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Glucoamylase Originating from *Schwanniomyces occidentalis* Is a Typical α-Glucosidase

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A starch-hydrolyzing enzyme from *Schwanniomyces occidentalis* has been reported to be a novel glucoamylase, but there is no conclusive proof that it is glucoamylase. An enzyme having the hydrolytic activity toward soluble starch was purified from a strain of *S. occidentalis*. The enzyme showed high catalytic efficiency (k_{cat}/K_m) for maltooligosaccharides, compared with that for soluble starch. The product anomer was α-glucose, differing from glucoamylase as a β-glucose producing enzyme. These findings are striking characteristics of α-glucosidase. The DNA encoding the enzyme was cloned and sequenced. The primary structure deduced from the nucleotide sequence was highly similar to mold, plant, and mammalian α-glucosidases of α-glucosidase family II and other glucoside hydrolase family 31 enzymes, and the two regions involved in the catalytic reaction of α-glucosidases were conserved. These were no similarities to the so-called glucoamylases. It was concluded that the enzyme and also *S. occidentalis* glucoamylase, had been already reported, were typical α-glucosidases, and not glucoamylase.

Key words: *Schwanniomyces occidentalis*; glucoamylase; α-glucosidase; product anomer analysis

It has been reported that yeast genus *Schwanniomyces* spp. secretes α-amylase and glucoamylase to hydrolyze soluble starch to glucose using the extracellular enzymes. The two α-amylase genes (*AMY1* and *AMY2*: *SWA1* and *SWA2*, respectively)[1–4] were cloned from *S. occidentalis* and *S. castellii*.

As for glucoamylase, the *S. occidentalis GAM1* gene,[5,6] encoding a starch-hydrolyzing enzyme, was cloned and expressed in *Saccharomyces cerevisiae*. The enzymes of *Schwanniomyces* spp., showing hydrolytic activities not only on soluble starch but also on maltose and isomaltose, have long been designated glucoamylase (GAM).[7–9] The amino acid sequence deduced from the *GAM1* gene represented a high level of similarity to those of human lysosomal α-glucosidase and rabbit intestinal sucrose-isomaltase, and no similarity to those of other fungal glucoamylases. Nevetheless, it has been concluded that *S. occidentalis* glucoamylase is a novel type of glucoamylase that might derive from the same ancestral gene as those of mammalian α-glucosidase, isomaltase, and sucrase.[6] In addition, indispensable confirmation, on the basis of the product anomer and the substrate specificity, has hardly been made as to whether *S. occidentalis* glucoamylase is glucoamylase or α-glucosidase.[10] In the classification of glycosyl hydrolases,[11] *S. occidentalis* glucoamylase is grouped into the family 31 and not into the family 15, implying the possibility that the glucoamylase is α-glucosidase. Therefore, we have looked upon the glucoamylase as α-glucosidase, because it exhibits high similarity in the amino acid sequences to many α-glucosidases from fungi, plants, and mammals.[12] It seems significant to ascertain whether the enzyme really is glucoamylase or not.

Glucoamylase (EC 3.2.1.3, 1,4-α-D-glucan glucohydrolase) and α-glucosidase (EC 3.2.1.20, α-D-glucoside glucohydrolase) are a group of typical exo-type hydrolases, which catalyze the splitting of α-glucosidic linkage at the non-reducing terminal side of the substrates, such as α-glucosides, maltooligosaccharides, and α-glucans, to release D-glucose. Various types of α-glucosidases[12] are widespread in microorganisms, plants, mammals, and insects. On the other hand, the distribution of glucoamylase is limited only to microorganisms. The two enzymes are not, apparently, distinguished in view of releasing only D-glucose, but can be essentially differentiated by the anomer type of the product D-glucose. α-Glucosidase produces α-D-glucose by retaining the anomeric configuration of substrate, and glucoamylase produces β-D-glucose by inverting. The two enzymes are quite different from

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each other in the reaction mechanism.

The present paper describes the purification, characterization, substrate specificity, and product anomer of an original enzyme purified from *S. occidentalis.* Additionally, the similarity in the primary structures between the original enzyme and *S. occidentalis* glucoamylase already reported, and small discrepancies in enzymatic properties between the original enzyme and the recombinant enzyme expressed in *Pichia pastris*, are discussed on the basis of the structure of *Escherichia coli* α-xylosidase, which is the only enzyme in the family 31 glycoside hydrolases whose three-dimensional structure has recently been solved.[13,14] The data on the product anomer and the substrate specificity provide conclusive evidence that *S. occidentalis* glucoamylase is a typical α-glucosidase, and not a so-called glucoamylase.

Materials and Methods

Yeast strain. The yeast strain used in this study was *Schwanniomyces occidentalis* ATCC26074. The use of *S. occidentalis* ATCC26076 was desirable, but unfortunately we could not obtain that strain.

Materials. Arabitol, maltose (SP-grade), *p*-nitrophenyl α-glucoside, *p*-nitrophenyl α-maltoside, trehalose, and soluble starch (SP-grade) were purchased from Nacalai Tesque Chemical Inc. (Kyoto, Japan). To remove possible impurities, maltose was further purified by repeated recrystallization, and soluble starch was washed with ice-cold water. α-Glucose was purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan); β-glucose, from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Malto-triose, -tetraose, -pentaose, -hexaose, and -heptaose, and isomaltose were kindly supplied by Nihon Shokuhin Kako Co., Ltd. (Fuji, Japan). Nigerose and kojibiose were enzymatically synthesized through transglucosylation by buckwheat α-glucosidase.[15] High maltotetraose syrup (TETRUP®) was kindly supplied by Hayashibara Biochemical Lab., Inc. (Okayama, Japan). Polypentone and yeast extract were purchased from Seikagaku Co. (Tokyo, Japan); endoglycosidase F (*N*-glycosidase F-freetype), from Boeringer Mannheim Biochemia (Mannheim, Germany); and trimethylsilylation reagent (TMSI-H) containing hexamethyldisilazane and trimethylchlorosilane in pyridine, from GL Sciences Inc. (Tokyo, Japan).

Enzyme assay. Maltase activity was determined by the glucose oxidase method[16] with our modification, using Glucose AR-II (Wako Pure Chemical Ind., Ltd.). The standard reaction mixture, containing 0.2 ml of 0.5% maltose, 0.2 ml of 0.1 M sodium acetate buffer (pH 5.0), and 0.1 ml of the enzyme solution in a total 0.5 ml volume, was incubated at 37 °C. One unit of the enzyme activity was defined as the amount of enzyme that hydrolyzed 1 µmol of maltose per min under the conditions just described.

Protein determination and amino acid sequencing. The enzyme concentration was spectrophotometrically determined on the basis of the value that $E^{1\%}_{1\,cm}$ at 280 nm was 13.1, which was calculated from the amino acid contents of protein hydrolyzate (6 M HCl, 24 h, and 110 °C) determined using JEOL JLC/500V equipped with the ninhydrin detection system. In the purification steps, however, the value, based on the assumption that $E^{1\%}_{1\,cm}$ at 280 nm was 10, was also used. The analysis of amino acid sequence in the N-terminal of the purified enzyme was carried out on an Applied Biosystems model 477A protein sequencer with an on-line phenylthiohydantoin analyzer (model 120A; Applied Biosystems Inc., Foster City, CA, U.S.A.).

Estimation of molecular weight. To estimate the molecular weight (M_r) of the purified enzyme, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to the method of Laemmli,[17] using 7.5% gel and the standard proteins (Invitrogen Corp., Carlsbad, CA, U.S.A.): myosin H-chain (M_r, 200,000), phosphorylase *b* (M_r, 97,400), bovine serum albumin (M_r, 68,000), ovalbmin (M_r, 43,000), and carbonic anhydrase (M_r, 29,000). Proteins were stained with Rapid CBB KANTO (Kanto Kagaku Co., Tokyo, Japan).

Gas-liquid chramatography (GLC). GLC of anomeric forms of the enzyme reaction products was conducted by the novel quantitative method described in our previous papers.[18,19] The reaction mixture consisting of 120 µl of 6.25 mM *p*-nitrophenyl α-maltoside or maltotriose and 30 µl of the original enzyme (0.95 µg) in 25 mM sodium acetate buffer (pH 5.0) was incubated at 25 °C for 4 min. The reaction mixture was immediately frozen with liquid nitrogen and then lyophilized at −50 °C. The freeze-dried sample was converted to the trimethylsilyl (TMS) compound by incubating with 0.05 ml of a TMSI-H reagent at 80 °C for 10 min, and then subjected to GLC. The TMS derivative of arabitol was used as the internal standard. The TMS derivatives of α- and β-glucose and the equilibrated maltose were also used as the standard sugars. The column temperature was elevated at a rate of 10 °C per min from 150 to 270 °C.

Culture media. Yeast cells were grown at 21 °C for 21 h with shaking in synthetic media, a modified PYN medium[7,20] containing 0.7% polypeptone, 0.6% yeast extract, 0.4% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, 0.2% $(NH_4)_2SO_4$, 2% $CaCO_3$, and 4% TETRUP®. Three liters of the culture medium was centrifuged at 22,000 × *g* for 60 min, and the supernatant (2,200 ml) was recovered as the crude enzyme solution.

Enzyme. An enzyme was purified from the culture supernatant (2,200 ml). To the supernatant (the crude enzyme solution), solid ammonium sulfate was slowly added with stirring up to 100% saturation (about 70 g of

ammonium sulfate per 100 ml of the solution), and the turbid solution was allowed to stand overnight in a cold room. The resulting precipitate was collected by centrifugation at 25,000 × *g* for 60 min, and then dissolved in 350 ml of 0.01 M sodium acetate buffer (pH 4.3). The solution was thoroughly dialyzed against the same buffer.

The dialyzed solution was applied to a DEAE-Sepharose CL-6B (2.6 × 31 cm) equilibrated with the same buffer (pH 4.3). Linear gradient elution was carried out by increasing the concentration of sodium chloride (0 to 0.5 M) in the same buffer; the flow rate was 24 ml/h, and each fraction, 10 ml (fractions 1 to 66) or 4 ml (fractions 67 to 178). The active fractions (nos. 143 to 149) were combined and concentrated up to 3 ml. The concentrate (3 ml) was subjected to gel-chromatography in a Bio-Gel P-200 column (2.1 × 40 cm) equilibrated with 0.01 M sodium acetate buffer containing 0.2 M sodium chloride (Fig. 1). The active fractions (nos. 33 to 45) were recovered and used as a purified enzyme preparation. The enzyme gave a broad and smeary band on the usual polyacrylamide gel electrophoresis. Such a band is often observed in the electrophoresis of glycoprotein. Therefore, the elution pattern is shown in Fig. 1, so as to confirm the homogeneity of the enzyme.

Cloning of a glycoside hydrolase family 31 enzyme gene. The gene encoding the enzyme was amplified from genomic DNA of *S. occidentalis* ATCC26074 by PCR, using the forward primer 5′-GAA ATT TTA AAA TGA ACT CAT GAC TG-3′ and the reverse primer 5′-AAA ACT AAA ATG ATA TCA TTC AAA TAA T-3′, which were designed from the sequence of *S. occidentalis* ATCC26076 glucoamylase (*GAM1*) gene (accession no. M60207). The PCRs were done independently

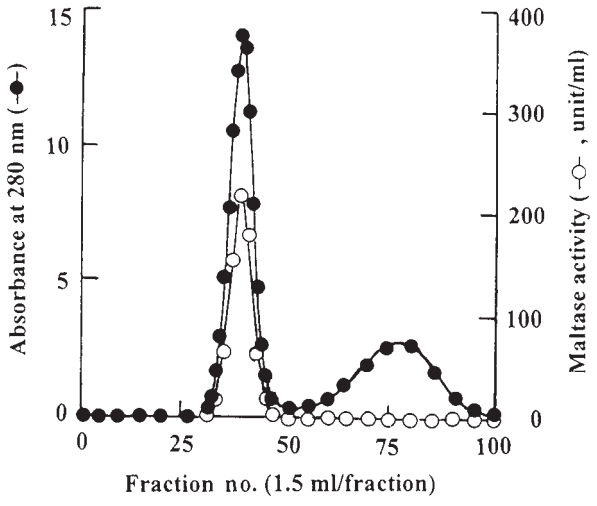

Fig. 1. Gel Chromatography of the Enzyme from *Schwanniomyces occidentalis* on a Bio-Gel P-200 Column.

Enzyme solution, 3 ml; column, 2.1 × 40 cm; equlibrium, 0.01 M sodium acetate buffer (pH 5.0) containing 0.2 M NaCl; elution, the same buffer; flow rate, 6 ml/h.

three times using KOD DNA polymerase (TOYOBO Biochem., Tokyo, Japan) with high fidelity. The amplified DNA fragment was ligated into pBluescript II SK (Stratagene, La Jolla, CA, U.S.A.) at the *Eco*RV site, and the generating plasmid pBS-SOG was sequenced by using 310 Genetic analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

Production of the recombinant enzyme. The pBS-SOG was amplified by PCR to construct an expression vector in *Pichia pastoris*, using the forward primer, 5′-ATT ATA **GAA TTC** ACC ATG ATT TTT C-3′ (containing a *Eco*RI site, bold characters), and the reverse primer, 5′-ATG TTA **GGG CCC** CCA AGT AAT GGT-3′ (containing an *Apa*I site, bold characters). The resulting fragment was digested by corresponding restriction enzymes and ligated into pPICZA (Invitrogen) at corresponding sites, developing the plasmid pPICZA-SOG encoding the *S. occidentalis* enzyme, with the His × 6 sequence added to its C-terminal end. The pPICZA-SOG was propagated in *E. coli* DH 5α, purified by a method with alkaline-lysis of the *E. coli* cell, and digested with *Bgl*II to generate a linear-form plasmid. The linear pPICZA-SOG (10 μg) was electroporated into *P. pastoris* GS115 cells (Invitogen) according to the supplier's protocol, and positive colonies were selected. The colonies selected were cultured to check for the enzyme secretion by 3-ml minicultures grown overnight at 30 °C in BMGY (13.4 g/l yeast nitrogen base without amino acids, 20 g/l peptone, 10 g/l yeast extract, 0.5% glycerol, 0.4 mg/l biotin, and 0.1 M potassium phosphate, pH 6.0) in 50-ml tubes. Cells were harvested, resuspended in 12 ml BMMY (13.4 g/l yeast nitrogen base without amino acids, 20 g/l peptone, 10 g/l yeast extract, 0.5% methanol, 0.4 mg/l biotin, and 0.1 M potassium phosphate, pH 6.0), and incubated for 96 h at 30 °C under vigorous shaking. After centrifugation, culture supernatants were tested for maltase activity, as described above.

Purification of the recombinant enzyme. A yeast transformant was grown in BMGY (200 ml in a 500-ml flask) up to 2.5 in OD_{600}, and the cells were harvested by centrifugation, resuspended in BMMY (200 ml in a 500-ml flask), and the culture was continued for 70 h at 30 °C. One ml of methanol was added every 24 h to maintain induction. To the culture supernatant (190 ml), ammonium sulfate was added up to 90% saturation and the suspension was allowed to stand overnight at 5 °C. The resulting precipitant was collected by centrifuge (20,000 × *g*, 15 min, 4 °C) and dissolved in 20 mM sodium phosphate buffer (pH 7.5) containing 500 mM sodium chloride, and loaded onto a Ni-Sepharose Fast Flow (Amersham Bioscience AB) column prepared according to the method in the supplier's manual, and equilibrated with the same buffer. The column was washed with 20 mM sodium phosphate buffer (pH 7.5), followed by washing with the same buffer (pH 6.0),

Table 1. Summary for Purification of *Schwanniomyces occidentalis* Enzyme

Procedure	Protein (mg)	Activity (unit)	Specific Activity (unit/mg)	Purification (-fold)
Culture medium	2,200[a]	3,000	1.3	1
Ammonium sulfate (100% saturation)	1,600[a]	3,300	2.1	1.6
DEAE-Sepharose CL-6B	290[a]	3,000	10	7.7
Bio-Gel P-200	92[b]	2,500	27	16

[a]Calculated under the assumption that $E^{1\%}_{1\,cm}$ at 280 nm was 10.
[b]Calculated according to the fact that $E^{1\%}_{1\,cm}$ at 280 nm was 13.1.

containing 500 mM sodium chloride. The absorbed components were eluted with 20 mM sodium phosphate buffer (pH 4.5) containing 500 mM sodium chloride. The active fractions containing the enzyme were pooled and dialyzed against 20 mM sodium acetate buffer (pH 4.5). The dialyzed solution (3.2 ml) was used as the recombinant enzyme. All purification steps were done at 4 °C.

Biochemical assay. Optimum pH was examined with 0.2% malose and 4.6 nM original enzyme or 1.9 nM recombinant enzyme in McIlvaine buffer (pH 2.5 to 8.0), at 37 °C for 5 min. For pH stability, 13.8 nM (original) or 19 nM (recombinant) of the enzyme was incubated at 4 °C for 24 h in 25 mM Britton–Robinson buffer at various pHs from 2.5 to 10.7. The pH-treated sample was added to a solution of 0.2% maltose and incubated at 37 °C in 200 mM sodium acetate buffer (pH 4.5). For thermal stability, 6.8 nM (original) or 8 nM (recombinant) of the enzyme was incubated for 15 min in 75 mM sodium acetate buffer (pH 4.5) at various temperatures from 25 to 60 °C, followed by measurement of the activity at pH 4.5. The kinetic constants were calculated by Lineweaver–Burk plot, Hanes–Woolf plot, and fitting to the Michaelis–Menten equation by nonlinear regression using the computer program Curve Expert 1.3, in which the initial velocities were measured under various concentrations from $0.5 \cdot K_m$ to $2 \cdot K_m$ in 40 mM sodium acetate buffer (pH 4.5) at 37 °C. The enzyme concentrations used were made to vary from 3.8 to 38 nM. Liberated glucose was measured by Glucose AR-II.

Results and Discussion

Purification of the enzyme from S. occidentalis culture medium

The purification procedure for the original enzyme is summarized as maltase in Table 1, in which the total activity in the procedure of salting-out with ammonium sulfate is higher than that in the crude enzyme solution. The reason why the activity increased by the procedure is obscure, but it seems reasonable to assume that an unknown inhibitor against maltase might be removed from the culture media by salting-out with ammonium sulfate, although the possibility cannot be ruled out of an inhibitor against glucose oxidase in Glucose AR-II. The

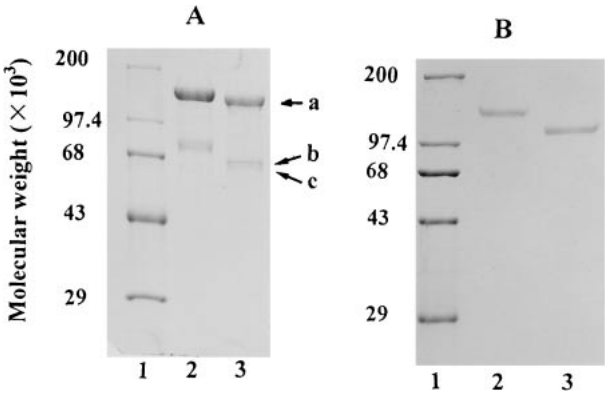

Fig. 2. SDS–PAGE of the Purified Original (A) and the Recombinant (B) Enzymes.

Lane 1 in A and B, molecular marker proteins; lane 2 in A, purified original enzyme; lane 3 in A, endogylcosidase F-treated enzyme; N-terminal amino acid sequences of **a**, **b** and **c**, APAXXIG, APAXXIG, and DGIWA, respectively; lane 2 and 3 in B, recombinant enzyme and endglycosidase F-treated recombinant enzyme.

purified enzyme preparation, which appeared to be chromatographically homogeneous (Fig. 1), was subjected to SDS–PAGE. As shown in Fig. 2, however, the enzyme preparation was separated into two components having the relative molecular masses (M_rs) of 130,000 and 69,000. Separation of the two components as active enzymes was attempted, but was unsuccessful by all chromatographic methods.

The molecular weights of the two components were diminished by treatment with endoglycosidase F, implying that the components were glycoproteins; the major component gave an M_r of 126,000, and the minor one, an M_r of about 66,000. Especially, the minor component indicated two sharp bands. The proteins corresponding to the three bands were transferred on PVDF membrane[21] from SDS–PAGE gel, and then subjected to amino acid sequence analysis. The N-terminal amino acid sequence for the band of the major component (band **a** in lane 3 of Fig. 2A, M_r, 126,000) was identified to be APAXXIG, and those for the upper and lower bands of the minor component (bands **b** and **c** in lane 3 of Fig. 2A, M_r, 66,000), were identified to be APAXXIG and DGIWA, respectively. The sequence of APAXXIG agreed with an internal sequence, 21-

QA**APASSIG**SSAS-, in the primary structures deduced from the DNA sequence and also from the ATCC26076 glucoamylase (*GAM1*) gene.[5,6,10] The -XX- amino acid residues, corresponding to -SS- in the deduced amino acid sequence, could not be identified by protein sequencer, probably because the serine residues were considered to be modified by the sugar chain of the *O*-linked type. The sequence of DGIWA also agreed with an internal sequence, 465-PF**DGIWA**DMN-, in the primary structure of *S. occidentalis* enzyme, as described below. These findings imply that the minor component consisting of two polypeptides may be generated from the major component by limited proteolysis. It is thought that the two polypeptides maintain a three-dimensional structure similar to the main component as the active enzyme even after the limited proteolysis. Consequently, the major and minor components could not be separated into each other without inactivation. The cleavage site corresponds to the loop region between α3 and β4, the backside of $(\beta/\alpha)_8$ barrel (Met408-Asp411 of *E. coli* α-xylosidase), in the three-dimensional structure of *E. coli* α-xylosidase, which is the only available structure in glycoside hydrolase family 31.[13,14] The region might be susceptible to digestion by protease, since it is exposed to the surrounding solution. Such limited proteolytic modifications have been often found in α-glucosidases,[22–24] although their physiological and functional significance has not been ascertained. Therefore, the enzyme preparation containing a small amount of the enzyme modified by limited proteolysis was used itself in the experiment described below.

Molecular cloning of S. occidentalis enzyme DNA

The gene encoding the enzyme was amplified by PCR using primers designed from the *S. occidentalis* ATCC26076 glucoamylase gene (accession no. M60207), and sequenced. The gene from start to stop codon had 2,883 base, encoding a protein of 960 amino acids with a calculated molecular mass of 10,6584.7 Da, and no intron was found. Figure 3 shows the alignment of the only amino acid sequence from the catalytic domain to the C-terminal domain of the *S. occidentalis* ATCC26074 enzyme, together with *S. occidentalis* ATCC26076 glucoamylase and *E. coli* α-xylosidase. It has been reported that, in glucoamylases, two Glu residues function as the acid and base groups in the catalytic reaction.[12] However, the common regions conserving such catalytic Glu residues could not be found in the primary structures of either *S. occidentalis* enzyme. The amino acid sequence showed high similarity with those of α-glucosidases in glycoside hydrolase family 31 enzymes, and two invariant amino acid residues, Asp472 and Asp640, which function as the catalytic nucleophile and acid/base, respectively, are conserved.[25] Therefore, the enzyme must hydrolyze or generate *O*-glucosidic bond through a retaining mechanism. The sequence of the strain ATCC26074 enzyme in the upper stream of Trp325 up to N-terminal Met1 was entirely identical with that of the strain ATCC26076 glucoamylase (accession no. M60207), but in the lower stream a few substitutions were found in the two strains. Almost all mutations are silent, but variants of A1682C, C1927G, and G2575T lead to amino acid substitutions, Tyr547 to Ser, Arg629 to Gly, and Ala845 to Ser. Moreover, a six-nucleotide insertion causes the generating of two amino acid gaps and some amino acid substitution, 436-PGYTVFPDFLAENIQEYMN-454 (ATCC26074) and 436-QVTLFSRFLSRKHSDMD-452 (ATCC26076).

The alignment with *E. coli* α-xylosidase as an enzyme in the family 31 glycoside hydrolases shows that the different residues must be located far from an active site pocket, as stated below (Fig. 3). Gly629 (26074) corresponds to Gly471 (yicI) in *E. coli* α-xylosidase residue, located in the back face of $(\beta/\alpha)_8$ barrel of the three-dimensional structure. Ser547 (26074) is situated in an extra region which has been found only in fungi α-glucosidases, and Ser845 (26074), in the C-terminal domain of the α-xylosidase. The region of Pro436 to Asn454 (26074) is comparable to that of Gln381 to Asp400 (yicI) in *E. coli* α-xylosidase, which is located in a portion of the protruded domain between β3 and α3 of the catalytic domain, and is deviated from the active site. Therefore, these substitutions would not affect the catalytic activity, especially, the reaction mechanism, such as retaining or inverting of product anomer.

Production and purification of the recombinant enzyme

Minicultures of *P. pastoris* transformations of resistance to zeocin were analyzed for α-glucosidase secretion by measuring maltase activity. Supernatants from transformants contained 0.213 ± 0.031 U/ml of α-glucosidase. In large-scale cultures using 200 ml of induction medium, the level of α-glucosidase was about 14.2 mg/l, estimated from the final specific activity of α-glucosidase, 29.5 U/mg. The purified recombinant enzyme was subjected to SDS–PAGE, and no limited proteolysis was observed in the recombinant enzyme (Fig. 2). This implies that protease involved in digestion of the original enzyme was absent in *P. pastoris* culture supernatant. The N-terminus of the recombinant enzyme was APAXXIGXX, the same as that of the original enzyme. That is, the signal peptidases derived from *S. occidentalis* and *P. pastoris* would recognize the substrate in the same manner.

Effects of pH and temperature

The effects of pH and temperature on enzyme activity were examined using maltose as substrate. The pH-optimum of the original enzyme was observed in a broad pH range from 3.5 to 5.0, and that of the recombinant enzyme was also observed in a broad pH range from 3.7 to 4.7. The original enzyme was stable in a range from pH 3.5 to 8.5, but appeared to be labile below pH 3.0.

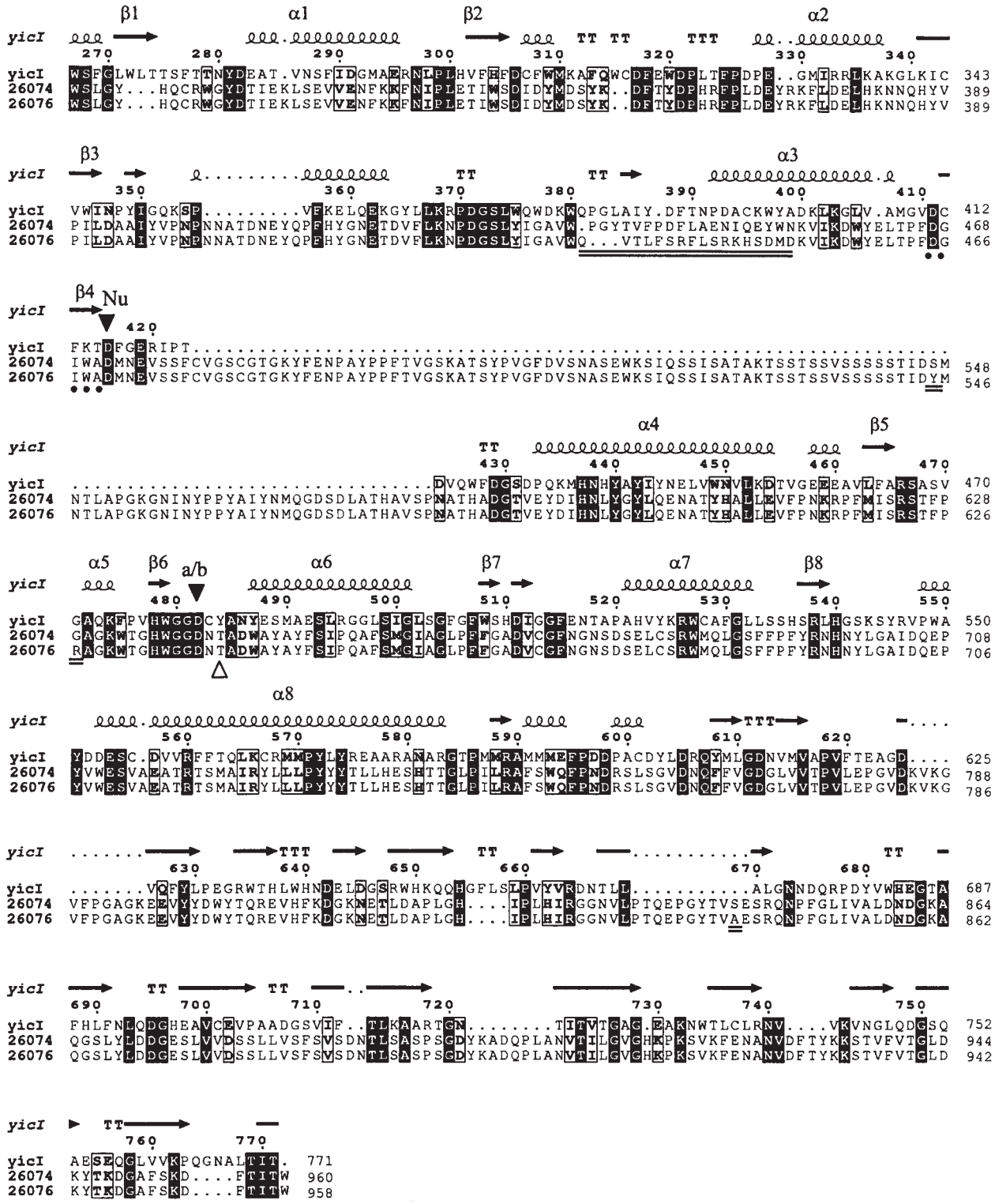

Fig. 3. Multiple Alignment for Catalytic and C-Terminal Domains of Glycoside Hydrolase Family 31 α-Glycosidases, from *E. coli* (yicI), *S. occidentalis* ATCC 26074 (26074), and *S. occidentalis* ATCC 26076 (26076).

Structural features are represented by squiggles (α-helices), arrows (β-strands), and T (β-turns) with element of the $(\beta/\alpha)_8$ barrel core numbered. Residues conserved among the three enzymes are expressed by reversal letters, and similar amino acids are boxed. The catalytic acidic-residues and Thr642 (26074) focused in context are indicated by closed and open triangles, respectively. The polymorphisms between ATCC 26074 and 26076 are displayed by double underlines. The N-terminus of the minor component of the original enzyme is indicated by dashed underline.

Table 2. Kinetic Parameters of Original and Recombinant Enzymes for Hydrolysis of Various Substrates

Substrate	Original			Recombinant		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
Maltose	190	0.096	2,000	210	0.13	1,600
Maltotriose	230	0.064	3,590	330	0.074	4,500
Maltotetraose	180	0.051	3,500	250	0.042	6,000
Maltopentaose	200	0.087	2,300	180	0.043	4,200
Maltohexaose	140	0.078	1,800	190	0.073	2,600
Maltoheptaose	140	0.11	1,270	190	0.083	2,300
Soluble starch	140	0.66	210	220	0.20	1,100
*p*NP α-glucoside	8.0	0.48	16.7	10.2	0.62	16.5
*p*NP α-maltoside	260	0.12	2,170	—*	—*	—*
Kojibiose	33	0.61	54.1	97	0.76	128
Nigerose	59	3.0	19.7	180	3.3	54.5
Isomaltose	130	1.6	81.2	140	1.2	117

*Not measured.

The recombinant enzyme was stable in a range from pH 3.1 to 8.1.

Both of the original and the recombinant enzymes were stable up to 45 °C, but lost the activity completely by incubation at 60 °C for 15 min.

These results show that there are a little or no differences in the effects of pH and temperature between the original and recombinant enzymes.

Substrate specificity of the enzyme

The kinetic parameters for the hydrolysis of various substrates by the original and the recombinant enzymes are listed in Table 2. The tendencies of substrate specificity toward maltooligosaccharides, glucobioses, and soluble starch were similar in the original and the recombinant enzymes. Neither enzyme favored soluble starch, as compared with maltooligosaccharides. The enzymes showed relatively low K_m values for glucobioses such as kojibiose, nigerose, and isomaltose, having α-1,2-, α-1,3-, and α-1,6-glucosidic linkages, respectively. The noteworthy difference between the original and recombinant enzymes was observed in hydrolysis of maltotetraose and maltopentaose, for which the k_{cat}/K_m values of the α-glucosidase from *P. pastoris* were 1.7- and 1.8-fold higher, respectively, than the original enzyme from *S. occidentalis* (Table 2). Additionally, the k_{cat}/K_m values of recombinant enzyme for maltotetraose were higher than that for maltotriose, meaning that, unlike the original enzyme, the recombinant enzyme possess positive subsite affinity at subsite +3. This variation appears to be caused by a difference in post-translational modification with a sugar chain. According to the three-dimensional structure of *E. coli* α-xylosidase and the alignment between the primary structures of the *S. occidetalis* enzyme and *E. coli* α-xylosidase, Asp233-Pro234 and Asn641-Thr642 (ATCC26074) may be situated near the subsite +3 and on the protein surface. Among them, Thr642 could be modified by *O*-glycosylation, and thus the difference in *O*-glycosylation might affect the affinity at subsite +3. It would be significant also to check the substrate specificity to learn whether the enzyme is glucoamylase or α-glucosidase. The distinction between glucoamylase and α-glucosidase has been explained by the substrate specificities for maltooligosaccharides and soluble starch.[12] In the case of α-glucosidase, the molecular activity k_{cat} value for the smallest substrate such as maltose in a series of maltooligosaccharides is not much different from the values for other maltooligosaccharides. In the case of glucoamylase, however, the k_{cat} values are highly dependent on the polymerization degree for substrate, resulting in remarkable differences in the values between maltose and other maltooligosaccharides,[12] and the catalytic efficiency (k_{cat}/K_m) for soluble starch is overwhelmingly large in comparison with any other substrates. The result of substrate specificity analysis strongly suggests that the enzyme is not glucoamylase, but is α-glucosidase.

Anomeric form of glucose liberated from substrates by the enzyme

Determination of the anomeric configuration of the product is significant for understanding the reaction mechanism of glycosidases. The anomeric form of glucose produced from *p*-nitrophenyl α-maltoside and maltotriose by the original enzyme was examined by GLC.[18,19] The chromatograms are shown in Figs. 4A and B. α-Glucose, *p*-nitrophenyl α-glucoside, and a trace amount of β-glucose, which appeared to occur through sponteneous mutarotation of the product α-glucose, were detected as the products from *p*-nitrophenyl α-maltoside (Fig. 4A). From maltotriose, α-glucose, maltose equilibrated between α- and β-anomers, and a small amount of β-glucose, which appears to be somewhat greater in Fig. 4B than in Fig. 4A, were detected as products. In this case, the maltose released from the non-reducing terminal side of maltotriose, in which β-anomer is usually greater than α-anomer, may be additionally cleaved into α- and β-glucose. Therefore, the amount of β-glucose detected in GLC appears

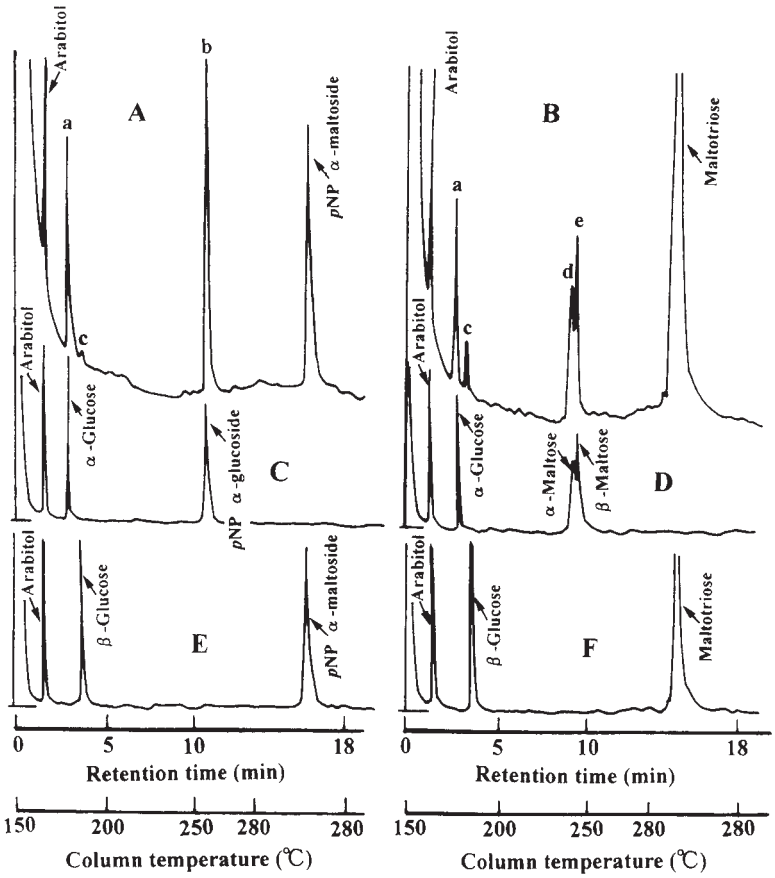

Fig. 4. Gas-Liquid Chromatograms of TMS Derivatives of Products from *p*-Nitrophenyl α-Maltoside (A) and Maltotriose (B) by *Shwanniomyces occidentalis* Enzymes.

C, D, E, and F are chromatograms of standard sugars. TMS derivative of arabitol was used as the internal standard. Column temperature was elevated at a rate of 10 °C/min from 150 to 270 °C, and kept constant at 280 °C. Symbols a, b, c, d, and e corresponds to α-glucose, *p*-nitrophenyl α-glucoside, α-maltose, and β-maltose, respectively.

to be over that of β-glucose occurring through spontaneous mutarotation from α-glucose. These results imply that the anomeric configuration of the glucose produced from *p*-nitrophenyl α-maltoside and maltotriose is α-glucose, and that the enzyme is a typical α-glucosidase.

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