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Improved expression of *Rhizopus oryzae* α-amylase in the methylotrophic yeast *Pichia pastoris*

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ABSTRACT

In our previous study, the α-amylase from *Rhizopus oryzae* (RoAmy) was expressed in *Escherichia coli* and *Saccharomyces cerevisiae* but the obtained recombinant RoAmy (rRoAmy) yields were too low. The aim of the present research was to obtain high-level expressions of RoAmy in the methylotrophic yeast *Pichia pastoris*. To this end, we constructed *P. pastoris* strains with the capability to express recombinant α-amylase under the control of constitutive glyceraldehyde-3-phosphate dehydrogenase (*GAP*) and methanol-inducible alcohol oxidase 1 promoters. The levels of inducibly expressed rRoAmy were higher than those of constitutively expressed. The maximal inducible rRoAmy expression levels for the Mut^+ strains (41.1 mg/l) were approximately eight times higher than those for the Mut^s strains and 24 times higher than those expressed under the control of the *GAP* promoter. For both inducible and constitutive expressions, the *S. cerevisiae* α-prepro sequence and the native signal sequence of RoAmy were used separately to direct the secretion of rRoAmy into the culture medium of *P. pastoris*. Low levels of intracellular amylase activities that had been detected after shake-flask fermentation indicated that both signal sequences could effectively direct the secretion of rRoAmy under all studied conditions. In addition, the secretion levels of rRoAmy directed with its own signal peptide were 7–10% higher than those directed by the α-prepro sequence.

Introduction

α-Amylase (1,4-α-D-glucan-glucanhydrolase; EC.3.2.1.1) belongs to family 13 (GH13) of the glycosyl hydrolases. It catalyzes the hydrolysis of the α-1,4 glycosidic linkages of glycogen, starch, or related polysaccharides to produce glucose, oligosaccharides, and dextrins [1,2]. Fungi are the most important sources of α-amylases used for starch degradation in various applications such as baking, brewing, and sweetener production [2–4]. Currently, almost all industrial fungal α-amylases are produced by *Aspergillus* strains [5]. These enzymes have moderate reaction conditions [2,6,7], with an optimal temperature between 50 and 55 °C and an optimum pH between 5 and 5.5. Amylase activity decreases sharply at temperatures above 60 °C or pH below 5. Thus, the application of traditional fungal amylases is restricted by this similarity in their properties. Therefore, the discovery of fungal α-amylases with unique properties, such as raw starch-digesting ability, high maltose-forming ability, and thermo- or acid-stability, is important to meet the demands from different fungal α-amylase-based industries.

Rhizopus oryzae, a filamentous fungus that is the most frequent etiologic agent of zygomycosis [8], is often used in the production of various fermented foods and alcoholic beverages in several Asian countries (e.g., China, Indonesia, and Japan) [9], and in industrial glucoamylase production [10]. The production of α-amylase by *R. oryzae* has also been described by Soccol et al. [9]. To the best of our knowledge, however, the heterologous expression of this α-amylase has not been reported yet.

Previously, we cloned a *R. oryzae* α-amylase gene (GenBank Accession No. HM234170) and obtained heterologous expression in *Escherichia coli* and *Saccharomyces cerevisiae*. The alignments of amino acid sequence indicated that RoAmy is distinct from most of the known α-amylases. Moreover, enzyme characterization showed that RoAmy has a high-level maltose-forming ability (74%, w/w), which makes it a promising enzyme for high-maltose syrup production. Nevertheless, the recombinant α-amylase expression is limited by the characteristics of the host stains or the construction methods.

Pichia pastoris is a methylotrophic yeast that has been used as a powerful eukaryotic expression system to produce large quantities of heterologous proteins of interest [11]. The most important advantages of using *P. pastoris* as a host for the production of α-amylase are the absence of amylolytic activity, the availability

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of strong promoters, the efficient heterologous protein expression system, and the low levels (0.5%) of secreted native proteins [12,13]. α-Amylases from different sources have been successfully produced in the *Pichia* expression system, such as from mouse [14], barley [15], *Bacillus amyloliquefaciens* [16], and *Bacillus subtilis* [17].

The aim of the present study was to improve the expression level of recombinant RoAmy (rRoAmy) in *P. pastoris*. The effects of different signal peptides and promoters on rRoAmy expression were also investigated. The highest yield of rRoAmy obtained in the culture medium by shake-flask fermentation was 41.1 mg/l, approximately 50- and 400-fold higher than those obtained with *S. cerevisiae* and *E. coli*, respectively.

Materials and methods

Strains, plasmids, and culture media

E. coli JM109 was obtained from the Culture and Information Center of Industrial Microorganisms of China Universities (http://www.cicim-cu.jiangnan.edu.cn/), and used for the propagation of plasmids. The methylotrophic yeast *P. pastoris* GS115 strain (*his4*, Mut$^+$, and wild-type) and the expression plasmids pGAPZC and pPIC9K were purchased from Invitrogen (Carlsbad, CA, USA). The recombinant plasmid pMD-*RoAmy* containing the *R. oryzae* α-amylase gene coding sequence was constructed in our previous research. Luria–Bertani medium (5 g yeast extract, 10 g peptone, 10 g NaCl, and 100 mg ampicillin/l) was used for the cultivation of *E. coli*. Yeast extract peptone dextrose (YPD), minimal dextrose (MD), minimal methanol (MM), buffered glycerol-complex (BMGY), and buffered methanol-complex (BMMY) media were prepared according to the *Pichia* expression manuals [18]. MM plates containing 0.5% soluble starch and YPD plates containing 0.5% soluble starch were used for screening potential *Pichia* transformants with higher levels of α-amylase expression.

Construction of expression plasmids

Expression vectors containing inducible and constitutive promoters were constructed to determine which expression mode was the most suitable for rRoAmy expression. Constructs wherein rRoAmy was expressed with either its own signal sequence or the *S. cerevisiae* α-mating factor prepro leader sequence [18] were used to investigate the effects of different signal sequences on the production level of rRoAmy in *P. pastoris* (Fig. 1). All recombinant plasmids were sequenced to confirm the absence of mutations in the target gene. The primers used for the construction of recombinant plasmids are listed in Table 1. The recombinant plasmids were constructed as follows:

(A) pAαRA. The mature RoAmy (*RA*) coding sequence was amplified using polymerase chain reaction (PCR) from pMD-*RoAmy* with primers P1 and P3. The PCR product was gel-purified, digested with *Sna*BI, and then ligated into the *Sna*BI site of the pPIC9K vector, which contains the complete α-mating factor prepro sequence and the alcohol oxidase 1 (*AOX1*) promoter.

(B) pAnRA. To remove the α-factor prepro sequence from the pPIC9K vector, the plasmid was digested with *Bam*HI and *Sna*BI and the larger fragment was gel-purified. After confirming by DNA sequencing that the α-factor prepro sequence had been removed, the α-amylase coding sequence containing its native signal sequence (*nRA*)—which was amplified from pMD-*RoAmy* with the primers P2 and P3 and digested with *Bam*HI and *Sna*BI—was ligated into the digested pPIC9K vector.

(C) pGαRA. The DNA fragment containing the α-factor secretion signal sequence and the mature RoAmy coding sequence (α*RA*) was amplified from pAαRA with primers P3 and P4, and then inserted into the pGAPZC vector at the *Sna*BI recognition site.

(D) pGnRA. The *nRA* DNA fragment amplified with P2 and P3 was inserted into the *Sna*BI site of the pGAPZC vector.

Transformation of P. pastoris

The inducible expression plasmids (pAαRA and pAnRA) and the constitutive expression plasmids (pGαRA and pGnRA) were linearized with *Bgl*II and *Avr*I, respectively. Both expression plasmids were then transformed into *P. pastoris* GS115 by electroporation according to the *Pichia* expression manuals [18,19], resulting in two inducible expression strains (GS115/AαRA and GS115/AnRA) and two constitutive expression strains (GS115/GαRA and GS115/GnRA) (Fig. 1). The transformants harboring the *AOX1* or *GAP* promoter-controlled *RoAmy* expression cassette were selected on the MD or YPD plates containing Zeocin (0.15 mg/ml), respectively.

Selection of transformants with higher expression of rRoAmy

The selection procedure was divided into two steps. In Step 1, clones of GS115/AαRA and GS115/AnRA with putative multiple copies of the RoAmy expression cassette were selected on the MD plates containing 1.0, 2.0, 5.0, and 10.0 mg/ml antibiotic G418, whereas clones of GS115/GαRA and GS115/GnRA were selected on the YPD plates containing 0.5, 1.0, 1.5, and 2.0 mg/ml Zeocin. In Step 2, after the above-mentioned plates were incubated at 30 °C for 3 days, the clones that survived the highest antibiotic concentrations were transferred onto the MM plates containing 0.5% soluble starch (for strains harboring the inducible expression cassette) or the YPD plates containing 0.5% soluble starch (for strains harboring the constitutive expression cassette) to determine their starch-degrading ability. In addition, clones of GS115/AαRA and GS115/AnRA with different methanol-utilization rates were selected on the MM plates containing 1% methanol according to the *Pichia* expression manual [18].

Determination of gene copy number by real-time PCR

Absolute quantification of the copy number was performed with the Chromo 4 system (MJ Research Inc., Watertown, MA, USA) using quantitative real-time PCR [20]. SYBR Green detection method and primers P5 and P6 were used for real-time PCR (Table 1). A 10-fold dilution series of plasmid pMD-*RoAmy* ranging from 10^7 to 10^3 copies per reaction was used to establish the standard curves for copy number determination, and 5 ng genomic DNA of the recombinant yeast was used to determine the gene copy number.

Expression of rRoAmy

Pichia transformants with six copies of expression cassettes were cultured in shake flasks to determine rRoAmy expression levels. For GS115/AαRA and GS115/AnRA, both the Mut$^+$ and Mut^s strains were inoculated in 100 ml BMGY medium in 500 ml baffled flasks and grown at 30 °C in a shaking incubator (220 rpm) until the cultures reached an OD_{600} of 4–6. The cells of Mut$^+$ strains were harvested and transferred into 100 ml BMMY medium in 500 ml baffled flasks, and methanol was added to a final concentration of 0.5% every 12 h to maintain induction for 160 h. The cells of Mut^s strains were harvested and transferred into 20 ml BMMY medium in 500 ml baffled flasks, and methanol was added to a final