Molecular cloning and heterologous expression of the isopullulanase gene from *Aspergillus niger* A.T.C.C. 9642

Hiroyoshi AOKI, Yopi and Yoshiyuki SAKANŌ*

Department of Applied Biological Science, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwaicho, Fuchu, Tokyo 183, Japan

Isopullulanase (IPU) from *Aspergillus niger* A.T.C.C. (American Type Culture Collection) 9642 hydrolyses pullulan to isopanose. IPU is important for the production of isopanose and is used in the structural analysis of oligosaccharides with α -1,4 and α -1,6 glucosidic linkages. We have isolated the *ipuA* gene encoding IPU from the filamentous fungi *A. niger* A.T.C.C. 9642. The *ipuA* gene encodes an open reading frame of 1695 bp (564 amino acids). IPU contained a signal sequence of 19 amino acids, and the molecular mass of the mature form was calculated to be 59 kDa. IPU has no amino-acid-sequence similarity with the other pullulan-hydrolysing enzymes, which are pullulanase, neopullulanase and glucoamylase. However, IPU showed a high amino-acid-sequence similarity with dextranases from

Penicillium minioluteum (61 %) and *Arthrobacter* sp. (56 %). When the *ipuA* gene was expressed in *Aspergillus oryzae*, the expressed protein (recombinant IPU) had IPU activity and was immunologically reactive with antibodies raised against native IPU. The substrate specificity, thermostability and pH profile of recombinant IPU were identical with those of the native enzyme, but recombinant IPU (90 kDa) was larger than the native enzyme (69–71 kDa). After deglycosylation with peptide-*N*-glycosidase F, the deglycosylated recombinant IPU had the same molecular mass as deglycosylated native enzyme (59 kDa). This result suggests that the carbohydrate chain of recombinant IPU differed from that of the native enzyme.

INTRODUCTION

Pullulan, an α -glucan produced by *Aureobasidium pullulans*, is used in various fields, including the food, pharmaceutical and chemical industries [1]. Pullulan is a linear polymer which is composed of maltotriose units with α -1,6 glucosidic linkages (Figure 1). Enzymes that hydrolyse pullulan have been classified into four groups on the basis of their cleavage sites on pullulan [2]. They are (i) pullulanase (EC 3.2.1.41) [1], (ii) *Thermoactinomyces vulgaris* α -amylase (TVA; EC 3.2.1.1) [3] and neopullulanase (EC 3.2.1.135) [4], (iii) isopullulanase (IPU) [5] and (iv) glucoamylase (EC 3.2.1.3) [1] (Figure 1). As pullulan-hydrolysing enzymes can precisely recognize the structural differences between α -1,4 or α -1,6 glucosidic linkages, they are applied in the structural analysis of oligosaccharides and polysaccharides.

IPU (pullulan 4-glucanohydrolase (isopanose-forming), EC 3.2.1.57) was purified from *Aspergillus niger* A.T.C.C. (American Type Culture Collection) 9642 [5]. IPU hydrolyses pullulan and panose (Glc α 1-6Glc α 1-4Glc) to produce isopanose (Glc α 1-4Glc α 1-6Glc), and glucose and isomaltose, respectively. However, IPU does not attack starch or dextran.

IPU was purified as an extracellular enzyme in a solid culture on wheat bran (extracellular IPU) and as a cell-bound enzyme from the cell wall of mycelia in a submerged culture (cell-bound IPU) [6]. Furthermore, cell-bound IPU was separated into two different glycosylated forms, IPU F1 (pI 5.0) and IPU F2 (pI 4.9) by Mono-P HR 5/20 chromatofocusing [7]. In the present paper we describe the cloning and nucleotide sequencing of the IPU

gene (*ipuA*) and show that the deduced amino acid sequence of IPU is similar to those of the *Penicillium* and *Arthrobacter* dextranases. We also characterized the *ipuA* gene expressed in *Aspergillus oryzae* M-2-3 and the enzymic properties of the expressed protein (recombinant IPU).

MATERIALS AND METHODS

Determination of internal amino acid sequence

IPU F2 was previously purified from mycelia of *A. niger* A.T.C.C. 9642 [7]. IPU F2 was partially digested with *Staphylococcus* V8 protease (Boehringer Mannheim) by the method of Cleveland et al. [8]. After the peptide fragments of IPU F2 were separated by SDS/15%-PAGE, they were electrotransferred to a PVDF membrane (Immobilon-P; Millipore Co.) and stained with Coomassie Brilliant Blue by the method of Matsudaira [9]. Peptide fragments on the membrane were cut out and analysed by automated Edman degradation on an ABI 477A protein sequencer (Applied Biosystems).

Cloning strategy of IPU cDNA and genomic DNA

(I) cDNA cloning from an *A. niger* A.T.C.C. 9642 cDNA library

Total RNA was isolated from mycelia of a submerged culture of *A. niger* A.T.C.C. 9642 by the method of Okayama et al. [10]. Poly(A)-rich RNA was purified from the total RNA using a

Abbreviations used: IPU, isopullulanase; PNGase F, peptide-*N*-glycosidase F; TVA, *Thermoactinomyces vulgaris* α -amylase; DIG, digoxigenin; GCG, Genetics Computer Group; ORF, open reading frame; A.T.C.C., American Type Culture Collection.

* To whom correspondence should be addressed.

The nucleotide sequence data reported in this paper have been submitted to the DDBJ/EMBL/Genbank nucleotide sequence database under the accession number D85240.

Figure 1 Action patterns of pullulan-hydrolysing enzymes

Arrows indicate the attack points of pullulan-hydrolysing enzymes on pullulan.

mRNA purification kit (Pharmacia Biotech). Double-strand cDNA was synthesized from poly(A)-rich RNA using an oligo(dT) primer and a cDNA synthesis kit (Pharmacia Biotech). A cDNA library was constructed in a λ gt11 vector (Stratagene). The cDNA library (1.5×10^5 plaques) was immunoscreened with a polyclonal rabbit antibody raised against purified native IPU F2 [7]. Three positive plaques (λ IP101, 201 and 401) were obtained and subcloned into pUC119 [11]. The nucleotide sequences of these clones were determined.

(II) Cloning of the IPU-encoding gene from an *A. niger* A.T.C.C. 9642 genomic DNA library

The genomic DNA of *A. niger* A.T.C.C. 9642 was purified by the method of Hynes et al. [12]. The genomic DNA was completely digested with *Eco*RI. The *Eco*RI fragments were separated by 0.8 % agarose-gel electrophoresis and 6.6–2.3 kb fragments were extracted from the gel and ligated into the *Eco*RI site of pUC119. The genomic DNA library (1.0×10^4 colonies) was screened by colony hybridization [11] using the cDNA of λ IP101 as a probe. The cDNA probe was labelled with digoxigenin (DIG)-dUTP using a DIG DNA labelling and detection kit (Boehringer Mannheim). Hybridization and detection of DIG-labelled probe was performed according to the manufacturer's instructions. A positive clone, pIPE, was analysed by restriction-endonuclease

Figure 2 Nucleotide sequences of primers used for PCR

The annealing regions and restriction enzyme sites of the primers are shown by broken and double underlining respectively. The start and stop codons of the isopullulanase gene are indicated.

digestion and Southern hybridization using the cDNA of λ IP101 as a probe. The *Hind*III--*Eco*RI region of the genomic DNA insert of pIPE was sequenced.

(III) Cloning of the full-length cDNA by PCR

A. niger total RNA was purified from mycelia by the acid guanidine thiocyanate/phenol/chloroform (AGPC) method [13]. Single-strand cDNA (25 μ l) was synthesized from 10 μ g of the total RNA using an AMV (avian myeloblastosis virus) Reverse Transcriptase First-strand cDNA Synthesis Kit (Life Science Inc., St. Petersburg, FL, U.S.A.) and an oligo(dT) primer according to the manufacturer's instructions.

PCR was performed using the first-strand cDNA as a template. The PCR reaction mixture (100 μ l) contained 1 μ l of the first-strand cDNA solution, 2.5 units of *Taq* DNA polymerase (TaKaRa), 2.5 mM dNTP, 10 μ g/ml RNase A, and 0.5 μ M IPUNT and RTR1 primers (Figure 2) in a standard reaction buffer. The PCR reaction mixture was incubated at 37 $^{\circ}$ C for 1 h to increase the efficiency of PCR prior to the PCR cycle. The PCR cycle program was 95 $^{\circ}$ C for 2 min (denaturation), 55 $^{\circ}$ C for 1 min (annealing), and 72 $^{\circ}$ C for 2 min (extension) for two cycles, followed by 33 cycles at an elevated annealing temperature (60 $^{\circ}$ C). The amplified cDNA of IPU (1.2 kb) was digested with *Eco*RI and *Xba*I, and subcloned into pUC119. This constructed plasmid was named pRT1, and the nucleotide sequence of the amplified cDNA was determined.

Southern-blot analysis

DNA was digested with restriction endonucleases, electrophoresed on an 0.8 % agarose gels, and alkaline-transferred to nylon membranes (Hybond N+; Amersham International). The cDNA probe subcloned from λ IP101 was labelled by DIG-dUTP using a DIG DNA labelling and detection kit. Hybridization and detection with the DIG labelled cDNA probe were carried out according to the manufacturer's instructions.

Nucleotide sequencing and analysis

DNA sequence analysis was carried out by the dideoxy-chain-termination method [14] using an AutoRead Sequencing Kit and an A.L.F. DNA Sequencer II (Pharmacia Biotech). Both strands were independently and completely sequenced. Nucleotide sequences were analysed with the Genetics Computer Group (GCG, Madison, WI, U.S.A.) sequence-analysis software package version 8.0.1. The hydrophobicity and hydrophilicity of the amino acid sequence of IPU was analysed by the PepPlot

Figure 3 Restriction-endonuclease map of the cloned genomic DNA and cDNA of isopullulanase-encoding gene from *A. niger* A.T.C.C. 9642

(A) genomic DNA; (B) cDNA. cDNAs in λ IP101-401 were obtained by immunoscreening the cDNA library; the cDNA in pRT1 was cloned by PCR using first-strand cDNA transcribed from *A. niger* A.T.C.C. 9642 mRNA. The white arrow represents the ORF of isopullulanase. Only the major restriction-endonuclease sites are shown.

program in the GCG software package. An amino acid sequences similarity search was performed using the tfasta program [15] on the GenBank DNA database (Release 95, June 1996). Percentages of identity and similarity in sequence alignments were calculated using the BestFit program in the GCG software package. The multiple alignment of amino acid sequences was performed by the PileUp program in the GCG software package.

Expression of the IPU-encoding gene in *A. oryzae*

The IPU gene (*ipuA*) was amplified by PCR using the pIPE as a template. PCR was performed using IPUNT and IPUCT primers in a similar manner to the amplification of the cDNA of IPU. The amplified *ipuA* gene was digested by *EcoRI* and *KpnI* and inserted into the polycloning sites after the *amyB* promoter in pTAex3, which is the expression vector in *Aspergillus* [16,17]. This constructed plasmid was denoted pIPFEX. *A. oryzae* M-2-3 (*argB*⁻) [18] was transformed by the pIPFEX using the methods of Iimura et al. [19]. Stable transformants were isolated by transferring sporulating colonies on a selection plate (Czapek–Dox medium) at least four times. The transformant that expressed the highest IPU activity was selected and used for the production of recombinant IPU.

Recombinant IPU was purified from culture supernatant of the *A. oryzae* transformant; the *A. oryzae* transformant was inoculated into 1 litre of YPM medium (1% yeast extract/2% polypeptone/1% maltose, pH 6.0) and cultivated at 28 °C for 2 days in a rotary shaker (120 rev./min). In YPM medium, maltose was the inducer for the *amyB* promoter of pIPFEX and was also a carbon source for growth. Culture supernatant was extracted from culture broth by filtration, and recombinant IPU was purified using the procedure for native IPU purification with slight modifications [7]. The recombinant IPU in the culture supernatant was precipitated with 60% acetone. The crude enzyme was purified by SP-Toyopearl 650M chromatography (Tosoh Co., Tokyo, Japan) and Superose 12 HR 10/30 gel chromatography (Pharmacia Biotech) using an FPLC® system (Pharmacia Biotech).

SDS/PAGE, Western blotting and deglycosylation analysis

SDS/8%-PAGE was performed using the method of Laemmli [20]. Protein bands were revealed by staining with Coomassie Brilliant Blue R-250 or a Silver Staining Kit (Kanto Chemical Co. Ltd, Tokyo, Japan). Western-blotting analysis using anti-(IPU F2) antibody was performed as described previously [21,22].

Deglycosylation of IPU with peptide-N-glycosidase F (PNGase F) [23] was performed as described previously [7].

Assay of substrate specificity of recombinant IPU

Pullulan and Dextran T-2000 were purchased from Hayashibara Biochemical Laboratory (Okayama, Japan) and Pharmacia Biotech respectively. Panose (Glc α 1-6Glc α 1-4Glc) was prepared as described previously [24]. $^2\text{H}_2\text{O}$ was purchased from Aldrich Chemical Co. Inc.

Purified recombinant IPU (1 mU) was added to 50 μ l of 1% substrate in 50 mM acetate buffer, pH 3.5, and incubated at 40 °C for 12 h. Digests were separated on a TLC plate (Kieselgel 60 F₂₅₄; Merck) and detected by spraying with 5% sulphuric acid in methanol as described previously [7].

The pullulan hydrolysate produced by recombinant IPU was identified by NMR; recombinant IPU (0.1 unit) was used to digest 5 ml of 1% pullulan in 50 mM acetate buffer, pH 3.5, at 40 °C for 12 h. After incubation, complete digestion was checked by TLC. The pullulan hydrolysate was evaporated to dryness and redissolved in 1 ml of $^2\text{H}_2\text{O}$. The ^1H NMR spectrum was recorded with a JEOL EX 270 spectrometer (270 MHz) at room temperature.

Other assay methods

The protein content was measured by the method of Lowry et al. [25], with BSA as a standard. IPU activity (pullulan-hydrolysing activity) was assayed as described previously [6]. Total sugar in protein was measured by the phenol/sulphuric acid method using mannose as a standard [26].

RESULTS

Isolation of the IPU-encoding gene from *A. niger* A.T.C.C. 9642

An *A. niger* A.T.C.C. 9642 cDNA library constructed in λ gt11 was screened with a polyclonal antibody against IPU F2 (native IPU). Three positive phage clones, λ IP101, 201 and 401, were obtained from 1.5×10^8 recombinant phages (Figure 3). Sequence analysis showed that λ IP101 contained a 945-bp cDNA insert. The cDNA inserts of λ IP201 and 401 were shorter than that of λ IP101. The cDNA insert of λ IP101 had one open reading frame (ORF) of 262 amino acids and its estimated molecular mass was 28.7 kDa. However, the molecular mass of deglycosylated native IPU is 59 kDa [7] so the cDNA of λ IP101 was almost a half that of the IPU gene.

The genomic DNA was digested with *ApaI*, *BamHI*, *EcoRI*, *HindIII*, *KpnI* or *PstI* and subjected to Southern-blot analysis using the DIG-labelled cDNA of λ IP101 as a probe. The

```

1  AAGCTTTGTCAGATATCCCCGTATAAACAGGTCTCTTGATATTATGTGGGCCCTAAATAAGATATGACTGGATTGATGATCAGGGGAATA 90
91  TCAGACCATCAATTACCCGTCAGGGGAAGCGGGAATTATCGGGAATTTATCGGCACATGACCCCTGAATGCAGGGATAACATCATGCC 180
181 GATCAGACCAATGCCGACCACGACAAGCCGACAAAGTCTTGAAGAACCAATATAGAGCACTGGTATTGCACTCGACAAAGATACAGATA 270
      primer IPUNT =====>
271  CCAAATCAATTTTCAACACCCGTTGCTACCATCGCTCTACAGGTATCTTCTTACCCCTTCCGCGGCCCTTCCAAGTGGCTCAAGCCGCT 360
      M R S T G Y L L T L S A A F O V A O A A
361  GTGACAGCCAACAACAGCCAACCTGCTGACCTGGTGGCATAAACACCGCGCAAAATCAACACTCAGACCCCGTTGCCGATGGCAATGTGCGT 450
      V T A N N S O L L T W W H N T G E I N T Q T P V A D G N V R
451  CAGTCGGGTCTGTACTCCGTCAAGGTCAGACTACTCCGGCTTCAAGCAGCCTCTATTACGATTCTTTGTCTATCTGGCCATTCCCGGC 540
      Q S G L Y S V K V Q T T P A S S S L Y Y D S F V Y L A I P G
541  AATGGCATGTGCGACAGTTTGCAGTACACCCAGGCTACAACACAGCCAGGATGGACTTCTTTTCTCTATTCCTATGACGCCACGGTG 630
      N G M S D Q L Q Y T Q G Y N Q T Q A W T S F L Y S H D A T V
631  AAAATCAGCCGAATGGTTCCTCTGCCAACAGCAACGTTGTCTATCGTCTACCTCACTGAACCTTCCAGTGAGATACGACAACAGAGC 720
      K I S R N G S S A N S N V V I R P T S L N F P V R Y D N Q S
721  GTCTACATCAGATTCTCTACAGTCCCACTGGGTATCGCTTTTTCAGTCGAGTTTCGATGATGATCTGATCTCCCTGGCACCAGCGGTGCG 810
      V Y I T V P Y S P T G Y R F S V E F D D D L I S L A P S G A
811  AGGCAACCTGAGAATGCACCTCTGATTTTCGCGAGCCCGTTTCGAGAAGTCTCGACCAAGCCCGAGCCAGGTTCCGCCAACTCCATCGCC 900
      R Q P E N A L L I F A S P F E N S S T K P Q P G S P N S I A
901  CCGGCACCGAGGAGTCTCTCGGCCTCAACACAACAGTGTCTTCAACAGTCGCTTCAACCCGGCGCTACTACTTCACTGGCCACGAT 990
      P A P G R V L G L N T T S A S T V V F N P G V Y Y F T G H D
991  CACATGGTGCTCAGCTCAAGTGTGACTTGGGTCTACTTCGCCCTGGCGCATATGTGAAAGCGCGGTGGAGTTCTGTCCACCGCATCG 1080
      H M V L S S S V T W V Y F A P G A Y V K G A V E F L S T A S
1081  GAGGTCAAAGCCAGCGCCATGGTGTCTTTCCGGAGAGCAGTACGCTTGGTATGCCGACCCGTGACGAAGGATACCAAAAGCCAGCGGG 1170
      E V K A S G H G V L S G E Q Y V W Y A D P D E G Y Q K A S G
1171  GCCAACAACAACGAGTACGAATGTGGCGCGGACCCCTGGGCAACAGCAGCCAGACATTGCTTCTGAATGGTGTACCGTTAGCGCCCT 1260
      A N N N G L R M W R G T L G N S S Q T F V L N G V T V S A P
1261  CCCTTCAACAGCATGGACTGGAGTGGAAACAGCTTGGACCTGATCACTTGGCGAGTCGATGATTACAAACAGGTGCGGCGCTTCTACGGT 1350
      P F N S M D W S G N S L D L I T C R V D D Y K Q V G A F Y G
1351  CAGACTGACGATTGGAAATGTACCTTGGCACCATTCTCCAGGATGTGTCTTACCACACCGACGACGCGCTGAAGATGTATTACTCG 1440
      Q T D G L E M Y P G T I L Q D V F Y H T D D D G L K M Y Y S
      <===== primer RTR1
1441  AACGTCACAGCGCGCAACATCGTCATGTGGAAGGAGCGTGGTCCGGTGGTGGAGTTTGGTTGGAGCCCGGAACACGAGAATGTC 1530
      N V T A R N I V M W K E S V A P V V E F G W T P R N T E N V
1531  CTCTTTGACAAATGTGATGTGATTACCAAGCATATGCGAATGCCGAAACACCCCTGGCATATTTGGTGGCGTCAACAACCTACCTGTAC 1620
      L F D N V D V I H Q A Y A N A G N N P G I F G A V N N Y L Y
1621  GCCCGGAGCGCCTTTTCGTCCAACCATTCAACGGGCAACAGCAACATGACAGTCCGAAACATCACCTGGTTCGAATTTCCGAGCGGAGGGT 1710
      A P D G L S S N H S T G N S N M T V R N I T W S N F R A E G
1711  TCCTCTCTGCGCCTCTTCCGTATCAACCAATCCAGAATCTGGACAATATCTCTATCAAGAATGTGTCGATTGAGTCTTCGAGCCTTTG 1800
      S S S A L F R I N P I Q N L D N I S I K N V S I E S F E P L
1801  TCTATCAATACCACGAGAGTTGGATGCCGTTGGTATGACCTCAACAACGAAAGCAGATCACCGTGACCGACTTTTCGATCGAGGGC 1890
      S I N T T E S W M P V W Y D L N N G K Q I T V T D F S I E G
      <===
1891  TTCACTGTTGGGAACACCAACATCACTGCGAGCAATGCTGCCAGTGTGGGGCGAATTTGATGGCGTTGATCCGCGCATGACAGGATCTGTG 1980
      F T V G N T T I T A S N A A S V G R I D G V D P A Y A G S V
      ===== primer IPUCT
1981  CACTATATAGATTAAAGTAGCGGCTTGTGTCGTATCAAGAATCGGATAGACTAGCCCATGCAATGGTAGTGACTCTGTGTATGCTGGG 2070
      H Y I D *
2071  TCTATATTCTAGTGGTAGAAGATTATCTCTACCCCGAGTTTGAATGCAAAACCTTGGTAAACACACCAACCAAAATACACCTTTGTCTCC 2160
2161  TCTAGTCCTTGATAAAACAACAGCTCAACTATCGCTAAACCCAAAAAACCCTCCGAATTC 2221

```

Figure 4 Nucleotide sequence and deduced amino acid sequence of the *ipuA* gene of *A. niger* A.T.C.C. 9642

Southern-blot analysis revealed that the cDNA probe hybridized to one strong band in each digest (results not shown); in the *Eco*RI digest the cDNA probe hybridized to a fragment of

approx. 3.3 kb. Therefore 6.6–2.3 kb *Eco*RI fragments of genomic DNA were used to construct a genomic DNA library as described in the Materials and methods section.

sequence of the *ipuA* gene and the cDNA of λ IP101 respectively (Figures 3 and 4). PCR was performed using a single-strand cDNA from *A. niger* A.T.C.C. 9642 and these primers. Only a single band (1.2 kb) was amplified in this PCR. The PCR product was subcloned into pUC119 and the resulting plasmid (pRT1) was sequenced. The amplified cDNA corresponded to positions 299–1485 in Figure 4.

Nucleotide sequence of the *ipuA* gene

The nucleotide sequences of both the genomic DNA and cDNA of the *ipuA* gene were determined (Figure 4). The full-length IPU cDNA was identical with the genomic DNA from position 299 to 2139 (Figure 4); the *ipuA* gene does not have an intron. The *ipuA* gene consists of 1695 bp coding a protein of 564 amino acids. In the putative amino acid sequence of IPU, Ala²⁰–Leu²⁹ and Asn¹⁷⁵–Asn¹⁸⁶ correspond to the N-terminal [7] and the internal amino acid sequences of native IPU F2 that were identified by Edman degradation. Hydropathy analysis by the Kyte and Doolittle algorithm [27] indicated that the N-terminal sequence from Met¹ to Ala¹⁹ is hydrophobic and this portion probably functions as a signal sequence. The mature IPU consisted of 545 amino acids and its molecular mass was calculated to be 59 kDa, which agrees well with that of deglycosylated native IPU [7].

In the 5' upstream region of the *ipuA* gene, a TATA box-like sequence, TATAGA, was found at –64 bp from the start codon and a putative CAAT motif was at –110 (Figure 4). The C+T rich motif, which is found between the TATA box and start codon in highly expressed genes [28], was not found in this sequence. The consensus sequence, AATAAA, which is related to the polyadenylation signal of higher eukaryotes, was not present in the downstream region of the *ipuA* gene. However, a similar sequence to the consensus polyadenylation signal, AAAATA, was found at +148 from the TAA stop codon (Figure 4).

Fifteen potential N-glycosylation sites [29,30], Asn-Xaa-Ser/Thr (Xaa all but Pro), were found in the amino acid sequence of IPU (Figure 4). IPU has only one cysteine residue and does not have any disulphide linkages.

Amino-acid-sequence similarity search of IPU

The deduced amino acid sequence of IPU was compared with amino acid sequences translated by the tfasta program [15] from DNA sequences on the GenBank DNA sequence database. The deduced amino acid sequence of IPU was very similar to the ORF of dextranase (EC 3.2.1.11) from *Arthrobacter* sp. (accession number D00834) [31] and the ORF of dextranase from *P. minioluteum* (accession number L41562) [32]. Amino acid sequence similarities between *A. niger* IPU and dextranases from *P. minioluteum* and *Arthrobacter* sp., were 61.0 and 56.0% respectively. Amino acid sequences of these three enzymes were compared using the PileUp program in the GCG software package (Figure 5). Seven conserved regions (Regions I–VII) are found in these three enzymes.

Heterologous expression of the *ipuA* gene in *A. oryzae*

The plasmid pIPFEX was constructed for the expression for the *ipuA* gene and introduced into *A. oryzae* M-2-3 as described in the Materials and methods section. *A. oryzae* M-2-3 did not show IPU activity at all, but the transformant released high pullulan-hydrolysing activity into the YPM medium. The recombinant IPU of the *A. oryzae* transformant was purified to show

Figure 6 SDS/PAGE analysis of recombinant isopullulanase

(A) Protein was separated on an 8%-polyacrylamide gel and the protein bands were subsequently stained with Coomassie Brilliant Blue R-250 (lanes 1 and 2) or detected using Western-blot analysis with anti-isopullulanase antibody (lanes 3 and 4). Lanes 1 and 3 were loaded with native isopullulanase (IPU) F2 (lane 1, 4 µg; lane 3, 2 µg). Lanes 2 and 4 were loaded with recombinant IPU (lane 2, 4 µg; lane 4, 2 µg). The positions of marker proteins (Low-molecular-weight Electrophoresis Calibration Kit; Pharmacia Biotech.) (molecular masses in Da) are indicated on the left. (B) Deglycosylation analysis of recombinant IPU. The native or recombinant IPU (0.1 µg) was treated with PNGase F (0.2 unit) at 37 °C for 12 h. Then these samples were subjected to SDS/8%-PAGE, and the gel was stained with a Silver Staining Kit (Kanto Chemicals). Lane 1, native IPU F2 (0.3 µg); lane 2, recombinant IPU (0.3 µg); lane 3, recombinant IPU + PNGase F; lane 4, native IPU F2 + PNGase F. The positions of molecular-mass (in Da) markers are indicated on the left.

a single band on SDS/PAGE. From 1 litre of culture supernatant of the *A. oryzae* transformant, 28.9 mg of purified recombinant IPU (yield 21.1%) was obtained. The specific activity of recombinant IPU was 37.5 units/mg, and this value was larger than that of native IPU (27.0 units/mg). The purified recombinant IPU was used in the following experiments.

Characterization of recombinant isopullulanase

The recombinant IPU was subjected to SDS/PAGE and Western-blotting analysis (Figure 6A). Recombinant IPU was immunoreactive to anti-(IPU F2) antibody. However, the estimated molecular mass of the recombinant IPU on SDS/PAGE was 91 kDa, which was larger than that of native IPU F2 (69 kDa) [7].

To analyse their carbohydrate chains, recombinant and native IPU were deglycosylated with PNGase F, which hydrolyses all types of N-glycan chains from glycoprotein, and subjected to SDS/PAGE (Figure 6B). After deglycosylation by PNGase F, deglycosylated recombinant and native IPU showed identical molecular masses (59 kDa). Total carbohydrate of recombinant IPU was assayed by the phenol/sulphuric acid method using mannose as a standard. The total carbohydrate content of recombinant IPU was 15.4% (w/w).

Recombinant IPU was most active at pH 3.5 and 40–45 °C. The stable pH range of recombinant IPU was between pH 3 and

Figure 7 Substrate specificity of recombinant isopullulanase

(A) TLC analysis of the substrate specificity of recombinant isopullulanase (IPU). A reaction mixture containing 50 μ l of 1% panose, 1% pullulan or 1% dextran in 50 mM acetate buffer, pH 3.5, and 1 μ l of the enzyme (1 munit) was incubated at 40 °C for 12 h. Samples (1 μ l) of the digests were spotted on to a TLC plate. Then the plate was developed twice using a solvent system of butan-1-ol/ethanol/water (5:5:3, by vol.). Separated carbohydrates on the plate were detected with 5% sulphuric acid in methanol. G1, glucose; IM2, isomaltose; IM3, isomaltotriose; IM4, isomaltotetraose; IM5, isomaltopentaose. Lane 1, panose; lane 2, pullulan; lane 3, dextran T-2000. '+' indicates that recombinant IPU was added to substrate, and '-' indicates that enzyme was not added to it. (B) ¹H NMR spectra of (i) panose; (ii) isopanose; and (iii) pullulan hydrolysate produced by recombinant isopullulanase.

7, at 4 °C for 12 h. Recombinant IPU was stable at up to 45 °C for 30 min. These stability and optimum conditions were almost identical with those for native IPU [7].

The recombinant IPU hydrolysed panose to glucose and isomaltose, and hydrolysed pullulan to only one oligosaccharide (Figure 7A). From the cleavage patterns of pullulan-hydrolysing enzymes (Figure 1), the pullulan hydrolysate was thought to be panose or isopanose, but panose and isopanose could not be distinguished by TLC. However, the ¹H NMR spectrum of the pullulan hydrolysate was identical with that of isopanose (Figure 7B), indicating that recombinant IPU hydrolyses pullulan to isopanose.

Although the deduced amino acid sequence of IPU shows high similarity to the amino acid sequence of dextranases, IPU did not attack dextran at all. This result agrees with the substrate specificity of the native enzyme [6].

DISCUSSION

In this paper we present the genomic DNA and cDNA sequences of IPU from *A. niger* A.T.C.C. 9642. The *A. niger* IPU gene (*ipuA*) is approx. 1.7 kb and does not have any intron. The *ipuA* gene encodes an IPU precursor consisting of a signal sequence of 19 amino acids and a mature enzyme of 545 amino acids (Figure 4).

That the cloned gene encoded IPU was confirmed in three ways: (i) N-terminal and internal amino acid sequences of native

IPU were found in the deduced amino acid sequence of the cloned gene; (ii) the protein encoded by the *ipuA* gene is immunoreactive against the anti-(native IPU) antibody; and (iii) the expressed protein had IPU activity and showed the same substrate specificity as the native enzyme.

A. niger A.T.C.C. 9642 produces cell-bound IPU and extracellular IPU [6]. The cell-bound and extracellular IPU are slightly different in their molecular masses, pI values and stabilities, but their substrate specificities and optimum-activity conditions are almost identical. In the present study, Southern-blot analysis showed that the IPU gene is a single-copy gene in the genome of *A. niger* A.T.C.C. 9642, indicating that the cell-bound and extracellular IPU are transcribed from the same gene.

The results of deglycosylation by PNGase F (Figure 6B) showed that both native and recombinant IPU were glycosylated at N-glycosylation sites, but that the structure of the carbohydrate chain of recombinant IPU was different from that of native IPU. Glycosylation can have many effects on a protein, and influences its solubility, resistance to proteolytic attack, *in vivo* activity, half-life and thermal- and pH-stability [33,34]. However, the differences in the carbohydrate chains of native and recombinant IPU did not contribute to their thermal- and pH-stabilities.

The amino acid sequences of *A. niger* IPU, *P. minioluteum* dextranase and *Arthrobacter* sp. dextranase have seven conserved regions (Figure 5), which may be important for their structure and function. In particular, Region III consists of hydrophobic amino acid residues and is probably part of the hydrophobic core

Figure 8 Schematic models of the active sites of isopullulanase and dextranase

(A) A schematic model of the active site of isopullulanase. Isopullulanase binds to a panose unit in pullulan. (B) A schematic model of the active site of dextranase. Dextranase binds to the isomaltotriose unit in dextran. The 'wedges' indicate the catalytic residues.

of the protein structure. These conserved regions are mainly located in the centre of the amino acid sequences.

The pullulan-hydrolysing enzymes, pullulanase, TVA and neopullulanase are members of the α -amylase family and have the α -amylase conserved regions (regions 1–4) [35]. IPU, pullulanase, TVA and neopullulanase hydrolyse pullulan endo-wise, but the deduced amino acid sequence of IPU is not similar to those of the α -amylase family members, and the α -amylase conserved regions are not found in the deduced amino acid sequence of IPU. These results suggest that the three-dimensional structure of IPU is different from that of α -amylase and that IPU was not a member protein of the α -amylase family. Dextran is mainly composed of α -1,6 linked homoglucan [36]. The deduced amino acid sequence of IPU is similar to those of two dextranases, but IPU does not hydrolyse dextran at all. IPU hydrolyses the α -1,4 glucosidic linkage of the panose unit and does not hydrolyse the α -1,6 glucosidic linkage in dextran [5–7] (Figure 8). Presumably some structural differences between IPU and the dextranases from *P. minioluteum* and *Arthrobacter sp.* cause IPU to hydrolyse pullulan rather than dextran. We think that IPU may be able to hydrolyse dextran if converted by site-directed mutagenesis.

We thank Dr. Katsuya Gomi (National Research Institute of Brewing, Hiroshima, Japan) for teaching us how to transform *Aspergillus* and for giving us pTAex3 and *Aspergillus oryzae* M-2-3. We used the GCG software and tfasta program in the DNA Information and Stock Center (National Institute of Agrobiological Resources, Japan) via the World Wide Web for the nucleotide and amino acid sequence analysis. We thank the DNA Information and Stock Center.

REFERENCES

- 1 Amylase Research Society of Japan (eds.) (1988) Handbook of Amylases and Related Enzymes: Their Source, Isolation, Methods, Properties and Applications, Pergamon Press, Oxford
- 2 Sakano, Y., Hiraiwa, S.-I., Fukushima, J. and Kobayashi, T. (1982) Agric. Biol. Chem. **46**, 1121–1129
- 3 Shimizu, M., Kanno, M., Tamura, M. and Suekane, M. (1978) Agric. Biol. Chem. **42**, 1681–1688
- 4 Amylase Research Society of Japan (eds.) (1995) Enzyme Chemistry and Molecular Biology of Amylases and Related Enzymes, CRC Press, Boca Raton, FL
- 5 Sakano, Y., Higuchi, M. and Kobayashi, T. (1972) Arch. Biochem. Biophys. **153**, 180–187
- 6 Sakano, Y., Taguchi, A., Hisamatsu, R., Kobayashi, S., Fujimoto, D. and Kobayashi, T. (1990) Denpun Kagaku **37**, 39–41
- 7 Aoki, H., Yopi, Padmajanti, A. and Sakano, Y. (1996) Biosci. Biotech. Biochem. **60**, 1795–1798
- 8 Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977) J. Biol. Chem. **252**, 1102–1106
- 9 Matsudaira, P. (1987) J. Biol. Chem. **262**, 10035–10038
- 10 Okayama, H., Kawauchi, M., Brownstein, M., Lee, F., Yokota, T. and Arai, K. (1987) Methods Enzymol. **153**, 3–28
- 11 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., pp. 4.44–4.45, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 12 Hynes, M. J., Corrick, C. M. and King, J. A. (1983) Mol. Cell. Biol. **3**, 1430–1439
- 13 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
- 14 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
- 15 Pearson, W. R. and Lipman, D. J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 2444–2448
- 16 Fujii, T., Yamaoka, H., Gomi, K., Kitamoto, K. and Kumagai, C. (1995) Biosci. Biotech. Biochem. **59**, 1869–1874
- 17 Huang, K.-X., Fujii, I., Ebizuka, Y., Gomi, K. and Sankawa, U. (1995) J. Biol. Chem. **270**, 21495–21502
- 18 Gomi, K., Iimura, Y. and Hara, S. (1987) Agric. Biol. Chem. **51**, 2549–2555
- 19 Iimura, Y., Gomi, K., Uzu, H. and Hara, S. (1987) Agric. Biol. Chem. **51**, 323–328
- 20 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
- 21 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
- 22 Burnette, W. M. (1981) Anal. Biochem. **112**, 195–203
- 23 Chu, F. K. (1986) J. Biol. Chem. **261**, 172–177
- 24 Sakano, Y., Kogure, M., Kobayashi, T., Tamura, M. and Suekane, M. (1978) Carbohydr. Res. **61**, 175–179
- 25 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
- 26 Fox, J. D. and Robyt, J. F. (1991) Anal. Biochem. **195**, 93–96
- 27 Kyte, J. and Doolittle, R. F. (1982) J. Mol. Biol. **157**, 105–132
- 28 Gurr, S. J., Unkles, S. E. and Kinghorn, J. R. (1987) Gene Structure in Eukaryotic Microbes, (Kinghorn, J. R., eds.), pp. 93–139, IRL Press, Oxford
- 29 Marshall, R. D. (1972) Annu. Rev. Biochem. **41**, 673–702
- 30 Gavel, Y. and von Heijne, G. (1990) Protein Eng. **3**, 433–442
- 31 Okushima, M., Sugino, D., Kouno, Y., Nakano, S., Miyahara, J., Toda, H., Kubo, S. and Matsushiro, A. (1991) Jpn. J. Genet. **66**, 173–187
- 32 Roca, H., Garcia, B. M., Margollez, E., Mateu, D., González, M. E., Raices, M., Cremata, J. A., Rodriguez, E., Garcia, R., Morera, V. et al. (1995) The European Patent Office (EPO), Eur. Patent Appl. 94203614.6; U. S. Patent Appl. 354618
- 33 Hart, G. W. (1992) Curr. Opin. Cell Biol. **4**, 1017–1023
- 34 Neustroev, K. N., Golubev, A. M., Firsov, L. M., Ibatullin, F. M., Protasevich, I. I. and Makarov, A. A. (1993) FEBS Lett. **316**, 157–160
- 35 Nakajima, R., Imanaka, T. and Aiba, S. (1986) Appl. Microbiol. Biotechnol. **23**, 355–360
- 36 Robyt, J. F. (1995) J. Appl. Glycosci. **42**, 53–67