Aspergillus niger ATCC 9642 isopullulanase (IPU) was heterologously expressed by Pichia pastoris GS115 under three different signal sequences of Saccharomyces cerevisiae acid phosphatase, S. cerevisiae α-factor prepro peptide, and A. niger isopullulanase. One-step purification using lectin Con A affinity chromatography yielded recombinant IPU (IPU-PP) with high purity. IPU-PP had a higher carbohydrate content than native IPU and IPU-AO expressed in A. oryzae M-2-3. IPU-PP hydrolyzed various substrates containing the structure of panose, which indicated a strict subsite recognition of the panose motif.

Key words: Aspergillus niger; heterologous expression; isopullulanase; Pichia pastoris

Aspergillus niger ATCC 9642 isopullulanase (abbreviated to IPU; EC 3.2.1.57, pullulan 4-glucanohydrolase) hydrolyzes pullulan to produce isopanose.1) IPU also hydrolyzes oligosaccharides containing a motif of panose (Glcα1→Glcα1→4Glc), and cleaves the α-1,4-glucosidic linkage of this motif. Among the pullulan-hydrolyzing enzymes,1–5) IPU is virtually the only isopanose-producing enzyme. Although Arthrobacter globiformis T6 isomaltodextranase, which hydrolyzes dextran to produce isomaltose, is also reported to hydrolyze pullulan to produce isopanose, it cannot be used for the production of isopanose, because the ratio of isopanose production to isomaltose production by this enzyme is only 0.18%.6) The primary structure of IPU is completely different from that of isomaltodextranase or other pullulan hydrolases, but surprisingly, is highly similar to that of several dextranases8–12) that do not hydrolyze pullulan.

To study this unique enzyme further, an efficient expression system is essential. Our previous study also showed that the proper carbohydrate chains are required for the activity of IPU.13) Although we have already reported the expression of IPU in Aspergillus oryzae M-2-3 (abbreviated to IPU-AO),7) the expression of IPU-AO was not stable, probably due to the complicated experimental procedures used. Recently, a methylotrophic yeast, Pichia pastoris, has been used frequently for heterologous expression,14–16) and this host-vector system allows simple genetic manipulation and cultivation. Here we report that the expression of IPU in P. pastoris (abbreviated to IPU-PP) is efficient and the enzyme can be purified with a simple procedure.

The IPU-PP expression system was constructed according to the manufacturer’s instructions for a Pichia Expression Kit (Invitrogen). For secreted protein expression, an appropriate signal peptide is a prerequisite. To test different signal peptides, we constructed three expression plasmids to secrete IPU-PP from different signals, namely, a signal peptide of S. cerevisiae acid phosphatase (pSig-PHO), an α-factor prepro peptide from S. cerevisiae (pSig-Alpha), and a signal peptide of IPU (pSig-IPU) (Fig. 1). The pGIP vector, a derivative of pGEX-4T-2 (Amer Sham Bioscience), contains the cDNA encoding the full-length IPU. The EcoR I-Not I fragment of pGIPA was subcloned into the expression Kit (Invitrogen). For secreted protein expression, an appropriate signal peptide is a prerequisite. To test different signal peptides, we constructed three expression plasmids to secrete IPU-PP from different signals, namely, a signal peptide of S. cerevisiae acid phosphatase (pSig-PHO), an α-factor prepro peptide from S. cerevisiae (pSig-Alpha), and a signal peptide of IPU (pSig-IPU) (Fig. 1). The pGIP vector, a derivative of pGEX-4T-2 (Amer Sham Bioscience), contains the cDNA encoding the full-length IPU. The EcoR I-Not I fragment of pGIPA was subcloned into the multiple cloning site of pPIC3.5 (Invitrogen), resulting in the plasmid pSig-IPU. The pIPA118- and pETIPA-vectors, the derivative of pUC118 and pET21a (+) (Novagen) respectively, contain cDNA encoding only the mature IPU. The EcoR I-Bam H I fragment of pIPA118- was subcloned into the multiple cloning site of the vector pHIL-S1 (Invitrogen), resulting in the plasmid pSig-PHO. The the EcoR I-Not I fragment of pETIPA-A was subcloned into the vector pPIC9 (Invitrogen), resulting in the plasmid pSig-Al-
At the top, a schematic diagram of the $Bgl\ II$ fragment of three IPU expressing plasmids used for transformation of $P.\ pastoris$ is shown. Under the diagram, the signal sequence of each plasmid is shown between the arrows followed by the mature sequence. The underline indicates the analyzed N-terminal sequence of each secreted IPU-PP. The bold letters indicate the mature sequence of native IPU from $A.\ niger$. $p\text{Sig-IPU}$ has the original signal sequence of $A.\ niger$ IPU. $p\text{Sig-PHO}$ and $p\text{Sig-Alpha}$ have the $Saccharomyces\ cerevisiae$ signal sequence of acid phosphatase and the $S.\ cerevisiae$ prepro peptide of $\alpha$-factor, respectively. S, signal peptide. ipuA-, mature IPU-encoding gene. HIS4, histidinol dehydrogenase-encoding gene. 5$'\text{PAOX1}$ and 3$'\text{AOX1}$.

All cloning procedures were done applying standard molecular biological techniques, and *Escherichia coli* strain JM109 was used for the multiplication of plasmids.

The following culture media used for the transformation of $P.\ pastoris$, selection of recombinant clones, and expression of IPU-PP were prepared according to the manufacturer's recommendations: Minimal dextrose medium (MD), minimal methanol medium (MM), buffered glycerol-complex medium (BMGY), and buffered minimal methanol (BMM). For cultures in liquid BMM, which contain methanol as an inducer and carbon source, methanol was added every 24 hours to a final concentration of 0.5\% (v/w). All cultivation was done at 30°C.

$P.\ pastoris$ GS115 was mixed with pSig-IPU, pSig-PHO, or pSig-Alpha linearized by $Bgl\ II$ and electroporated in a 0.2-cm electroporation cuvette using a Bio Rad Gene Pulser according to the manufacturer's instructions. Immediately after the pulsing, 1 ml of cold 1 M sorbitol was added to the *Pichia* cells, and plated on MD containing 1 M sorbitol. After cultivation, all transformants were patched or replica plated on both MM and MD plates to select the colonies growing more slowly in MM than MD, and such colonies were cultivated in liquid BMM. The secretion of IPU-PP in the supernatant of BMM was detected by the pullulan-hydrolyzing activity assay and immunoblot analysis. $P.\ pastoris$ harboring pSig-IPU, pSig-PHO, and pSig-Alpha, which secrete IPU-PP from different signal peptides respectively, were obtained.

To evaluate the most efficient signal peptide, secretion of the three IPU-PPs was compared. Each of these $P.\ pastoris$ was inoculated in 250 ml of BMGY and cultured for 2 days. Then the cells were collected by centrifugation, suspended in 100 ml of BMM, and separated into three portions to be cultured in respective flasks. Every day, just before the addition of methanol, 0.5\% (v/v) of the broth was taken out to measure IPU activity and protein concentration. IPU-PP secreted from the signal of acid phosphatase and $\alpha$-factor increased pullulan-hydrolyzing activity to 16 and 15 units per milliliter of BMM respectively, but from the signal of IPU, pullulan-hydrolyzing activity reached to only 12\% of other signals (Fig. 2A). The signal peptide of IPU is not suitable for the secretion of protein from $P.\ pastoris$ relative to the other two signal sequences.

The N-terminal sequences of the three recombinants were analyzed using a protein sequencer (Applied Biosystems Model 476A). Because $P.\ pastoris$ secretes very small amounts of proteins other than IPU, a dialyzed culture broth (against 20 mM acetate buffer (pH 3.5)) was directly used for the analyses. Each dialysate of culture broth (0.5 to 1.0 ml) was blotted on Immobilon-P Transfer Membrane (MILLIPORE) using Bio-Dot SF (Bio Rad). The analyzed sequence of the pSig-IPU product was Ala-Val-Thr-Ala-Asn-Asn (Fig. 1), which matched with the first six amino acid residues of the N terminus of the $A.\ niger$ IPU. The sequence of the pSig-PHO product was Arg-Glu-Phe-Met-Ala. The prepro peptide of $\alpha$-factor was found to give two different N-terminal sequences, Glu-Ala-Glu-Ala-Tyr and Ala-Tyr-Val-Glu-Phe (Fig. 1). Since the signal sequence of acid phosphatase gave uniform N-terminus and was found to secrete IPU with high pullulan-
Fig. 2. Expression and Purification of IPU-PP.
A. Three IPU-PP-expressing P. pastoris were compared for the expression of IPU activity in the culture medium. Circle, IPU-PP expressed from signal of acid phosphatase. Triangle, IPU-PP expressed from prepro peptide of α-factor. Square, IPU-PP expressed from signal of IPU. One unit of IPU activity was defined as the activity that increased the reducing power equivalent to 1 μmol of glucose in 1 minute at 40°C. B. Purified IPU-AO (lane 1), IPU-PP (lane 2), and IPU-PP treated with PNGase F (lane 3) were put onto 8% SDS-PAGE. The left lane (M) is the high molecular weight marker, and the numbers at the left indicate molecular weight.

Hydrolyzing activity, it was chosen to be used for further experiments as IPU-PP.

IPU-PP from 100 ml of BMM showed a single band on SDS-PAGE. IPU-PP was purified on a HiTrap Con A Sepharose HP column (Amersham Bioscience) with 0.5 M methyl α-D-mannopyranoside, for the purpose of excluding the BMM components. The purified IPU-PP was dialyzed against 50 mM acetate buffer (pH 3.5). From 100 ml of culture supernatant (18 U/ml BMM), 16 mg of purified IPU-PP was obtained with a specific activity of 25.2 U/mg. But the total recovery of IPU activity in this step was only 13%. Some other purification could give a better recovery and is now being tested.

Since IPU-PP uses yeast as the host organism, the carbohydrate chain is predicted to be different from those of native IPU and IPU-AO which are expressed from Aspergillus. The results of SDS-PAGE showed a band for IPU-PP at 104 k (Fig. 2B). The removal of the carbohydrate chain with Peptide-N-glycosidase F (PNGase F, NEB) reduced the molecular weight of IPU-PP to approximately 60 k, which is the deduced molecular weight of the primary structure (Fig. 2B). After deglycosylation, the carbohydrate of IPU-PP was extracted with chloroform and measured by a modified phenol-sulfuric acid method using mannose as the standard. The carbohydrate content of IPU-PP relative to the molecular weight of IPU-PP was found to be 41%, which was higher than the value for native IPU (12–15%) and IPU-AO (34%). Possibly, this difference is due to the changes in the composition, structure, or number of the chains attached to the protein.

A report by Padmajanti et al. demonstrated that the existence of a carbohydrate chain is important for the activity of IPU. To test the effects of the carbohydrate chain on the substrate specificity of IPU-PP, various oligosaccharides were hydrolyzed by IPU-PP and the patterns of the hydrolysate were examined. The oligosaccharides (panose, 6'-α-isomaltosylmaltose (isomaltotriosylglucose, IMTG), 6'-α-maltosylmaltose (MMal), 4'-α-isomaltosylisomaltose (IMIM), 6'-α-glucosylmaltotriose (isomaltosylmaltose, IMM), and 4'-α-panosylpanose (panosylpanose, PAPA)) were enzymatically prepared from pullulan (Hayashibara), dextran (Amersham Bioscience), and maltose (Hayashibara) as described previously (Fig. 3A). These oligosaccharides and pullulan were hydrolyzed by IPU-PP, and the products were analyzed by thin-layer chromatography (Fig. 3B). The substrates listed in Fig. 3, except for IMIM and PAPA, had been previously tested to be hydrolyzed by IPU-AO or native IPU at the α-1,4-glucosidic linkage of the panose motif. IPU-PP hydrolyzed all of these substrates at the same site in the panose motif (Fig. 3B), and also IPU-AO hydrolyzed IMIM and PAPA (data not shown). Thus, an increase in the amount of carbohydrate chain did not affect the substrate specificity of IPU-PP. The ability of IPU to hydrolyze various substrates with the motif of panose indicates a strict subsite recognition of panose, but the recognition of glucose residues of reducing and non-reducing ends of the panose is not as strict.

The optimum pH and temperature of the purified IPU-PP were the same as those of IPU-AO. The pH
stability of IPU-PP had a slightly wider range of than that of IPU-AO, since IPU-PP retained pullulan-hydrolyzing activity after treatment at pH between 2.4 to 7.0 at 4°C for 12 hours. In this study, we have constructed an expression system for recombinant IPU with P. pastoris which secrete virtually IPU only and purified the enzyme in one step. When compared with native IPU and IPU-AO, IPU-PP had a high carbohydrate content but its substrate specificity for the panose motif was invariant.

References


