Construction of a Shuttle Vector for Heterologous Expression of a Novel Fungal α-Amylase Gene in Aspergillus oryzae

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Introduction

Filamentous fungi are useful industrial microorganisms capable of producing various kinds of important commercial products. In particular, Aspergillus oryzae is well known as one of the popular hosts for homologous and heterologous protein expression, as it has numerous advantages [23]. First, A. oryzae can secrete high levels of protein directly into the growth medium [26], which reduces the complexity of downstream extraction and purification operations. Second, unlike other microbial expression systems such as Escherichia coli [13], A. oryzae possesses the ideal capability of generating proper eukaryotic posttranslational modifications, such as glycosylation and protein folding. A. oryzae is also generally recognized as safe (GRAS) by the US Food and Drug Administration [3] owing to its long history of usage in traditional fermented food production for over 1,000 years. Because of the recent availability of its genome sequence [21] and advanced technologies in genetic engineering, A. oryzae has become widely used as a tool of protein engineering to achieve higher enzyme productivity or desired characteristics. A. oryzae is an extremely efficient producer of α-amylase [31], glucoamylase [15, 16], and α-glucosidase [24]. Among these amylolytic enzymes, the Taka-amylase (amyB) promoter has been most widely used for expression of foreign genes effectively and efficiently [33]. Therefore, the promoter and terminator of Taka-amylase have been considered as the strongest elements in the construction of expression vectors for A. oryzae to produce homologous and heterologous proteins.
Alpha-amylases (endo-1,4-α-D-glucan glucohydrolase; E.C. 3.2.1.1) are extracellular starch-degrading enzymes that randomly catalyze the hydrolysis of the 1,4-α-D-glucosidic linkages between adjacent glucose units in the linear amylose chain. Alpha-amylases from different origins have been extensively studied, including those from animals, plants, and microbes. Among them, fungal amylases play a significant role in biopharmaceutical applications [30], and are also useful tools in medical and clinical chemistry, food processing, etc. So far, many kinds of amylase genes have been successfully cloned and heterologously expressed. Alpha-amylase from Penicillium sp. has also been well studied. Alpha-amylase from P. expansum could produce very high levels of maltose (74%) from starch [7], and alpha-amylase produced by P. chrysogenum showed high enzymatic activity when cultivated in liquid media containing maltose (2%) [2]. Alpha-amylase from P. camemberti was obtained using orange waste as a substrate and the specific activity achieved 154.2 units/ml/mg protein after a trial of purification [25]. However, to the best of our knowledge, few reports have been presented about research into the cloning and expression of Penicillium sp. α-amylase in a heterologous system.

Because of the importance of Aspergillus as a cell factory for production of recombinant proteins [9], the construction of an Aspergillus expression system has attracted a lot of interest. In this study, we present the development of an improved plasmid for heterologous expression in A. oryzae, based on the usage of an orotidine-5-phosphate carboxylase (pyrG)-deficient mutant, PF2, and a binary fungal expression vector, pAsOP, which contains the functional A. nidulans pyrG gene flanked by matel elements as a selection marker [6] and the promoter and terminator of the amyB gene controlling the expression of heterologous proteins. In theory, pAsOP, which contains a pyrG expression cassette, could be transformed into any pyrG-deficient fungus strain (such as Aspergillus) effectively. To test the functionality of the newly constructed vector, the green fluorescent protein gene (gfp) was cloned into pAsOP in the form of a fusion gene with A. oryzae α-amylase (amyB), and introduced into A. oryzae to test GFP expression. Furthermore, we present the cloning of a novel fungal α-amylase gene from Penicillium sp. and the first demonstration of its heterologous expression in A. oryzae PF2, which greatly increased the α-amylase activity of the host A. oryzae. Since the binary vector in this study can efficiently insert a foreign gene into the A. oryzae genome, it will therefore likely contribute to scientific research on gene function and expression, as well as industrial protein production.

Materials and Methods

Strains and Media
A. oryzae RIB40 (ATCC 42129) was used as the DNA donor and starting strain. The recipient strain for transformation was PF2, a mutant strain deficient in orotidine-5'-phosphate decarboxylase selected by 5-fluorouracil (5-FOA) resistance from A. oryzae RIB40 in our laboratory. Briefly, PF2 was isolated as an ultraviolet irradiation-induced mutant by exposing A. oryzae RIB40 wild-type spores to 3 min of a 15 W laminar-flux germicidal lamp at a distance of 30 cm to 30–40% survival, followed by selection for 5-FOA resistance (1.0 g/l) [11] on minimal medium (MM) plates (described below) containing 10 mM uridine and 0.25% TritonX-100. Two of the five 5-FOA-resistant mutants obtained were identified as uridine auxotrophs, and the transformant that showed recovery of uridine autotrophy caused by the plasmid pAsOP was identified as a pyrG mutant, named as PF2, and confirmed not to be a revertant through repeated phenotypic observation. Penicillium sp. 3-5 is an amylase-producing strain isolated from soil taken from a flour factory in China and selected by our laboratory. The 18S rDNA sequence has been assigned to GenBank (Accession No. KP256500). The 18S rDNA was amplified using universal primers 18sf and 18sR, shown in Table 1. E. coli DH5α was used for the construction and propagation of plasmids. pBC-hygro was a generous gift from P. Silar [29]. pANE, stored in our laboratory, contained the orotidine-5'-phosphate carboxylase gene (pyrG) from A. nidulans. E. coli was grown in Luria-Bertani (LB) medium at 37°C. A. oryzae was inoculated in 5x DPY fermentation medium containing 10% dextrin, 5% polypeptone, 2.5% yeast extract, 0.5% KH₂PO₄ and 0.05% MgSO₄·7H₂O. MM plates containing 0.6% NaNO₃, 0.05% KCl, 0.08% KH₂PO₄, 1.04% K₂HPO₄, 0.05% MgSO₄·7H₂O, and 1% glucose were used for transformation of A. oryzae PF2 with required supplements (0.1% uridine and 20.54% sucrose for maintaining the osmotic pressure). Chloramphenicol was added at a concentration of 34 μg/ml when it was needed.

Chemicals and DNA Manipulation

All restriction enzymes were purchased from Fermentas (Fermentas, Shanghai, China). Polymerase chain reaction (PCR) procedures were performed using PrimeStar DNA polymerase purchased from TaKaRa (TaKaRa, Shanghai, China). Ligations were performed overnight at 16°C using T4 DNA Ligase (Fermentas). Genomic DNA was extracted from A. oryzae RIB40 and various transformants, and prepared using the E.Z.N.A. Fungal DNA Midi Kit (Omega Bio-Tek, Norcross, GA, USA). The plasmids were prepared using a TIANprep Mini kit (TianGen Biotech, Beijing, China). Primer synthesis and DNA sequencing were performed by the HuaDa Gene company (HuaDa, Shanghai, China). Primers used in this study are listed in Table 1.

Construction of the Shuttle Expression Vector pAsOP

The starting plasmid, pBC-hygro (Fig. 1A), was a pBluescript II SK(+) based vector with a Cm⁰ resistance gene. The plasmid pBC,
Table 1. Primer sequences used for PCR amplification.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Specificity</th>
<th>Nucleotide sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18sF</td>
<td>18S rDNA</td>
<td>AGCCGCGCAAATTACCAATTC</td>
</tr>
<tr>
<td>18sR</td>
<td>18S rDNA</td>
<td>GCCCTCCAAATGTCCTGTTAAG</td>
</tr>
<tr>
<td>A1</td>
<td>amyB promoter</td>
<td>CGGCTCGAG(Xho)</td>
</tr>
<tr>
<td>A2</td>
<td>amyB promoter</td>
<td>CGGCTCGAG(Xho)</td>
</tr>
<tr>
<td>B1</td>
<td>amyB terminator</td>
<td>AAGGAAAAAACGGCGCCGC(NotI)AGGGTAGAGGATATAT</td>
</tr>
<tr>
<td>B2</td>
<td>amyB terminator</td>
<td>AAGGAAAAAACGGCGCCGC(NotI)AATCTTGAGGACCAT</td>
</tr>
<tr>
<td>C1</td>
<td>pyrG expression cassette</td>
<td>GACCGGCCCATATG(NotI)AGTTCTTGCTCCTCAAGTGTTGACCAAATAGTTCTCGAGA</td>
</tr>
<tr>
<td>C2</td>
<td>pyrG expression cassette</td>
<td>GACCGGCCCATATG(NotI)AAACAGAATAATATGGTGAACACTGGAAGGCAAAGAACTCCCTTTAGTCAATA</td>
</tr>
<tr>
<td>M1</td>
<td>amyB ORF</td>
<td>GCTTCTAGA(Xho)</td>
</tr>
<tr>
<td>M2</td>
<td>amyB ORF (fused with gfp)</td>
<td>ACTCATACCGCGCTCTCTT(Kex2)</td>
</tr>
<tr>
<td>M3</td>
<td>amyB ORF (fused with PcAmy)</td>
<td>GCTGCGACCGCGCTCTCTT(Kex2)</td>
</tr>
<tr>
<td>G1</td>
<td>gfp gene</td>
<td>TCGAAGAGAAGCGCTG(Kex2)ATGATGAAAGGAAAGA</td>
</tr>
<tr>
<td>G2</td>
<td>gfp gene</td>
<td>GCTTCTAGA(Xho)</td>
</tr>
<tr>
<td>P1</td>
<td>PcAmy gene</td>
<td>ATGGTTCTAGGCTCGTTCCGCTGCC</td>
</tr>
<tr>
<td>P2</td>
<td>PcAmy gene</td>
<td>CTATGATGACGACAGCCGGAGCCC</td>
</tr>
<tr>
<td>P3</td>
<td>Mature PcAmy gene</td>
<td>TCGAAGAGAAGCGCTG(Kex2)</td>
</tr>
<tr>
<td>P4</td>
<td>Mature PcAmy gene</td>
<td>GCTTCTAGA(Xho)</td>
</tr>
</tbody>
</table>

Sequences displayed in underline, boldface, or italics indicate restriction/Ke2 site, mate1 element, and His-Tag sequence, respectively. PCR: polymerase chain reaction; ORF: open reading frame.

derived from pBC-hygro with the BssHIII site deleted, was used as a backbone to construct the binary expression vector. PCR amplification of the strong promoter (PamyB, 698 bp) and the terminator (TamYB, 897 bp) of the A. oryzae α-amylase gene was carried out with primers A1/A2 and B1/B2 (Table 1), respectively. gfp and PcAmy fusion proteins with α-amylase (amyB) were generated by PCR amplification using primer pairs M1/M2 and M1/M3 (Table 1) for gfp and PcAmy, respectively. Genomic DNA of A. oryzae RIB40 was used as the template in the reaction. The PCR products of PamyB, digested with XhoI, and TamYB, digested with NotI, were inserted at the XhoI/NotI site of plasmid pBC in the correct orientation to generate plasmid pAsO.

The A. nidulans pyrG (1,477 bp) locus was PCR amplified from pANE with primers C1/C2 (Table 1), which contained 5’ and 3’ pyrG sequences flanked by a 40bp mate1 element and a NotI restriction site. The vector pAsO was digested with NotI to delete the redundant sequence, and the linear vector was dephosphorylated and ligated to the NotI-digested PCR pyrG product. The resulting plasmid (pAsOP) was validated by DNA sequencing (Fig. 1B).

Cloning and Sequence Analysis of the PcAmy Gene

Penicillium sp. 3-5 was incubated in DPY medium containing 2.0% dextrin overnight at 30°C. After collection of the mycelia, the samples were frozen and ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted from the samples with the E.Z.N.A. Fungal RNA Kit (Omega Bio-Tek). Reverse transcription was carried out using the PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa). The product of first strand cDNA synthesis was used as a template for gene amplification. The primers P1/P2 (Table 1) were used to amplify the α-amylase of Penicillium sp. 3-5 according to the gene sequence of P. chrysogenum Wisconsin 54-1255 hypothetical α-amylase mRNA (XM_002565132). Gene amplifications were performed by PCR as follows: initial denaturing at 95°C for 5 min, followed by 30 cycles of 30 sec at 95°C, 30 sec at 55°C, and 1 min 30 sec at 72°C. The PCR product, PcAmy (GenBank: KJ413971.1), was subcloned into vector pMD19T (Simple) (TaKaRa) to generate the plasmid pPcAmy. Using the SignalP website (http://www.cbs.dtu.dk/services/SignalP/), predictions were made on whether PcAmy contains a signal peptide.

Construction and Transformation of Expression Vectors pAsOP-GFP and pAsOP-PcAmy

To test whether the newly constructed plasmid performs its designed function, the coding region of the green fluorescent protein gene (gfp) was used as a reporter gene. GFP was fused with amyB and cloned into the expression vector pAsOP as follows: Using the template pBNS1-GFP [14], gfp (717 bp) was amplified with the primer pair G1/G2 (Table 1). The PCR product of the amyB gene, amplified by primer pair M1/M2, was fused with gfp by fusion PCR with a Kex2 cleavage site along with three consecutive glycine residues (Lys-Arg-Gly-Gly). After gel extraction, the PCR product was digested by XhoI, reclamed, and
ligated with vector pAsOP, which had been digested by the same restriction endonuclease. The recombinant plasmid was named as pAsOP-GFP (Fig. 1C).

The putative mature peptide sequence of the PcAmy gene was obtained through PCR using primer pair P3/P4 (Table 1). A His-Tag sequence was added at the C-terminus for purification purposes. The PCR product of PcAmy was fused with amyB. A sequence encoding the Kex2 cleavage-site (Lys-Arg-Gly-Gly-Gly) was inserted between PcAmy and amyB, and the fusion gene was recovered and purified. After XbaI digestion, the 3.0 kb amyB-PcAmy was subcloned into the XbaI site of pAsOP to generate the shuttle expression vector pAsOP-PcAmy (Fig. 1D).

After confirmation by restriction endonuclease digestion and sequence analysis (HuaDa), plasmids pAsOP-GFP and pAsOP-PcAmy were transformed into A. oryzae PF2 through PEG-CaCl₂ using the mediated method as previously described [17].

Transformants were selected according to the high capability of growing on MM plates, and then confirmed by PCR. The transformants were subcultured for at least two generations and then examined for heterologous expression of the gfp and PcAmy genes.

Measurement of GFP Fluorescence
About 10⁶ conidia of recombinant A. oryzae transformed with pAsOP-GFP were cultured in 100 ml Erlenmeyer flasks containing 20 ml of 5× DPY (pH 5.5) medium. Cultures were incubated at 30°C on a shaker rotating at 200 rpm. A. oryzae RIB40 was used as the negative control. Approximately 24 h after inoculation, the supernatant and the mycelia were collected by centrifugation at 10,000 rpm for 10 min at 4°C. The pelleted cells were resuspended in 100 mM citric acid-sodium citrate buffer (pH 5.0). Both the supernatant and the cells were analyzed for GFP expression on a microtiter plate reader (GENios Pro; Tecan, San Jose, CA, USA), using an excitation wavelength of 485 nm and an emission wavelength of 535 nm [19].

α-Amylase Enzyme Activity Assays
Approximately 10⁶ conidia of the PcAmy-expressing transformant or negative control RIB40 were inoculated into 20 ml of 5× DPY (pH 5.5) medium and cultured at 30°C for 3 to 6 days. The α-amylase activity was estimated on the basis of the decrease in soluble starch used as the reaction substrate, as described previously [18, 36]. A mixture of 0.5 ml of appropriately diluted enzyme and 5 ml

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Fig. 1. Schematic illustration of pBC-hygro, and binary expression vectors pAsOP, pAsOP-GFP, and pAsOP-PcAmy.
(A) The schematic map of pBC-hygro. (B) pAsOP was constructed using a pBC-hygro backbone, containing a PamB (A. oryzae amyB promoter), TamyB (A. oryzae amyB terminator), and pyrG gene expression cassette flanked by mate1 elements. (C) pAsOP-GFP was generated by insertion of the fusion gene amyB-gfp. (D) pAsOP-PcAmy was generated by insertion of the fusion gene amyB-PcAmy.
of 0.5% (w/v) soluble starch dissolved in 0.1 M citric acid-sodium citrate buffer (pH 5.0) was incubated at 30°C. As a negative control, 0.5 ml of 0.1 M citric acid-sodium citrate buffer (pH 5.0) was added instead to allow calculation of the total amount of soluble starch. The reaction was terminated by the addition of 5 ml of chilled 0.1 M HCl. Decreased amounts of soluble starches were detected by OD₆₆₀ using an ultraviolet spectrophotometer. One unit of α-amylase activity or dextrinogenic activity was defined as the amount of enzyme that hydrolyzed 1 mg of soluble starch in 5 min under the above conditions. The protein concentration was determined according to the Bradford method [4].

The method for determination of intracellular enzyme activity was as follows: Mycelia were cultivated in 5× DPY medium, collected by vacuum filtration, and washed several times with 0.1 M citric acid-sodium citrate buffer (pH 5.0). The pellets were pulverized in liquid nitrogen three times, and suspended in 15 ml of citric acid-sodium citrate buffer with slow shaking at 4°C for 2 h, followed by centrifugation at 8,000 rpm to obtain the supernatant. The intracellular enzyme fraction was used for purification and enzymatic activity determination, as described above.

**Purification and Western Blot Analysis of PcAmy**

Approximately 300 mg of protein from the fermentation supernatant of the recombinant strain was resuspended in 1 ml of NPI solution with 10 mM imidazole. A. oryzae RIB40 culture supernatant was used as a negative control. Resuspended protein was loaded onto Ni–NTA spin columns (Qiagen, Hilden, Germany). Recombinant PcAmy plus His-Tag was purified as described by the manufacturer. A 10 µl aliquot of the culture supernatant was loaded onto a sodium dodecyl sulfate polyacrylamide gel. After electrophoresis, protein bands were transferred to a polyvinylidenedifluoride membrane (CWBIO, Shanghai, China) using a semi-dry blotting system (Bio-Rad Laboratories, Berkeley, CA, USA). The membrane was immunostained using a mouse anti-His antibody, a goat anti-mouse IgG antibody conjugated with horseradish peroxidase (Beyotime, Shanghai, China), and an eECL Western Blot Kit (CWBIO, Shanghai, China).

**Characterization of PcAmy**

The optimal pH for PcAmy function was assayed at 30°C in reaction medium with adjusted pH 3.0 to 8.0 (100 mM Gly–HCl buffer for pH 3.0, 100 mM citrate-sodium citrate buffer for pH 4.0–5.0, 100 mM sodium phosphate buffer for pH 6.0–7.0, 100 mM Tris-HCl for pH 8.0–9.0). pH stability profiles were defined as the residual activity after incubating enzymes at different pH buffers at 30°C for 30 min. The optimum temperature of PcAmy was detected between 20°C and 70°C at pH 5.0. The thermal stability of the enzyme was then evaluated at different temperatures from 20°C to 50°C (pH 5.0). Samples were withdrawn when incubating at various temperatures for 30 min and placed on ice before the residual activities were assayed. All values were based on the average of triplicate measurements.

**Sequences Defined in This Study**

The GenBank sequence accession number for the gene PcAmy is KJ741397.1. The GenBank accession number for the 18S rDNA sequence of *Penicillium* sp. 3-5 is KP256500.

**Results**

**Construction and Transformation of the Binary Expression Vector**

The binary expression vector for *A. oryzae* was constructed based on the pBC-hygro plasmid. The pBC-hygro plasmid (Fig. 1A) was derived from the pBC-SK+ bluescript vector. Many studies of vector construction based on pBC-hygro have illustrated that this vector can be effectively transformed into a variety of filamentous fungi, such as *Aspergillus sydowii* [27], *Aspergillus fumigatus* [35], *Thermomyces lanuginosus* [10], and *Podospora anserine* [22], among others. However, as the wild-type *A. oryzae* is strongly resistant to hygromycin B, a pyrG mutant strain was chosen as a host strain, necessitating replacement of the hygromycin B resistance cassette with a pyrG expression cassette. As shown in Fig. 1B, pAsOP was constructed as a binary vector from scratch. The vector contains elements required for maintenance in *E. coli*, and the selectable marker pyrG allows screening for pyrG phenotype mutants when the vector is inserted into fungal chromosomes. To facilitate self-excising of the pyrG marker with 5-FOA resistance [28], repeated matel elements were placed at both ends of the pyrG expression cassette. In order to boost heterologous protein expression to a higher level, a strong amyB promoter and terminator were used in the vector.

To improve heterologous protein production in *A. oryzae*, an effective strategy has been shown to consist of expression of the heterologous protein as a fusion protein with a secretory protein from host cell [32]. In this study, the amyB promoter followed by the open reading frame (ORF) encoding the homologous secretory protein α-amylase of *A. oryzae* was chosen for fusion with target proteins [20]. Expression vectors pAsOP-GFP (Fig. 1C) and pAsOP-PcAmy (Fig. 1D) were constructed through insertion of fusion genes gfp and PcAmy with amyB into the multiple cloning site of pAsOP. Both vectors were introduced into the host strain PF2 by employing the PEG–CaCl₂-mediated transformation method. Positive transformants of pAsOP-GFP (G1) and pAsOP-PcAmy (P1) were selected based on the pyrG selection marker and confirmed by PCR. About 10⁶ spores from G1 and P1 were inoculated into 20 ml of 5× DPY for liquid fermentation.
GFP Expression in pAsOP-GFP-Transformed *A. oryzae*

The *gfp* gene was used as a reporter to test the functionality of the newly constructed vector pAsOP in *A. oryzae*. As shown in Fig. 2, recombinant *A. oryzae* cultures presented remarkable green fluorescence in the mycelia whereas no fluorescence was observed from cultures of the parent strain RIB40. The results indicated that the expression vector pAsOP was capable of expressing the foreign gene, and the recombinant protein showed good biological activity.

Cloning and Sequence Analysis of *PcAmy*

Based upon the genome sequence of *P. chrysogenum* Wisconsin S4-1255, the *PcAmy* gene, encoding a putative \(\alpha\)-amylase, was obtained using PCR amplification from *Penicillium* sp. 3-5, which was screened by our laboratory.

**Fig. 2.** Expression of GFP in transformant G1.
(A) The mycelia of G1 were detected under an Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan) with an excitation wavelength of 485 nm. Green fluorescence was clearly observed in mycelia of G1. (B) The mycelia of G1 were observed under natural light.

**Fig. 3.** Sequence analysis of *PcAmy* and *PcAmy*.
(A) Nucleotide sequence of *PcAmy*. (B) Amino acid sequence of *PcAmy*. (C) Alignment of *PcAmy* sequence with multiple \(\alpha\)-amylase sequences from different sources (*Penicillium roqueforti*, GenBank: CDM35475.1; *Aspergillus sojae*, GenBank: BAM28635.1; *Aspergillus oryzae*, GenBank: AAA32708.1; *Aspergillus kawachii*, GenBank: BAD01051.1; and *Aspergillus niger*, GenBank: CAK44693.1); a black border indicates calcium ion-binding sites; an asterisk indicates catalytic sites.
Nucleotide and amino acid sequences are shown in Figs. 3A and 3B.

Sequence analysis revealed that the sequence of the PcAmy ORF consisted of 1,485 nucleotides encoding a protein of 495 amino acid residues. BLAST analysis (National Center for Biotechnology Information, Bethesda, MD, USA) revealed that the amino acid sequence of PcAmy shared the highest identity (86.16%) with the P. roqueforti α-amylase (GenBank: HG792018.1), suggesting that PcAmy was a novel α-amylase. The homologies with α-amylase from A. oryzae (GenBank: BAA00336.1) and A. kawachii (GenBank: BAD01051.1) were only 67.24% and 67.71%, respectively. Using the SignalP website (http://www.cbs.dtu.dk/services/SignalP/), a potential signal peptide was predicted...
to be contained within amino acids 1–18 of the N-terminal region of PcAmy. Thus, the mature protein consisted of 477 amino acids with a calculated molecular mass of 52.1 kDa and an isoelectric point of 4.72 (http://web.expasy.org/compute_pi/).

PcAmy was compared with multiple α-amylases from different sources according to their amino acid sequences as deposited in the NCBI website: Penicillium roqueforti, GenBank: CDM35475.1; Aspergillus sojae, GenBank: BAM28635.1; Aspergillus oryzae, GenBank: AAA32708.1; Aspergillus kawachii, GenBank: BAD01051.1; and Aspergillus niger, GenBank: CAK44693.1. The amino acid sequence alignments are shown in Fig. 3C. The results suggested that Asp-224, Glu-248, and Asp-315 might act as catalytic sites, while Asn-139 and Asp-193 could play important roles in facilitating calcium ion binding.

Production of Recombinant PcAmy in A. oryzae

Using the newly constructed plasmid pAsOP, the mature form of PcAmy was fused with amyB and expressed under the control of the amyB promoter in order to promote the high-efficiency expression of PcAmy in A. oryzae. After transformation, a positive strain (P1) was selected for fermentation. As presented in Fig. 4, purified protein from the supernatant of the P1 culture showed a clear band on a western blot, whereas the supernatant from the negative control RIB40 showed no band. The western blot results demonstrated that the His-tagged PcAmy was expressed in A. oryzae and secreted into the culture medium. The P1 supernatant achieved a high α-amylase activity of 431.9 U/ml at 30°C, which was an improvement of 62.3% compared with that of the parent strain RIB40 (266.1 U/ml). Comparatively, in the mycelia of recombinant PcAmy, the total amylase activity was 132.19 U/ml, 62.1% higher than RIB40 intracellular amylase activity. After purification the PcAmy amylase activity in mycelia was about 3.7% of the total recombinant amylase activity in the supernatant, which indicated that the recombinant amylase PcAmy had been secreted into the supernatant and very little PcAmy was retained in the mycelia.

Purification and Characterization of PcAmy

To harvest the culture supernatant of P1, mycelia were removed by filtration using a 0.45 μm filter. The culture medium was adjusted to pH 7.0; and the recombinant PcAmy protein was purified using a Ni-NTA Sepharose column (Qiagen). The purified PcAmy was used to examine the characteristics of the PcAmy enzyme.

The optimum pH for PcAmy was 5.0 (Fig. 5A) and PcAmy was stable in the range of pH 4.0–6.0. The residual activities were over 85% control values after incubation at the pH range 4.0–6.0 for 30 min (Fig. 5B). PcAmy exhibited optimum activity at 30°C and showed relatively good stability between 20°C and 30°C (Fig. 5C). The residual activity of PcAmy was over 80% when incubated at 20–40°C for 30 min (Fig. 5D). The activity decreased rapidly when the reaction temperature was higher than 40°C. The specific activity of purified PcAmy was 2,124.6 U/mg at the optimal condition.

Discussion

Because of its strong ability to secrete high levels of correctly folded, modified, and functional proteins into the culture medium, A. oryzae has received considerable interest worldwide and has become one of the most favored hosts for expression of a wide range of heterologous proteins, especially eukaryotic proteins. Therefore, construction of a high-efficiency A. oryzae cloning and expression vector has received widespread attention, and is urgently needed for the large-scale investigation of gene functions in fungi. In order to achieve this goal, the identification of a strong promoter for the expression of exogenous genes, for utilization in the construction of a binary fungal expression vector, has become a very important concern.
A. oryzae has very robust starch-degrading activity; thus, its amylase system has inspired the interest of researchers. The use of strong amylase promoters for industrial application in the production of homologous and heterologous proteins presents a very rational choice. We proposed to take advantage of this system to construct an expression vector to boost foreign protein expression in A. oryzae. In particular, we constructed a shuttle plasmid, pAsOP, for protein expression using the promoter and terminator of the Taka-amylase (\textit{amyB}) gene. In addition, it is both preferable and convenient to include a single selectable marker within the expression vector to achieve gene overexpression or sequential gene deletions. Therefore, the plasmid pAsOP was constructed to carry the selectable marker \textit{pyrG} flanked by repeated matel elements to facilitate self-excising of the \textit{pyrG} marker with 5-FOA resistance. With further development and application of this system, we expect to be able to introduce a variety of genes into the genome of the A. oryzae host without the limitation of including a desired selectable marker.

Green fluorescent protein has been widely used as a convenient marker in both prokaryotic and eukaryotic cells. In this study, to analyze the inducible expression of an enzyme-reporter in germinating spores of a filamentous fungus, we tested the heterologous expression of the fusion construct \textit{amyB-gfp} in A. oryzae under the control of the \textit{amyB} promoter. When fungal secretory enzymes were fused with GFP, intense fluorescence was observed near (or at) the tip, cell wall, and septa regions of \textit{Aspergillus} hyphae [1]. Detection of GFP requires only irradiation with blue light and can be rapidly observed and analyzed [34]. The results of fluorescence measurements indicated that the \textit{gfp}
gene was successfully expressed in \textit{A. oryzae}, which demonstrated the applicability of the newly constructed system. However, GFP, originally isolated from the jellyfish \textit{Aequorea victoria} \cite{5}, has been used as a reporter for monitoring gene expression, and it may be necessary to produce an improved codon usage for \textit{Aspergillus} to enhance the expression of GFP at the transcriptional level \cite{8}. Furthermore, the increasing production of extracellular protease during conidial growth might further decrease GFP activity in the supernatant \cite{12}. Although we confirmed that most of the foreign PcAmy protein had been secreted into the medium through measurement of amylase activity, the amount of extracellular GFP was minimal and it was difficult to observe fluorescence in the supernatant.

Alpha-amylase is one of the most versatile enzymes utilized in a broad range of industrial processes. Diverse \(\alpha\)-amylases from various genera have been isolated and well studied. Members of the genus \textit{Penicillium} can also synthesize \(\alpha\)-amylases, yet few \textit{Penicillium} sp. sequences have been reported to date. Here, we identified a novel \(\alpha\)-amylase gene, \textit{PcAmy}, from \textit{Penicillium} sp. Using our constructed vector system, we expressed the \(\alpha\)-amylase from \textit{Penicillium} sp. in a heterologous host for the first time. Furthermore, the enzymatic properties of PcAmy were explored. Alpha-amylase PcAmy had an optimum temperature of 30\(^\circ\)C, and relatively high activity and stability between 20\(^\circ\)C and 30\(^\circ\)C. The application of a cold-adapted PcAmy enzyme could offer considerable potential to the biotechnology industry, such as the detergent and food industries, because it could assist substantially in preventing modification of heat-sensitive substrates and products at lower temperatures. Cold-adapted enzymes also spur commercial interest through energy savings, as they do not require expensive heating steps and can function in cold environments, as well as increase reaction yields. Valuable insight into the molecular basis of this characteristic may be provided by determination of the crystal structure of PcAmy in future studies. It is worthwhile to note that after the introduction of PcAmy into \textit{A. oryzae}, the enzyme activity at 30\(^\circ\)C increased by 62.24\% compared with the host strain RIB40; neither was the enzyme activity of the transformant lower than the host strain when assayed at 50\(^\circ\)C. Therefore, the transformants could be applied to catalyze the hydrolysis of substrates on a broader temperature scale than those reagents currently available, and could be of benefit to more extensive fields.

In summary, the newly developed vector could be efficiently transformed into the genome of \textit{A. oryzae}, and it effectively expressed heterologous proteins. The vector pAsOP has shown potential use in fungi and as an excellent candidate for scientific research or industrial production. Meanwhile, a novel \(\alpha\)-amylase, PcAmy, from \textit{Penicillium} sp. has been identified and successfully expressed in \textit{A. oryzae}. To the best of our knowledge, this is the first report of \(\alpha\)-amylase from \textit{Penicillium} sp. being successfully expressed and purified in a heterologous host system. The heterologous expression of PcAmy efficiently enhances the \(\alpha\)-amylase activity of the \textit{A. oryzae} host and increases its range of application.

Acknowledgments

This work was funded by The National Natural Foundation of China (No. 31201296), the National High Technology Research and Development Program of China (No. 2013AA102109; No. 2012AA020403), and the Fundamental Research Funds for the Central Universities. We thank Baoshan Zhang (NIH, Bethesda, MD, USA) for his assistance with preparation of the manuscript.

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