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Expression and characterization of a *Grifola frondosa* hydrophobin in *Pichia pastoris*

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ABSTRACT

Hydrophobins are small secreted proteins produced by filamentous fungi. Being amphipathic and self-assembling, hydrophobins have drawn great attention since their discovery. The increase of production can reduce the cost and open up several new applications of hydrophobins. We successfully expressed recombinant Class I hydrophobin HGFI (rHGFI) by using pPIC9 vector with an alcohol oxidase 1 promoter in *Pichia pastoris*. Tricine-SDS–PAGE and Western blotting demonstrated that rHGFI, an 8 kDa protein, was secreted into the culture medium. The culture conditions of the transformant strain were optimized by controlling the methanol concentration and induction time. Ultrafiltration and reverse-phase high performance liquid chromatography were used to perform a large-scale purification of rHGFI. A stable production of rHGFI around 86 mg/L was achieved after the two-step purification. X-ray photoelectron spectroscopy and water contact angle measurements indicated that the functional rHGFI could self-assemble on hydrophobic siliconized glass and Teflon as well as on hydrophilic mica surfaces. A methylthiazol tetrazolium assay showed that rHGFI film could facilitate human aortic smooth muscle cell proliferation due to its cytocompatibility.

Introduction

Hydrophobins are small proteins that are produced by filamentous fungi [1,2]. These proteins contain eight conserved cysteine residues that form four intramolecular disulfide bonds [3]. The most important feature of hydrophobins is that they can form an amphipathic membrane of 10-nm-thick, reversing the properties of the interface coated by them [4,5]. Hydrophobins are classified into two classes based on physical properties and sequence similarity [6]. Class I hydrophobins can form highly insoluble membranes that can resist boiling in 2% (w/v) sodium dodecoyl sulfate (SDS) [4], while the membranes formed by Class II hydrophobins are soluble in SDS and do not have the characteristic rodlet morphology [7].

Hydrophobins make great contributions to fungal development and growth [8]. In fungi, the hydrophobic surface with a hydrophobin coating can facilitate the attachment of hyphae to hydrophobic surfaces, aerial growth of the hypha, dispersal of aerial spores, and proper gas exchange in fungal air channels [9–11]. Moreover, some hydrophobins are implicated in the pathogenicity of several fungi, which are involved in the interaction between the pathogenic fungi and host plants [12].

The self-assembly of hydrophobins is interesting for many applications [13,14], which include personal care and emulsions [15,8], separation technologies [16], biosensors [17] and the gushing factor detection for beer production [18,19]. However, the increasing demand for hydrophobins leads to a challenge in terms of the production and purification of these proteins [20]. The production of Class II hydrophobins seems much easier than that of Class I hydrophobins based on their different assembly characteristics. A reasonable production level of a Class II hydrophobin (600 g/ml) was obtained by constructing a *Trichoderma reesei* HFBI-overproducing strain that contains three copies of the *hfbI* gene [21]. However, efforts to overcome the limitations in the production of Class I hydrophobins have not yet produced satisfactory results. For instance, a homologous overproduction of the Class I hydrophobin SC3 in *Schizophyllum commune* was hampered by gene silencing and the occurrence of the *thn* gene mutation. Moreover, it has been reported that bacterial hosts could not be used to produce functional Class I hydrophobins [22]. The methylotrophic yeast *Pichia pastoris* is considered as an efficient expression system for proteins containing cysteine residues. It can produce soluble proteins that have the right post-translational maturation at high levels [23]. Therefore, expressing hydrophobins in yeast could be a feasible way to produce the hydrophobins with biological activity.

Hydrophobin HGFI[1], from the edible mushroom *Grifola frondosa*, is a Class I hydrophobin identified by our group [24]. In our previous

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[1] *Abbreviations used*: rHGFI, recombinant class I hydrophobin HGFI; WCA, water contact angle; RP-HPLC, reverse-phase high performance liquid chromatography; XPS, X-ray photoelectron spectrometry; MTT, methylthiazol tetrazolium; DLD, delay line detector; SMCs, human aortic smooth muscle cells; PCL, polycaprolactone; TCPS, tissue culture plates.

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studies, native HGFI has been found to have surface binding and modifying activities, capable of functionalizing carbon nanotube, immobilizing antibodies and modifying different surfaces with high efficiency. Significant amounts of protein are needed to further explore more properties and applications of HGFI. In this study, the recombinant Class I hydrophobin HGFI (r-HGFI) was expressed by using the pPIC9 vector in *P. pastoris*. Soluble r-HGFI from *P. pastoris* was firstly desalted and concentrated by ultrafiltration, and then further purified by reverse-phase high performance liquid chromatography (RP-HPLC). X-ray photoelectron spectrometry (XPS) and water contact angle (WCA) analysis confirmed that the biological activity of rHGFI was preserved after the two-step purification. The cytocompatibility of rHGFI film was also confirmed by methylthiazol tetrazolium (MTT) assay. To our knowledge, this is the first demonstration of production of Class I hydrophobin in a yeast expression system, indicating *P. pastoris* is an ideal expression system for expression of functional hydrophobins.

Materials and methods

Strains, vectors and reagents

P. pastoris strain GS115, *Escherichia coli* strain DH5α and plasmid pPIC9 were obtained from Invitrogen (Beijing, China). The pET-28a-*hgfI* vector was preserved by our laboratory, which was constructed to facilitate cloning of *hgfI* gene. The construction procedure of this vector was as follows: the coding region of *hgfI* gene was amplified by PCR directly from its full length cDNA using 5′ primer (5′-AAACATATGACCCCTGTCCGCCGC-3′) and 3′ primer (5′-CCCGAATTCTCAGACGTTAACCGGAACACAT-3′). The amplified DNA fragment was digested with *Nde*I and *Eco*RI enzymes and inserted into the *Nde*I and *Eco*RI sites of pET-28a expression vector (Novagen, China), resulting in recombinant vector named as pET-28a-*hgfI*. All restriction enzymes, Takara Ex Taq DNA polymerase, DNA marker, and protein marker were purchased from TaKaRa (Dalian, China). All primers were synthesized by Invitrogen (Beijing, China).

Vector construction

The *hgfI* coding sequence was amplified by PCR using pET-28a-*hgfI* vector as the template. A *Xho*I restriction site sequence was added to the N-terminal primer (5′-CGCTCGAGAAAAGACAA-CAGTGCACCACTGGC-3′) and an *Eco*RI restriction site sequence was added to the C-terminal primer (5′-CCCGAATTCTCAGA CGTTAACCGGAACACAT-3′). Moreover, the upstream primer contained the KEX2 protease recognition sequence locating after the *Xho*I site. The PCR reaction was carried out using the following reaction cycles in a Peltier Thermal Cycler (MJ Research, USA): initial denaturation at 94 °C for 5 min followed by 30 consecutive cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 45 s, extension at 72 °C for 1 min, and then final extension at 72 °C for 10 min.

The amplified *hgfI* gene was gel-purified by high pure PCR product purification kit (TaKaRa, Dalian, China). After digestion with *Xho*I and *Eco*RI, the purified product was inserted into the pMD18-T cloning vector. The positive clone was confirmed by clone PCR with the above primers and the correct gene insertion was verified by the restriction enzyme digestion and sequencing.

The pMD18-T-*hgfI* was extracted from an overnight liquid culture of one positive clone. The construct was digested with *Xho*I and *Eco*RI and cloned into the same enzyme-digested pPIC9 vector using T4 DNA ligase. The resulting plasmid pPIC9-*hgfI* contained the *hgfI* gene under the control of alcohol oxidase 1 promoter and was in-frame to the α-factor secretion signal (Fig. 1). Finally, pPIC9-*hgfI* was electrotransformed into *E. coli* DH5α for the amplification of the recombinant plasmid and the positive colonies were selected. The pPIC9-*hgfI* plasmid was purified and subjected to DNA sequence analysis.

Transformation of P. pastoris cells

The plasmid pPIC9-*hgfI* was linearized with *Stu*I restriction enzyme, and then was transformed into *P. pastoris* GS115 His^- cells by electroporation. Transformants were screened for methanol utilization by patching 70 colonies on Minimal Dextrose (MD) dishes and Minimal Methanol (MM) dishes incubated at 30 °C for 4 days, and scored as Mut^S (methanol utilizing slow) or Mut^+ (methanol utilizing plus). Integration of *hgfI* into the genome of *P. pastoris* was confirmed by PCR. Colony PCR was performed using the primers: 5'AOX1 (5'- GACTGGTTCCAATTGACAAGC -3') and 3'AOX1 (5'-GCAAATGGCATTCTGACATCC -3').

Selection and optimization of expression of rHGFI

For selection of rHGFI expression, 13 clones of His^+ Mut^+ transformed strains were selected and stored at −80 °C in yeast extract peptone dextrose medium containing 15% glycerol. Each clone was patched on an MD dish and incubated at 30 °C for 4 days. A single colony of each clone was used to inoculate 25 ml of buffered minimal glycerol in a 100 ml flask and grown at 30 °C in a shaking incubator at 250 rpm until the cultures reached an optical density (OD) of 6.0 at 600 nm. Cells were then harvested by centrifugation at 3000*g*, re-suspended in 10 ml of buffered MM medium and transferred to 100 ml flasks. The cultures were maintained at 28 °C in a shaking incubator and supplemented daily with 100% methanol to a final concentration of 0.5%. The culture was collected at 96 h and centrifuged. The resulting supernatant was analyzed by RP-HPLC analytical system. The clone with the highest production of rHGFI was chosen to optimize the production of proteins by controlling the menthol concentrations (0.5%, 0.7%, 1.0%, 1.2% and 1.5%) and induction times (24, 36, 48, 60, 72, 84, 96 and 120 h).

Purification of rHGFI by ultrafiltration and RP-HPLC

Large-scale cultivation (1 L) was performed after the optimization of rHGFI expression. When the cultivation was over, the culture was centrifuged at 7500*g* to get the supernatant. The

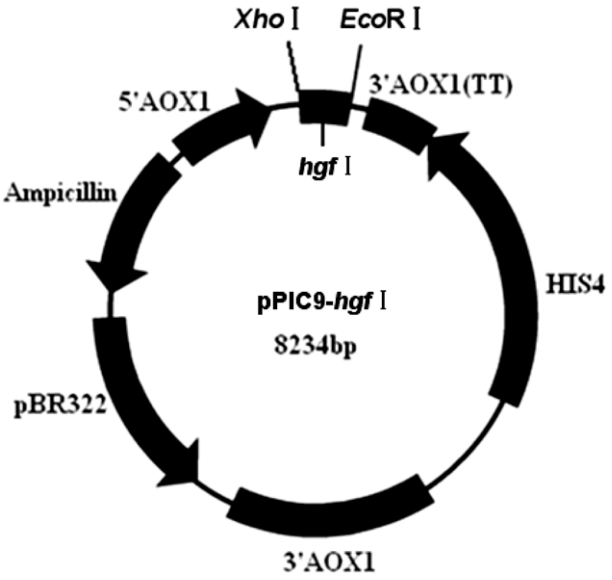

Fig. 1. Schematic representation of the pPIC9-*hgf I*.