Deglycosylated Isopullulanase Retains Enzymatic Activity

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Isopullulanase (IPU) from *Aspergillus niger* ATCC 9642 is a cell-bound glycoprotein that hydrolyzes pullulan into isopanose. The sugar content of the recombinant enzyme expressed in *Aspergillus oryzae* M-2-3 decreased from 33.8 to 2.1% by 13 h-treatment with endoglycosidase H (Endo H), and deglycosylated rec-IPU had 65% of the original activity. 3 Deg-IPU (prepared by 3 h-treatment of Endo H; 6.8% sugar) showed the same substrate specificities, optimum pH and optimum temperature as native IPU and rec-IPU, while its kinetic parameters, k_o and k_o/K_m values for pullulan, and K_m , k_o and k_o/K_m values for panose decreased with deglycosylation, except K_m for pullulan. The oligosaccharide chains of rec-IPU were typed using lectin-peroxidase reagents and classified as hybrid- and/or high-mannose types.

Isopullulanase (IPU; EC 3.2.1.57, pullulan 4-glucanohydrolase) from *Aspergillus niger* ATCC 9642 is a pullulan-hydrolyzing glycoprotein.¹⁾ IPU differs from other pullulan-hydrolyzing enzymes, such as *Klebsiella pneumoniae* pullulanase (EC 3.2.1.41, α -dextrin-6-glucanohydrolase)²⁾ and *Thermoactinomyces vulgaris* pullulan-hydrolyzing α -amylase (TVA; EC 3.2.1.1, 1,4- α -D-glucanohydrolase),³⁾ in that it produces isopanose, while pullulanase and TVA hydrolyze pullulan to produce maltotriose and panose, respectively, and are not glycoproteins. The substrate specificity of IPU is very strict,⁴⁾ making the enzyme extremely useful for determining the structure of $\alpha(1\rightarrow4)$ and $(1\rightarrow6)$ -oligosaccharides.⁵⁾

Because *A. niger* ATCC 9642 produces only a

small amount of IPU,⁶⁾ the *ipuA* gene encoding IPU was cloned and expressed in *Aspergillus oryzae* M-2-3.⁷⁾ The productivity was increased as a result, and the molecular mass (91 kDa) and sugar content (33.8%)** of the rec-IPU differed from those of native IPU (69–71 kDa and 12.4–15.3%). Aoki *et al.*⁷⁾ reported that native IPU from *A. niger* and rec-IPU from *A. oryzae* have similar molecular mass after deglycosylation, and suggested that native IPU and rec-IPU have different carbohydrate contents. Tago *et al.*⁸⁾ and Okada *et al.*⁹⁾ reported that *Arthrobacter globiformis* T6 produced extracellular isopullulanase activity, but nobody researched carbohydrate contents and functions of isopullulanase except our colleagues.

The structure and function of a glycoprotein may be characterized by not only its amino acid sequence, but also the arrangement of its sugar chains.¹⁰⁾ Aoki *et al.* digested carbohydrate chains of native IPU from *A. niger* and rec-IPU in *A. oryzae* with peptide-*N*-glycosidase F (PNGase F) to obtain deglycosylated IPUs which had, however, no enzyme activity.^{7,11)}

The aim of this work is to release the carbohydrate moieties of rec-IPU under nondenatured conditions to obtain an active deglycosylated rec-IPU and to analyze the type of oligosaccharide chains

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** The sugar content, 15.4%, reported previously by Aoki *et al.*⁷⁾ was corrected to 33.8% in this report.

Abbreviations: Con A, concanavalin A; RCA, *Rhizinus communis* agglutinin; WGA, wheat germ agglutinin; OVA, ovalbumin; IPU, isopullulanase; Endo H, endoglycosidase H; PNGase F, peptide-*N*-glycosidase F; TAA, Taka-amylase A; POD, horseradish peroxidase; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; EDTA, ethylenediamine tetraacetic acid; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); TBS, 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl; Tris, (hydroxymethyl) amino methane.

of rec-IPU using lectin-peroxidase reagents.

MATERIALS AND METHODS

Materials. Native IPU and rec-IPU were prepared as described previously.^{7,11} Peptide-*N*-glycosidase F (PNGase F), endoglycosidase H (Endo H), horseradish peroxidase (POD) and transferrin were purchased from Boehringer Mannheim. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and glucose oxidase (GOD) were obtained from Wako Pure Chemical Industries, Ltd. The Superose 12 HR 10/30 column, FPLC system, and ovalbumin (OVA) were purchased from Amersham Pharmacia Biotech. Pullulan (Mw=64,000) was obtained from Hayashibara Biochemical Laboratories Co., Ltd. Panose was prepared as described previously.¹² POD-ConA, -WGA and -RCA reagents were purchased from Honen Corporation. Immobilon-P transfer membrane was obtained from Millipore Corporation. Taka-amylase A (TAA) was obtained from Sankyo Co., Ltd., and Tween 20 was obtained from Sigma Chemical Co. All other chemicals were of analytical grade.

Assay of enzyme activities. Pullulan-hydrolyzing activities of enzyme preparations were determined as described previously.¹ Panose-hydrolyzing activity was determined using panose instead of pullulan as substrate under the same conditions used to determine pullulan-hydrolyzing activity as follows; panose and the IPU preparation were incubated at 40°C and the amount of glucose liberated was measured by the glucose oxidase/oxidase method.¹³ Kinetic parameters of the enzyme activity for pullulan and panose were measured to within 10% of the values for the hydrolyses of individual substrates. Products from pullulan and panose by the enzyme were analyzed as described previously.^{7,11}

Analyses of carbohydrate and protein. The protein content was measured by the method of Lowry *et al.*, with bovine serum albumin as the standard.¹⁴ Total sugar in the protein was measured by the phenol-sulfuric acid method using mannose as the standard.¹⁵

Deglycosylation of rec-IPU with PNGase F. Deglycosylation of rec-IPU was done in two ways

as follows. 1) The denaturing conditions: Four microliters of rec-IPU (0.96 $\mu\text{g}/\mu\text{L}$) was mixed with 0.4 μL of 10% SDS, and the mixture was heated for 5 min in a boiling water bath. After cooling, 4 μL of 0.5 M phosphate buffer (pH 7.2) containing 50 mM EDTA, 5% 2-mercaptoethanol and 2.5% Triton X-100, 1 μL of PNGase F (50 mU/ μL) and 0.6 μL of milli-Q-water were added to the boiled mixture. After incubation at 37°C for 12 h, the molecular weight of deglycosylated IPU was measured by SDS-PAGE according to the method of Laemmli.¹⁶ 2) The nondenaturing conditions: Four microliters of rec-IPU (0.96 $\mu\text{g}/\mu\text{L}$) was incubated with a mixture of 4 μL of 0.5 M phosphate buffer (pH 7.2) containing 50 mM EDTA, 1 μL of PNGase F (50 mU/ μL) and 1 μL of milli-Q-water at 37°C for 12 h. The residual activity of deglycosylated IPU was measured as described previously¹ and the molecular weight was measured by SDS-PAGE.¹⁶

Deglycosylation of rec-IPU with Endo H.

Ten microliters of rec-IPU (0.9 $\mu\text{g}/\mu\text{L}$) was incubated with 0.8 μL of Endo H (1 mU/ μL) in 50 mM acetate buffer (pH 3.5) at 37°C for an appropriate length of time, changing the pH (pH 6.0–6.5) as described by Yu *et al.*¹⁷ and Trimble *et al.*¹⁸ Individual samples incubated for 1 min, 5 min, 15 min, 30 min, 60 min, 90 min, 3 h, or 13 h were frozen to stop the enzyme reaction. The molecular weights and the residual activities of the deglycosylated IPU were determined as described above.

One-hundred-fifty microliters of rec-IPU (0.9 $\mu\text{g}/\mu\text{L}$) was incubated with 12 μL of Endo H (1 mU/ μL) under the conditions described above, and 8 samples taken at different time points were purified separately on a Superose 12 HR 10/30 column. Purified deglycosylated IPU were subjected to SDS-PAGE and measured for residual activity as described above.

SDS-PAGE and transfer to nitro-cellulose membrane. Enzyme preparations were subjected to SDS-PAGE according to the method of Laemmli.¹⁶ Proteins on a gel were transferred to nitro-cellulose membranes in 10 mM Tris-HCl buffer (pH 7.4) plus 150 mM NaCl (TBS) at 47 mA for 1 h. The nitro-cellulose membrane-blotted

protein was washed in washing solution (TBS + 0.05% Tween 20) and reacted with POD-lectin reagents as follows.¹⁹⁾

Detection of the oligosaccharide chains of rec-IPU.

The membrane was placed in blocking solution (TBS + 5% BSA), and the solution was shaken gently at room temperature for 1 h. POD-ConA, -WGA and -RCA reagents obtained from Honen Corp. were separately diluted to 10 $\mu\text{g}/\text{mL}$ with TBS, and the diluted reagents used for the characterization of oligosaccharides. After decanting the blocking solution, individual dilute POD-lectin reagents were added to membranes, and the membranes soaked in the reagents were shaken at room temperature for 1 h. Before a membrane was treated with POD-Con A, 1 mM of each Ca^{2+} , Mn^{2+} , and Mg^{2+} were added into POD-Con A reagent.¹⁹⁾ After treatment with the lectin reagent, the membrane was washed thoroughly with the washing solution and stained with 4-chloro-1-naphthol in cooled methanol. Before treatment of the membrane with POD-RCA, the membrane was incubated with 25 mM sulfuric acid at 80°C for 1 h to remove the sialic acid at the non-reducing end of the sugar chains,¹⁹⁾ and then washed with milli-Q-water and the washing solution.

RESULTS AND DISCUSSION

Deglycosylation of IPU by glycosidases and activity of deglycosylated IPU.

To ensure that PNGase F can digest the oligosaccharide chains of rec-IPU, it was first reacted with denatured rec-IPU boiled for 5 min in 0.1% SDS. The sugar chains were removed and a single band of deglycosylated IPU was seen (Fig. 1A). To obtain an active deglycosylated IPU, rec-IPU was digested with PNGase F under nondenaturing conditions. Two bands appeared on SDS-PAGE. PNGase F (5 mU/ μL) could not cleave all of the oligosaccharide chains from rec-IPU without SDS (Fig. 1A), and the activity of the deglycosylated rec-IPU decreased to 30% of the original rec-IPU. Rec-IPU deglycosylated without SDS using a higher concentration of PNGase F (20 mU/ μL) had a molecular mass of 59 kDa, a value close to that calculated from the nucleotide sequence of

Fig. 1. Deglycosylation of rec-IPU by PNGase F (A) and Endo H (B and C).

(A) SDS-PAGE of IPU and deglycosylated IPU on 10% gel stained with Coomassie Brilliant Blue. Lane 1, non-denatured rec-IPU; lane 2, denatured rec-IPU treated with PNGase F; lane 3, non-denatured rec-IPU treated with PNGase F. (B) SDS-PAGE of deglycosylated IPU, following incubation, on 10% gel stained with Coomassie Brilliant Blue. Lane 1, rec-IPU (0.48 $\mu\text{g}/\mu\text{L}$) without incubation with Endo H; lane 2 to lane 9, rec-IPU (0.48 $\mu\text{g}/\mu\text{L}$) incubated with Endo H (0.04 mU/ μL) for 1 min, 5 min, 15 min, 30 min, 60 min, 90 min, 3 h and 13 h, respectively; lane 10, Endo H. (C) Residual activity (open square) and sugar content (closed square) of deglycosylated IPU at each deglycosylation step.

ipuA, and showed less than 5% of the original activity. Rec-IPU deglycosylated in the presence of SDS had a molecular mass of 59 kDa and showed no activity. These results suggested that a detergent like SDS or excess PNGase F (20 mU/ μL) is essential for the complete removal of oligosaccharide chains from rec-IPU, and that the sugar chains prevent loss of activity or stability.

Endo H could readily eliminate oligosaccharide chains from rec-IPU in the absence of SDS. Changes in the molecular weight, sugar content, and residual activity of the enzyme on treatment

with Endo H are shown in Fig. 1 B, C. After 3 h-treatment with Endo H, the molecular mass and sugar content of the deglycosylated enzyme (3 deg-IPU) decreased to 64 kDa from 91 kDa and to 6.8% from 33.8%, respectively, and the residual activity was 65% of the original. Although the Endo H-treatment continued for 13 h, no further effects on molecular mass (61 kDa), residual activity (65%) or sugar content (2.1%) were observed. This deglycosylated IPU (13 deg-IPU) was deduced to have 6 to 10 GlcNAc residues per molecule, from the data of a 2.1% sugar content ($61,000 \times (2.1/100)/204 \div 6$) and the difference in molecular weight between deglycosylated IPU (13 deg-IPU) and completely deglycosylated IPU obtained from SDS-PAGE ($(61,000 - 59,000)/204 \div 10$). These residual GlcNAc residues may be anchored to Asn residues of the IPU molecule. It is assumed that the oligosaccharide chains liberated by Endo H contribute to the manifestation of enzyme activity or maintenance of enzyme stability. An active deglycosylated IPU having more than 60% of the original activity could thus be obtained using the above method.

Enzymatic characterization of deglycosylated IPU.

The enzymatic properties of deglycosylated IPU were determined using 3 deg-IPU. The enzyme had an optimum pH of 3.5, optimum temperature of 35–40°C, pH stability at 3.0–7.0 and thermal stability up to 40°C. These properties were similar to those of native IPU and rec-IPU,^{1,4,7,11)} except for the pH stability. Substrate specificity of 3 deg-IPU on pullulan and panose was the same as native IPU and rec-IPU.^{7,11)} It is suggested that the deglycosylation of IPU had no significant effects on the molecular structure of the enzyme.

Rec-IPU (33.8% sugar content) was compared with 30 min-deg-IPU (prepared by 30 min-treatment with Endo H; 13.8% sugar), 3 deg-IPU (6.8% sugar) and 13 deg-IPU (2.1% sugar) for kinetic parameters (Table 1). The IPU on pullulan had similar K_m values, but their molecular activity (k_o) and k_o/K_m values decreased with deglycosylation. The K_m and k_o values for panose also reduced with a sugar content-decreased, while the

decrease of k_o was larger than that of K_m . These results indicated that the presence of oligosaccharide chains affected the catalytic efficiency of IPU. The elimination of sugar chains from IPU affected the hydrolysis of panose more, especially the k_o , than that of pullulan, suggesting that deg-IPUs prefer polysaccharide over oligosaccharide as the substrate.

Oligosaccharide chains of rec-IPU and estimation of glycosylation sites.

Rec-IPU was incubated with three kinds of POD-lectins (POD-ConA, -WGA and -RCA) as described in MATERIALS AND METHODS, and the results were compared with three standard glycoproteins (TAA, OVA, and transferrin) (Fig. 2 and Table 2). ConA reacted with IPU and all three standards. But after the treatment of the glycoproteins with Endo H, transferrin alone reacted with POD-ConA to stain with 4-chloro-1-naphthol. WGA reacted with IPU, OVA and transferrin, but not with TAA. Moreover RCA tests were done with IPU and the three standard glycoproteins after removal of sialic acid at the non-reducing end of the

Table 1. Kinetic parameters of IPU on pullulan and panose.

IPU (sugar content (%))	Pullulan			Panose		
	K_m (mM)	k_o (s ⁻¹)	k_o/K_m (s ⁻¹ mM ⁻¹)	K_m (mM)	k_o (s ⁻¹)	k_o/K_m (s ⁻¹ mM ⁻¹)
Rec-IPU (33.8)	0.88	42.1	48	39.2	27.4	0.69
30 min-deg-IPU (13.8)	0.65	41.0	63	31.0	4.2	0.14
3 deg-IPU (6.8)	0.56	27.1	48	19.7	3.8	0.19
13 deg-IPU (2.1)	0.81	18.1	22	17.5	2.2	0.13

Table 2. Reactions of *N*-glycoproteins with lectin-peroxidase reagents.

Procedure:	A	B	C	D
	Endo H ↓		Removal of Sialic acid ↓	
Stained with:	Con A	Con A	WGA	RCA
Glycoprotein	Staining			
IPU	+	—	+	—
TAA	+	—	—	—
OVA	+	—	+	—
Transferrin	+	+	+	+

Lectin blotting on nitro-cellulose membrane

1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4

SDS-PAGE

1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4

Fig. 2. Detection of IPU and *N*-glycoproteins by lectin-peroxidase reagents.

Glycoproteins on SDS-polyacrylamide gels were electrophoretically transferred to nitrocellulose sheets and detected with POD-Con A (A), POD-Con A after treatment of Endo H (B), POD-WGA (C) and POD-RCA after removal of sialic acid (D). Lane 1, rec-IPU; lane 2, TAA; lane 3, OVA; lane 4, transferrin.

sugar chains. Only transferrin was positive. The results showed that rec-IPU is a glycoprotein of high mannose-type and/or hybrid-type, but not complex-type.

At which of the 15 N-X-S/T sequons do the 6 to 10 GlcNAc residues anchor on 13 deg-IPU? IPU has 8 N-X-S sequons and 7 N-X-T sequons.⁷⁾ From comparisons of hydrophobicity, hydrophilicity, and positive or negative charge of amino acids at the second and third positions of consensus sequences,²⁰⁻²⁵⁾ the N115GS, N186SS, N210TT, N305SS, N448HS, N455MT, N460IT, N486IS, N503TT and N535TT sequons of IPU were identified as possible sites for *N*-glycosylation.

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糖鎖を除去したイソプラナーゼは
酵素活性を保っている

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細胞結合型の *Aspergillus niger* のイソプラナーゼ (IPU) は糖蛋白質であり、プルランを加水分解し、イソパノースを生成する酵素である。 *Aspergillus oryzae* M-2-3 を宿主として、*A. niger* IPU 遺伝子を発現させて得た recombinant IPU (rec-IPU) の糖鎖含量は endoglycosidase H (Endo H) で 13 時間処理すると、33.8% から 2.1% に減少し、糖鎖除去した IPU の活性は rec-IPU の活性の 65% を保持していた。 3 Deg-IPU (Endo H で 3 時間処理して調製された酵素標品; 6.8% 糖含量) の基質特異性や至適 pH・温度は rec-IPU や native IPU と変わらないが、反応速度論定数は、プルランに対する K_m 値を除いて、プルランに対する k_o , k_o/K_m 値、とパノースに対する K_m , k_o , k_o/K_m 値は糖鎖含量が減るにつれ減少した。 また、レクチンプロット法によって、糖鎖の種類を検討した結果、rec-IPU の糖鎖はハイブリッド型糖鎖、もしくはハイブリッド型と高マンノース型糖鎖であると分類された。