_m and V_{max} values for soluble corn starch of 0.27± 0.026 mg/ml and 0.068±0.007 mg/min, respectively. RoAmy exhibited extremely high affinity (K_m=0.22±0.02 mM) for maltotriose, which is almost four times higher than that of *P. expansum* amylase (K_m=0.76 mM) [27], 13 times higher than that of Fungamyl (K_m=2.9 mM) [27] and 35 times higher than that of *Thermomonospora curvata* amylase (K_m=7.7 mM) [28].

The effects of various metal ions on RoAmy activity are shown in Table 1. The RoAmy activity was slightly inhibited by 1 mM Cu^{2+} and Fe^{2+} but strongly inhibited by 5 mM Cu^{2+} and Fe^{2+}. Ca^{2+} and EDTA had no effect to the activity and heat stability (data not shown) of RoAmy. Traditionally, Ca^{2+} has been considered necessary for the maintenance of the structure of the active site of α-amylases and thus in maintaining the activity and stability of α-amylases [29]. However, unlike many of the α-amylases [1], RoAmy showed no response to added Ca^{2+} and EDTA in terms of activity and heat stability, which indicated that RoAmy was possibly a Ca^{2+}-independent α-amylase.

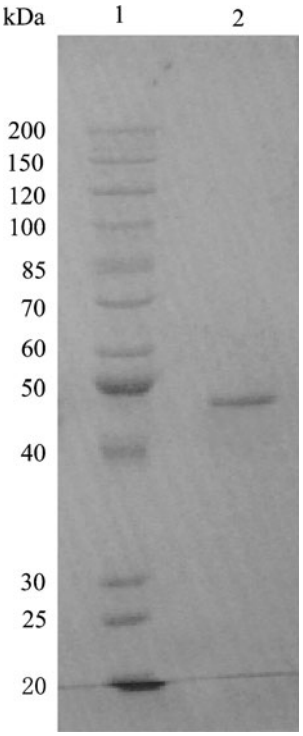

Fig. 3 SDS-PAGE analysis of RoAmy. *Lane 1* molecular mass standard. *Lane 2* purified RoAmy

RoAmy Action on Oligosaccharides

RoAmy activity was the highest on wheat starch and corn starch, but only 55% of the soluble corn starch activity was observed with amylopectin as substrate. Pullulanase activity was negligible (Table 2). After incubation of RoAmy on different starch substrates

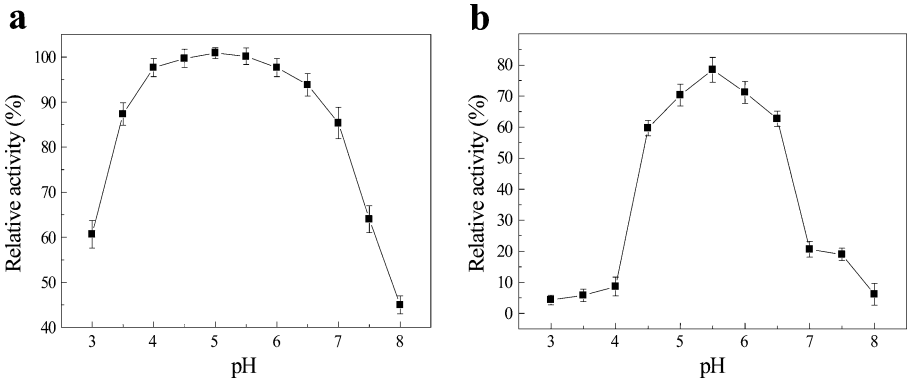

Fig. 4 Effect of pH on RoAmy. **a** Activity profile assays were conducted in 0.2 M citric acid–Na_2HPO_4, buffer at 55 °C for 5 min; **b** relative stability was determined by incubating RoAmy at 55 °C for 10 min. Residual activity was assayed in 0.2 M citric acid–Na_2HPO_4, buffer (pH 5) at 55 °C for 5 min. The data are given as mean ± standard deviation of three independent experiments

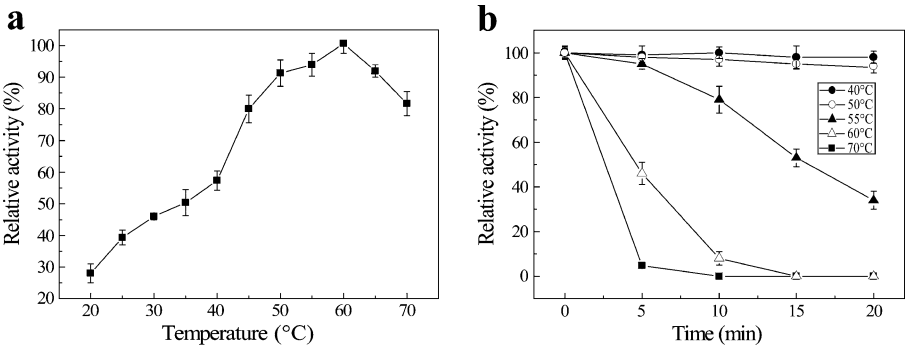

Fig. 5 Effect of temperature on RoAmy activity. **a** Activity profile assays were conducted in 0.2 M citric acid–Na_2HPO_4, buffer at pH 5 for 5 min; **b** relative stability was determined in 0.2 M citric acid–Na_2HPO_4 buffer (pH 5) for up to 20 min. Residual activity was assayed in 0 2 M citric acid–Na_2HPO_4, buffer (pH 5) at 55 °C for 5 min. The data are given as mean ± standard deviation of three independent experiments

at 40 °C for 12 h, the only detectable end products were glucose and maltose, with maltose accounting for 67–74% (*w*/*w*) of the total (Table 3). After 2 h incubation, some maltotriose and maltotetraose remained in some hydrolyzates suggesting that RoAmy might hydrolyze cassava, rice, and potato starches at a slower rate than soluble corn starch, sweet potato,

Table 1 Effects of metal ions on RoAmy activity

Compounds	Concentration (mM)	Relative activity (%)
None	–	100[a]
$MgCl_2$	1	101±2
	5	98±3
$CuSO_4 \cdot 5H_2O$	1	94±4
	5	50±3
$CoCl_2 \cdot 6H_2O$	1	104±5
	5	98±5
$MnCl_2 \cdot 4H_2O$	1	99±2
	5	98±2
$ZnSO_4 \cdot 7H_2O$	1	99±2
	5	102±3
$CaCl_2$	1	103±4
	5	106±2
$FeCl_2 \cdot 4H_2O$	1	86±3
	5	12±2
$FeCl_3 \cdot 6H_2O$	1	95±4
	5	78±3
EDTA	1	101±2
	5	100±2

The data are presented as mean ± standard deviation of three independent experiments

[a] Activity against soluble corn starch (1%) was measured using 0.1 U/ml enzyme in 0.2 M citric acid–Na_2HPO_4 buffer (pH 5) at 55 °C for 5 min, and the relative activity measured without added metal ion was calculated as 100%

Table 2 Ability of RoAmy to digest polysaccharides

Polysaccharides	Relative activity (%)
Soluble corn starch	100[a]
Cassava starch	104±3
Potato starch	97±2
Sweet potato starch	110±2
Wheat starch	133±3
Corn starch	129±2
Rice starch	93±3
Amylopectin	55±2
Pullulan	0.6±0.2

The data are presented as mean ± standard deviation of three independent experiments

[a] The relative activity measured against soluble corn starch (1%) by using 0.1 U/ml enzyme in 0.2 M citric acid–Na_2HPO_4 buffer (pH 5) at 55 °C for 5 min was calculated as 100%

wheat, and corn starches. A number of maltogenic α-amylases from actinomycetes [28, 30–32] have been reported to form high levels of maltose, ranging from 53% to greater than 80% (*w*/*w*). However, the only reported fungal α-amylase capable of producing exceptionally high levels of maltose (74%, *w*/*w*) from starch was by a strain of *P. expansum* [27]. The commonly used industrial *A. oryzae* α-amylase [33] only produces syrups with 50–60% (*w*/*w*) maltose content. In contrast, the RoAmy enzyme could produce a maltose content of 67–74% (*w*/*w*), which is comparable to that produced by maltogenic α-amylases of other microorganisms [27, 28, 31].

Table 3 Analysis of end products formed by RoAmy on starches (1%, *w*/*v*)

Polysaccharides	Constituents of end products (% (*w*/*w*))[a]							
	2 h[b]				12 h			
	G1[c]	G2	G3	G4	G1	G2	G3	G4
Soluble corn starch	10±1	62±3	5±2	ND	12±2	67±3	ND	ND
Cassava starch	10±1	62±2	4±1	6±3	10±3	68±2	ND	ND
Sweet potato starch	10±2	62±1	3±1	ND	10±3	66±1	ND	ND
Wheat starch	11±1	63±2	2±1	ND	11±1	69±2	ND	ND
Rice starch	10±1	62±2	2±3	3±2	14±2	73±4	ND	ND
Potato starch	10±2	60±3	5±1	1±1	14±3	74±2	ND	ND
Corn starch	10±2	59±2	6±2	ND	8±2	68±3	ND	ND

RoAmy (1,000 U/g starch) was incubated with starches in 0.2 M citric acid–Na_2HPO_4 buffer (pH 5) at 40 °C for up to 12 h, and the end products were quantitatively analyzed by HPLC. The data represent mean ± standard deviation of triplicate determination

ND not detected or the content is negligible

[a] Constituents of end products (% (*w*/*w*)) is defined as (concentration of end-product/original starch concentration)×100%

[b] Two and 12 h are the incubation time for digestion of starches by RoAmy

[c] G1, G2, G3, and G4 represents glucose, maltose, maltotriose, and maltotetraose, respectively

RoAmy Action on Maltotriose

The kinetics of maltotriose hydrolysis by RoAmy was examined in more detail as maltotriose has been found to be a significant byproduct of maltose production from starch by α-amylases [28]. The end products formed by RoAmy catalysis of maltotriose were found to be concentration dependent when the hydrolysis was examined over time (Table 4). At an initial low maltotriose concentration (2 mM), approximately equimolar concentrations of glucose and maltose were formed without accumulation of higher maltooligosaccharides (apart from the initial maltotriose substrate). This suggests that unicatalytic reactions predominate at low maltotriose concentrations. As maltotriose concentrations increased (20–200 mM), maltose became the principle product and the ratio of glucose/maltose increased from 1:2.2 to 1:4. However, the concentration of maltotetraose and maltopentaose also accumulated pointing to RoAmy possessing multicatalytic transferase activity at higher maltotriose concentrations.

Collins et al. [28] and McMahon et al. [31] suggested that the low affinity of α-amylases for maltotriose may be one of the reasons for the high maltose levels produced by *T. curvata* and *Streptomyces* sp. α-amylases. Doyle et al. [27] compared the levels of maltose produced by *P. expansum* α-amylase and Fungamyl and in contrast to other reports concluded that the higher levels of maltose produced by *P. expansum* α-amylase may be due to its greater affinity for maltotriose. However, the amounts of maltose produced by RoAmy with high affinity for maltotriose were similar to the enzymes with lower affinity for maltotriose [27, 28]. Therefore, the affinity for maltotriose may not be the main reason why an α-amylase forms high amounts of maltose.

At high maltotriose concentrations (20–200 mM), the accumulation of maltooligosaccharides higher than maltotriose and a shift in the G1/G2 ratio away from unity could only have arisen from synthesis reactions. This indicates that multicatalytic reactions may have contributed to the production of higher oligosaccharides of maltose from starches. For

Table 4 End products formed by the action of RoAmy on maltotriose

S_0[a] (mM)	Time (h)	Product concentration (mM)					Ratio
		G1[b]	G2	G3	G4	G5	G1/G2
2	0.5	1.2±0.1	1.6±0.3	1±0.1	0	0	1:1.3
	2.0	1.2±0.1	1.6±0.2	0.9±0.2	0	0	1:1.3
20	0.5	6±0.7	18±0.5	7±0.4	0	0	1:3
	2.0	9±0.6	20±2	3±0.4	0.12±0.02	0	1:2.2
40	0.5	8±0.9	32±2	15±0.2	0	0	1:4
	2.0	15±1	40±6	5.9±0.9	0.31±0.03	0.24±0.07	1:2.6
100	1.0	41±3	118±4	6±0.4	0.11±0.03	0	1:2.9
	5.0	51±2	123±2	0.98±0.3	0	0.25±0.02	1:2.4
200	1.0	54±3	203±5	45±2	0.27±0.06	0	1:3.8
	5.0	103±4	240±7	1.7±0.1	0.17±0.02	0.22±0.06	1:2.3

RoAmy (10 U/ml) was incubated with varying concentrations of maltotriose in 0.2 M citric acid–Na_2HPO_4 buffer (pH 5) at 40 °C for up to 5 h, and the end products were quantitatively analyzed by HPLC. The data represent mean ± standard deviation of triplicate determinations.

[a] S_0 is the initial substrate concentration

[b] G1 to G5 represent glucose to maltopentaose, respectively

multicatalytic reactions, condensation reactions would lead to the accumulation of maltohexaose whereas maltotetraose and maltopentaose are products of transfer reactions [28]. Therefore, the multicatalytic reactions of RoAmy act on maltotriose appear to be dominated by transfer reactions as no maltohexaose was detected.

Conclusions

In this study, a newly identified fungal α-amylase from *R. oryzae* was successfully expressed in *P. pastoris* at a high level (382 mg/l). This enzyme exhibits high levels of maltose (74%) forming ability, which is comparable to other α-amylases [27, 28, 31]. However, RoAmy differed from other α-amylases in that it had an extremely high affinity for maltotriose and no maltotriose remained after starch hydrolysis. The end-product profile of the action of RoAmy on maltotriose indicates that transfer reactions might account for the production of high levels of maltose from starches [33]. Though high affinity for maltotriose by this enzyme might not be the main reason for the high levels of maltose produced, it could explain why no maltotriose remained after 12 h starch hydrolysis. In industrial processes, starches are saccharified by fungal α-amylases in combination with β-amylases, debranching enzymes, or pullulanases to yield high-maltose syrups. This work suggests that high-maltose content mixtures could be obtained by using RoAmy alone. The Ca^{2+}-independent, high optimum temperature (60 °C) properties make this enzyme imminently suitable in high-maltose syrup production.

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