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Purification and Characterization of a New Type of α-Glucosidase from *Paecilomyces lilacinus* That Has Transglucosylation Activity to Produce α-1,3- and α-1,2-Linked Oligosaccharides

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A fungus producing an α-glucosidase that synthesizes α-1,3- and α-1,2-linked glucooligosaccharides by transglucosylation was isolated and identified as *Paecilomyces lilacinus*. The cell-bound enzyme responsible for the synthesis was extracted by suspension of mycelia with 0.1 M phosphate buffer (pH 8.0), and the extract was purified. The molecular weight and the isoelectric point were estimated to be 54,000 and 9.1, respectively. The enzyme was most active at pH 5.0 and 65°C. The enzyme hydrolyzed maltose, nigerose, and kojibiose. The enzyme also hydrolyzed soluble starch and amylose with the rate toward maltose. *p*-Nitrophenyl α-glucoside and isomaltose were not good substrates. The enzyme had high transglucosylation activity to synthesize oligosaccharides containing α-1,3- and α-1,2-linkages. At an early stage of the reaction, considerable maltotriose, 4-*O*-α-nigerosyl-D-glucose, and 4-*O*-α-kojibiosyl-D-glucose were synthesized. Afterwards, nigerose and kojibiose were accumulated gradually with glucose as an acceptor.

Key words: α-glucosidase; *Paecilomyces lilacinus*; kojibiose; nigerose; transglucosylation

α-Glucosidases (EC 3.2.1.20, α-D-glucoside glucohydrolase) are typical exo-type carbohydrolases, which liberate α-glucose from the nonreducing terminals of substrates such as maltose, isomaltose, and phenyl α-glucoside. The enzymes transfer glucosyl groups from the substrates onto acceptors, but the profiles of the products depend on the enzymes. An enzyme from *Aspergillus niger*, for instance, yields mostly α-1,6-linked oligosaccharides such as isomaltose, isomaltotriose, and panose when glucose or maltose are the acceptors.[1)] A *Penicillium purpurogenum* enzyme synthesizes mostly α-1,4-linked maltotriose from maltose.[2)] There are few studies of the α-glucosidases that synthesize oligosaccharides containing α-1,2-, or α-1,3-glucosidic linkages (Fig. 1). Chiba *et al.*[3)] reported that the buckwheat enzyme produces nigerose (α-1,3-linked glucobiose) and

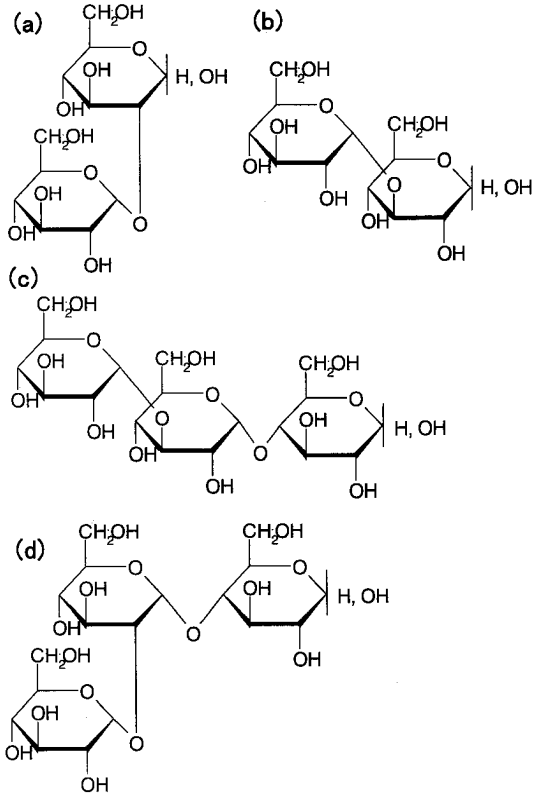

Fig. 1. Oligosaccharides Containing α-1,3- and α-1,2 Glucosidic Linkages.
(a) Kojibiose, (b) nigerose, (c) 4-*O*-α-nigerosyl-D-glucose, and (d) 4-*O*-α-kojibiosyl-D-glucose.

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Abbreviations: ABEE, *p*-aminobenzoic acid ethyl ester; IEF, isoelectric focusing

kojibiose (α-1,2-linked glucobiose) during reaction with soluble starch. An enzyme from an *Acremonium* sp. was found to synthesize nigerose and 4-*O*-α-nigerosyl-D-glucose from maltose.[4] These two oligosaccharides containing α-1,3-glucosidic linkages have several practical uses: they make the salty taste of foodstuffs more mellow, and prevent anthocyanins, color substances in food materials, from oxidating or discoloring.[5] Immunopotentiating the T-helper 1-like immune response may be their physiological activity.[6,7] Two oligosaccharides containing an α-1,2-glucosidic linkages, kojibiose and 4-*O*-α-kojibiosyl-D-glucose, synthesized by kojibiose phosphorylase,[8] have low cariogenicity[9] and digestibility.[10] In view of the practical usefulness of these rare oligosaccharides and of the few reports on the α-glucosidases that synthesize them, we started screening of microbes for the α-glucosidase. The most difficult problem was the separation and identification of the oligosaccharides with different linkages in the many samples obtained by the screening. To solve the problem, we used a method described in our preceding paper:[11] the saccharides were reacted with *p*-aminobenzoic acid ethyl ester (ABEE), and the derivatives were then analyzed by HPLC. Suitable HPLC conditions were found for identifying a number of α-linked glucooligosaccharides including ones containing α-1,2- and 1,3-glucosidic linkages.[11] The method (termed ''ABEE conversion'' in this study) allowed the simultaneous analysis of complex mixtures of reducing saccharides even in microbial culture broth or enzyme reaction mixtures. We used this method to find microorganisms that produces oligosaccharides with α-1,2- or α-1,3-glucosidic linkages. A fungus identified later as *Paecilomyces lilacinus* was obtained as a producer of an α-glucosidase that synthesized much oligosaccharides containing α-1,3-glucosidic linkage from maltose by a transfer reaction at an early stage. In addition, the enzyme accumulated oligosaccharides containing α-1,2-glucosidic linkage during the prolonged reaction. This paper describes the purification and some properties of this new α-glucosidase.

Materials and Methods

Materials. Glucose, maltose, trehalose, *p*-nitrophenyl α-glucoside, and soluble starch were purchased from Nacalai Tesque Chemical, Inc. (Kyoto, Japan). Kojibiose, nigerose, isomaltose, maltotriose, maltotetraose, maltohexaose and glycogen (from oyster) were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Amylose, (Hayashibara Biochemical Laboratories, Inc., Okayama, Japan), maltopentaose and α-cyclodextrin (Ensuiko Sugar Refining Co., Ltd., Yokohama, Japan) were obtained from commercial sources. 4-*O*-α-Nigerosyl-D-glucose was prepared by a method described in a previous paper.[11] 4-*O*-α-Kojibiosyl-D-glucose was the gift of the Hayashibara Biochemical Laboratories, Inc. Glucoamylase (Glutase 6000) was kindly supplied by Hankyu Bioindustry Co., Ltd. (Osaka). Potato dextrose was purchased from (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). All other chemicals were of the highest grade commercially available.

Screening of microorganisms. Microorganisms were isolated from soil samples in agar medium plates containing 0.2% maltose, 0.05% NH_4NO_3, 0.05% K_2HPO_4, 0.01% KCl, 0.01% $MgSO_4$, and 1.5% agar (pH 7.0). The isolates were cultivated aerobically at 30°C for 72 h in a liquid medium (10 ml) containing 1% maltose, 1% polypepton, 0.05% K_2HPO_4, 0.01% KCl, and 0.01% $MgSO_4$ (pH 7.0). The culture broth (50 μl) was incubated with 50 μl of 20% maltose containing 0.01% NaN_3 at 40°C for 72 h. After the mixtuere was boiled, the transglucosylation products were checked by TLC and the ABEE conversion as described below.

Cultivation of Paecilomyces lilacinus. The selected fungal isolate, *Paecilomyces* sp., was used to inoculate 5 ml of potato dextrose agar in a slant containing 0.4% potato extract, 2% glucose, and 1.5% agar, and incubated at 30°C for 2 days, by which time mycelia had grown. Sterilized water (15 ml) was added to the slant and the test tube was vortexed vigorously. A 0.5 ml portion of the suspension of mycelia was withdrawn and added to 150 ml of a medium containing 5% maltose, 1% peptone, 0.05% K_2HPO_4, 0.01% KCl, and 0.01% $MgSO_4$, adjusted to pH 7.0, in 500 ml shaking flasks. After cultivation for 3 days with reciprocating shaking at 80 rpm and 35°C, the mycelia were harvested by being filtering with Toyo Roshi filter paper No. 2. About 500 g (wet weight) of mycelia was obtained from 10-liter cultures.

Analytical methods. TLC was done with a TLC plate (Kiesel Gel 60, Merck, Darmstadt, Germany) and a solvent system of ethyl acetate-acetic acid-water (3:1:1, v/v). The spot of sugars was detected by spraying of the plate with 50% (w/w) sulfuric acid in methanol and its being heated at 150°C. ABEE conversion was done by the method of Yasuno *et al.*[12] with an ABEE labeling kit (Seikagaku Corporation, Tokyo). ABEE derivatives were analyzed by HPLC under conditions optimized for analysis of α-linked glucooligosaccharides as described in our previous paper[11] with the temperature at 30°C instead of 25°C. Native PAGE was done with an 7.5% acrylamide gel in β-alanine-acetic acid buffer (pH 4.5) as described by Reisfeld *et al.*[13] SDS-PAGE was done in 7.5% (w/v) polyacrylamide gel in the presence of 0.1% SDS as described by Laemmli.[14] Protein was

stained with Quick-CBB (Wako). Deglycosylation was done by the incubation of *N*-glycanase (Glycopeptidase F, Takara Shuzo Co., Ltd., Kyoto) with the heat-denatured enzyme with 0.5% SDS at 40°C for 24 h.

The isoelectric point of the enzyme was estimated by isoelectric focusing (IEF) on a Phastgel IEF 3-9 with the Phastsystem (Amersham-Pharmacia Biotech K.K., Tokyo).

Enzyme assay. α-Glucosidase activity was assayed at 50°C in a reaction mixture (2.5 ml) containing 20 mM maltose and 50 mM sodium acetate buffer (pH 5.5). The optimum pH of the enzyme was at about 5.0 in this experiment but the enzyme assay was done at 5.5 in this paper. The reaction was stopped by the addition of 125 μl of 0.2 M NaOH and the sample was neutralized with 125 μl of 0.2 M acetic acid. The glucose liberated was measured with a Glucose B-test Wako kit (Wako). One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 μmol of maltose (2 μmol of glucose as product) per minute under these conditions. Protein was measured by the method of Lowry *et al.*[15] with bovine serum albumin as the standard.

Effects of pH and temperature. The effects of pH on the activity were assayed at 50°C in a reaction mixture containing maltose (20 mM), the enzyme (0.01 unit/ml), and 100 mM of Britton-Robinson buffer (pH 2.8–9.1). The pH stability of the enzyme was analyzed with enzyme that had been incubated in Britton-Robinson buffer (pH 1.5–10.2) at 4°C for 24 h. The effects of temperature were assayed at various temperatures in 50 mM sodium acetate buffer (pH 5.5). The temperature stability of the enzyme was analyzed with the enzyme which had been incubated at various temperatures for 20 min.

Effects of various metal ions and chemicals. The enzyme (100 mU/ml) was incubated with various metal ions (Ca^{2+}, Hg^{2+}, Mg^{2+}, Na^{2+}, Mn^{2+}, or Zn^{2+}, all of 1.0 mM) or EDTA (1.0 mM) at 40°C for 1 h. The mixtures were diluted 100 times with 50 mM acetate buffer (pH 5.5) and the residual activity was measured under the standard assay conditions.

Analysis of transglucosylation products. Transglucosylation products were measured in mixtures (2.5 ml) containing 30% (w/v) maltose, and 50 mM sodium acetate buffer (pH 5.5) kept at 50°C for 24 h. After such mixtures were boiled for 10 min, the oligosaccharides in the reaction mixture were put through ABEE labeling and analyzed by HPLC.

Purification of α-glucosidase from Paecilomyces lilacinus.

Step 1: Extraction. The enzyme of the fungus (500 g, wet weight) was solubilized by suspension of mycelia in 400 ml of 0.1 M phosphate buffer, pH 8.0, at 5°C for 24 h with gentle stirring. The suspension was centrifuged at 20,200 × *g* and the supernatant was collected. This procedure repeated two more times and the extracts were combined. The solution was dialyzed against 50 mM sodium acetate buffer (pH 5.5).

Step 2: SP-Toyopearl column chromatography. The dialyzed enzyme solution (1,570 ml) was put on an SP-Toyopearl 550M column (3 × 25 cm, Tosoh corporation) equilibrated with 50 mM acetate buffer (pH 5.5). The enzyme was eluted with a linear gradient of NaCl concentrations from 0 to 0.75 M (each, 600 ml). The active fractions (eluted at about 0.5 M) were collected (80 ml). The specific activity of this active fraction was 3.02 units/mg protein, 62-fold that of the crude extract.

Step 3: Butyl-Toyopearl column chromatography. Solid $(NH_4)_2SO_4$ was added to the enzyme solution till a concentration of $(NH_4)_2SO_4$ become 1.5 M. The solution was put on a Butyl-Toyopearl 650M column (1.8 × 25 cm, Tosoh) equilibrated with 20 mM acetate buffer (pH 5.5) containing 1.5 M $(NH_4)_2SO_4$. The enzyme was eluted with a linear gradient of $(NH_4)_2SO_4$ concentrations from 1.5 to 0 M (each, 500 ml). The active fractions (eluted at about 0.75 M) were collected (30 ml) and concentrated with polyethylene glycol (mean molecular weight, 2×10^4). The specific activity of this active fraction was 10.0 units/mg protein.

Step 4: Sephacryl S-100 column chromatography. The concentrated sample (3.0 ml) was put on a Sephacryl S-100 column (3 × 100 cm, Amersham-Pharmacia) with 20 mM acetate buffer (pH 5.5) containing 0.1 M NaCl. The enzyme was eluted with the same buffer at a flow rate of 0.2 ml/min. The active fractions (75 ml) were collected. The specific activity of this active fraction was 12.2 units/mg protein.

Estimation of molecular weight by gel filtration. The enzyme solution (0.2 ml) was put on a Superdex 200 HR (Amersham-Pharmacia) column equilibrated with 50 mM acetate buffer (pH 5.5) containing 0.1 M NaCl, and eluted. The molecular weight of the enzyme was estimated by comparison of the retention time with the times for standard proteins, lysozyme (14,500), chymotrypsin (25,000), ovalbumin (45,000), and bovine serum albumin (67,000).

Results and Discussion

Screening and identification of the fungal isolate that produces α-glucosidase

Of the microorganisms grown on the agar plate containing maltose, about 500 strains were selected

on the basis of their production of transglucosylation products from maltose. The products were further analyzed by ABEE conversion. An isolate, HKS-124, was found to synthesize considerable nigerose and 4-*O*-α-nigerosyl-D-glucose. The strain was a fungus and grew well on 2% potato dextrose, 2% malt extract (2% malt extract and 2% agar, pH 5.6), and 2% Czapek-yeast agar media (3% sucrose, 0.5% yeast extract, 0.01% K_2HPO_4, 1.5% agar, 0.1% Czapek concentrate, which contained $NaNO_3$ (30%), KCl (5.0%), $MgSO_4 \cdot 7H_2O$ (5.0%), $FeSO_4 \cdot 7H_2O$ (0.1%), $ZnSO_4 \cdot 7H_2O$ (0.1%), and $CuSO_4 \cdot H_2O$ (0.05%); pH 6.2), forming colonies at least 60 mm across during 2 weeks of culture at 25°C. The color of the colonies was white in the first several days and gradually became purple about 1 week of cultivation on potato dextrose. The fungus had typical penicillus structure, stretching lemon-shaped conidia from slender necking sterigmata. The strain was identified as *Paecilomyces lilacinus* on the basis of detailed morphological observations and analysis of the 28S-rDNA sequence by NCIMB Japan (Shizuoka, Japan).

Cultivation

Paecilomyces lilacinus HKS-124 grew well in liquid media containing glucose, maltose, sucrose, lactose, dextrin, or starch as carbon sources. Among the saccharides, the addition of maltose at 5% (w/v) gave the most α-glucosidase activity (not shown). Peptone (1.0%) also was effective as a nitrogen source in terms of enzyme production.

The activities in the broth supernatant and cell extract of the mycelia which was prepared by sonic disruption, are shown in Table 1. The enzyme activity in the extract reached a maximum at the 4th day of cultivation and then decreased. On the other hand, the enzyme activity in the supernatant had risen each time it was assayed. The enzyme seemed to leak from the mycelia into the culture broth as cultivation continued, and the enzyme could easily be extracted from mycelia so the enzyme seemed to be cell-bound and was probably liberated by autolysis.

During the cultivation of the fungus on maltose, transglucosylation products including nigerose and 4-*O*-α-nigerosyl-D-glucose had accumulated in the medium by day 3. This results suggested that not only the cell-free enzyme but also the mycelia could be used for the production of oligosaccharides with α-1,3-glucosidic linkages.

Purification of the cell-bound α-glucosidase from Paecilomyces lilacinus

The enzyme bound to the mycelia of *Paecilomyces lilacinus* was easily extracted by the following procedure: washed mycelia (500 g) were incubated with 0.1 M phosphate buffer (pH 8.0) at 5°C for 24 h with occasional mixing. This procedure was repeated two more times, and 80% of the activity was recovered compared with the solubilization with sonication. Buffers of lower pHs than 5 were not effective for the extraction and those of higher pHs than 9 slightly inactivated the enzyme. Thus the extraction was done at about pH 8. The specific activity of the enzyme preparation thus prepared was almost twice that obtained by sonic disintegration of the mycelia.

From the extract, the enzyme was purified through three steps of column chromatography and the specific activity was increased 255-fold with a yield of 21% (Table 2). The purified enzyme migrated as a single protein band in native PAGE (Fig. 2(A)). However, in SDS-PAGE, the enzyme gave two major protein bands (molecular weights, 60,000 and 54,000) and one minor band (52,000). When the enzyme was treated with *N*-glycanase, these heterogeneous bands disappeared and a major protein band appeared at the molecular weight of 52,000 (Fig. 2(B)). By gel filtration on a Superdex 200 HR column, the molecular weight was estimated to be 54,000. These results indicated that the enzyme was a glycoprotein without subunits and the heterogeneity may have arisen from a difference in molecular weights of carbohydrate chains. The isoelectric point of the *Paecilomyces* enzyme was about 9.1 by IEF. The optimum pH and temperature of the enzyme were about pH 5.0 and 65°C. The stability of the enzyme forward pH and temperature was between 3 to at least 9 and below 60°C. The enzyme retained 70% of activity at 65°C. The enzyme was inactivated by Hg^{2+} and Zn^{2+} (residual activity; 19% and 48%, respectively). EDTA and metal ions tested had neither inhibition nor activation effects.

Table 1. α-Glucosidase Activity and Growth of *Paecilomyces lilacinus*

Cultivation (days)	Enzyme activity (units/ml)		Wet weight of mycelia (mg/ml)
	Supernatant	Cell extract*	
3	1.46	31.8	23.8
4	8.27	51.7	42.8
5	15.8	48.5	45.9
6	25.6	33.2	42.0

* Activity per milliliter of culture broth.

Table 2. Purification of Intracellular α-Glucosidase from *Paecilomyces lilacinus*

Procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
Extraction	5830	284	0.0487	100	1.0
SP-Toyopearl	57.9	175	3.02	61.6	62.0
Butyl-Toyopearl	8.32	83.0	9.98	29.2	205
Sephacryl S-100	4.85	60.1	12.4	21.2	254

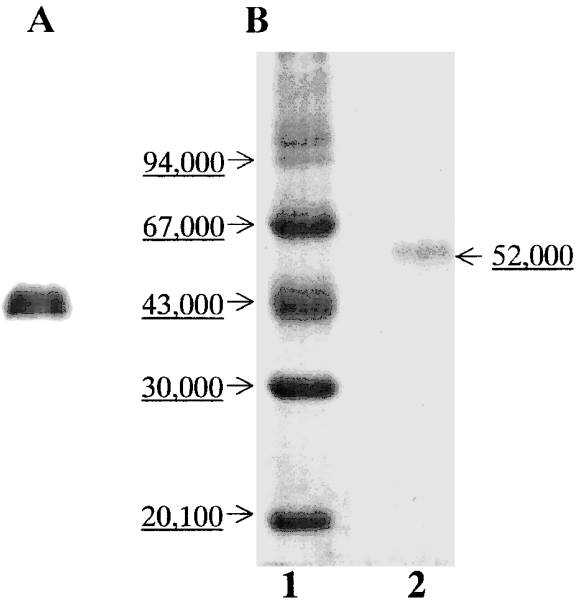

Fig. 2. Native and SDS-PAGE of the *Paecilomyces lilacinus* α-Glucosidase.

(A) Native PAGE of the purified enzyme. (B) SDS-PAGE of the deglycosylated enzyme. Lane 1, marker proteins (from top): phosphorylase *b* (molecular weights: 94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactoalbumin (14,400). Lane 2, deglycosylated enzyme.

Table 3. Substrate Specificity of *Paecilomyces lilacinus* α-Glucosidase[1]

Substrate	Relative activity[2] (%)	K_m (mM)	V_{max} (mM/min)
Maltose	100	0.16	51.3
Maltotriose	26.1	n.d.[3]	
Maltotetraose	11.3	n.d.	
Maltopentaose	30.1	n.d.	
Maltohexaose	46.5		
Kojibiose	85.4	1.44	56.2
Nigerose	183	0.14	54.6
Isomaltose	0.9	0.86	0.4
Trehalose	0	n.d.	
Sucrose	0	n.d.	
p-Nitrophenyl α-glucoside	0.35	n.d.	
Soluble starch	69.9	2.8[4]	813[5]
Amylose	80.5	n.d.	
Glycogen	9.60	n.d.	

[1] Enzyme activity was assayed at pH 5.5 but the optimum pH of the enzyme was at about 5.0.
[2] The enzyme (0.01 U) was incubated with an oligosaccharide (10 mM), starch, amylose, glycogen (0.4%), and *p*-nitrophenyl α-glucoside (1 mM) at 50°C for 10 min. The liberated glucose was measured by the glucose oxidase method as described in Materials and Methods. The following were inert as substrates: dextran, cellobiose, α-cyclodextrin, pullulan, inullin.
[3] n.d., "not determined".
[4] mg/ml.
[5] mg/ml·min.

The enzyme from an *Acremonium* sp., an enzyme that produces oligosaccharides containing α-1,3-linkages ("nigerooligosaccharides"[6]), has a larger molecular weight (240,000 by gel filtration) than our *Paecilomyces* enzyme, and a ternary structure composed of subunits of two sizes (128,000 and 53,000 by SDS-PAGE).[16] Moreover, the isoelectric point of our enzyme was different from that of the *Acremonium* enzyme (pI 2.7).[16] On the basis of these properties, we expected the protein structures of the *Paecilomyces* and *Acremonium* enzymes to be quite different.

Substrate specificities of the enzyme

The α-glucosidase was reacted with various substrates and the hydrolysis velocities relative to the velocity on maltose were compared (Table 3). Enzyme activity was assayed at pH 5.5 but the optimum pH of the enzyme was near 5.0. The enzyme preferably hydrolyzed nigerose, maltose, and kojibiose. Glucans such as soluble starch and amylose also were hydrolyzed to a considerable extent. Activities toward a series of maltooligosaccharides were generally less than activities toward glucobioses. The results suggested that the enzyme was a α-glucosidase. However, less *p*-nitrophenyl α-glucoside and isomaltose were hydrolyzed less than the above glucobioses. α,α-1,1-Linked trehalose and α,β-1,2-linked sucrose were not degraded at all.

The pattern of substrate specificities indicated that the enzyme acted not only on homogeneous oligosaccharides but also on glucans such as starch and glycogen. The fungal α-glucosidases from *Aspergillus niger*,[1] *Aspergillus oryzae*,[17] and an *Acremonium* sp.[16] had low or no activity toward glucans. An enzyme obtained from the mycelia of *Mucor javanicus*[18] has as much activity as the *Paecilomyces* enzyme although it produced no oligosaccharides containing α-1,2- or α-1,3-linkages.

Of the α-linked glucobioses, kojibiose and nigerose were hydrolyzed as fast as maltose. Isomaltose was decomposed much more slowly than maltose. K_m for maltose, kojibiose, nigerose, and isomaltose were 0.16, 1.44, 0.14, and 0.86 mM respectively. *Mucor javanicus* α-glucosidase has similar specificity; relative activities toward 1,2-, 1,3-, 1,4-, and 1,6-linked glucobioses are 25, 95, 100, and 0%, respectively.[18] The *Paecilomyces* enzyme had much higher relative activity forward nigerose (183%). Such hydrolysis specificity may be related to the production of more oligosaccharides containing α-1,3-linkages in the transglucosylation reaction as described below.

Transglucosylation activity of the enzyme

The purified enzyme was allowed to react with 30% (w/v) maltose and the products were analyzed by ABEE conversion. Figure 3 shows a typical chromatogram of transglucosylation products by the ABEE conversion. The retention time of each peak was compared with the reaction time of authentic

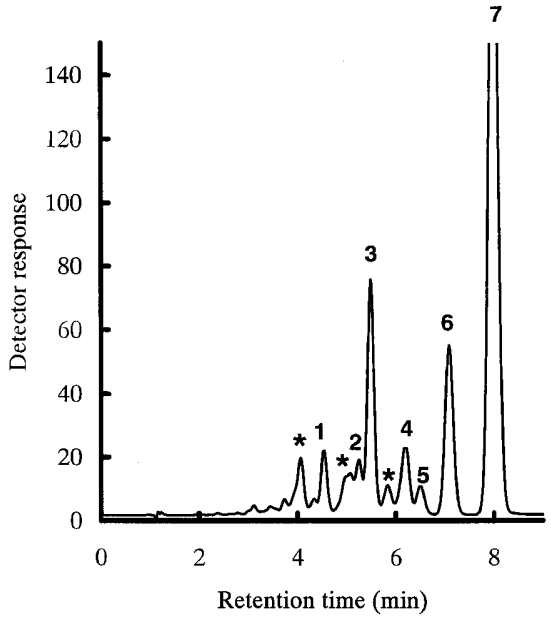

Fig. 3. Chromatogram of Transglucosylation Products from Maltose by *Paecilomyces lilacinus* α-Glucosidase.

A reaction mixture (1 ml) containing 30% (w/v) maltose and 0.23 unit of the enzyme in 20 mM acetate buffer (pH 5.5) was incubated at 50°C for 24 h. Saccharides in the reaction mixture were analyzed by the ABEE conversion described in Materials and Methods. Peaks of 1, 2, 3, 4, 5, 6, and 7 were 4-*O*-α-kojibiosyl-D-glucose, kojibiose, nigerose, 4-*O*-α-nigerosyl-D-glucose, maltotriose, maltose, and glucose respectively. The peaks with an asterisk are unknown reducing saccharides. Note that the peak area almost indicates molar concentrations of each saccharide. Non-reducing saccharides, if any, could not be detected by this method.

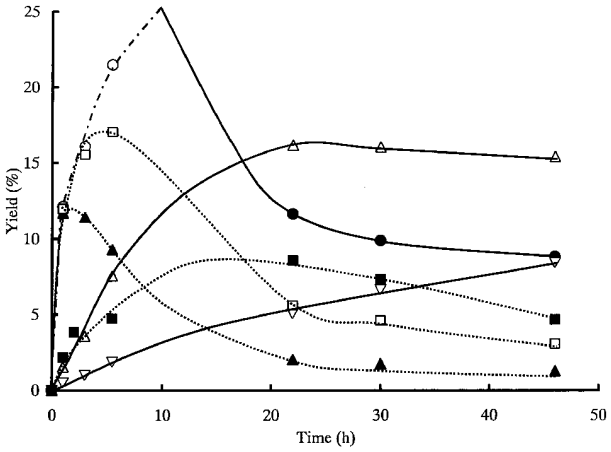

Fig. 4. Changes in Time during Transglucosylation.

The transglucosylation reaction was started by the addition of 0.5 ml of enzyme solution (12 units of enzyme activity) into 0.5 ml of a maltose solution (0.6 g/ml) in 40 mM acetate buffer (pH 5.5) at 50°C. The final concentrations of maltose and the enzyme were 30% (w/v) and 10 units/g maltose, respectively. Samples (10 μl) were taken at various times and the reaction stopped by boiling of the mixture for 10 min. The sample was derivatized with ABEE and analyzed by HPLC. The Y axis shows the yield of oligosaccharides from 100 g of the substrate. The composition of the saccharides is plotted. ○, glucose; ●, maltose; △, nigerose; ▽, kojibiose; ▲, maltotriose; □, 4-*O*-α-nigerosyl-D-glucose; ■, 4-*O*-α-kojibiosyl-D-glucose.

saccharides under two different conditions for HPLC (acetonitrile concentration in the mobile phase, 10.5%, or 12%) to identify the components of the products. Among the reaction products, at least three trisaccharides, 4-*O*-α-kojibiosyl-D-glucose, 4-*O*-α-nigerosyl-D-glucose, and maltotriose, were synthesized with maltose as an acceptor, although panose was not formed. As disaccharides, nigerose and kojibiose also were formed, and isomaltose was scarcely detected (less than 1% of all disaccharides). Disaccharides were separated from the reaction mixture by activated carbon column chromatography and the mixture was analyzed by ^{13}C NMR. Comparison of the spectrum with the spectra of authentic nigerose and kojibiose showed that the disaccharides mixture contained these two disaccharides (not shown). There were no signals corresponding to α-α trehalose. There were several unidentified peaks, presumed to be tri- or tetrasaccharides. Trisaccharides such as nigerotriose (3^2-*O*-α-D-glucosyl-D-nigerose) and kojitriose (2^2-*O*-α-D-glucosyl-D-kojibiose) and tetrasaccharides other than maltotetraose could not be identified because of standard saccharides were not available.

Figure 4 shows the course of transglucosylation catalyzed by the enzyme with 30% maltose as the substrate. In less than 10 h of the start of the reaction, the synthesis of 4-*O*-α-nigerosyl-D-glucose and maltotriose reached the maximum yield of about 17% and 12%, respectively, and afterward, these substances were degraded rapidly. 4-*O*-α-Kojibiosyl-D-glucose was produced more slowly to give an approximate yield of 8.6% at 22 h, and then it decreased slightly. Nigerose accumulated (the yield at 22 h, 16%) with the decrease of maltose and the trisaccharides. Kojibiose also was produced to reach an yield of 8.4% at 46 h. During prolonged incubation (72 h), the yield of nigerose decreased slightly and that of kojibiose increased further to 12% and 14%, respectively (not shown).

Nigerose and kojibiose seemed to be formed from three reactions. In the first one, the disaccharides were produced by transglucosylation of glucose with the maltose remaining in the reaction system as a donor. The second one was also a transglucosylation, although the enzyme transferred glucose residues from trisaccharides such as 4-*O*-α-nigerosyl-D-glucose, 4-*O*-α-kojibiosyl-D-glucose, or maltotriose, which also were transfer products, to glucose. The third one was reverse hydrolysis reaction of glucose to form disaccharides. The glucose concentration may be not so high at an early reaction stage as reverse reaction can occur, although it is possible to form nigerose and kojibiose in reverse reaction, especially after long reaction time.

Chiba *et al.*[3] reported that an α-glucosidase from

buckwheat synthesizes nigerose and kojibiose from starch. In addition, the plant enzyme accumulates isomaltose. The *Paecilomyces* enzyme was different in causing no synthesis of 1,6-glucosidic linkages. The enzyme from the *Acremonium* sp. was the only enzyme that produces oligosaccharides containing α-1,3-glucosidic linkages from maltose or maltooligosaccharides.[4] The *Paecilomyces* enzyme reported here was similar to the *Acremonium* enzyme in its production of oligosaccharides containing α-1,3-glucosidic linkages. Our enzyme can be clearly distinguished from the *Acremonium* enzyme by the production of oligosaccharides containing α-1,2-glucosidic linkages in addition to ones containing α-1,3-glucosidic linkages. To our knowledge, there are no reports describing microbial α-glucosidases that synthesize considerable amounts of oligosaccharides containing both α-1,2- and α-1,3-glucosidic linkages in transglucosylation reactions.

Kojibiose and nigerose are contained in sake, honey, and beer, but at low concentrations. The α-glucosidase of *Paecilomyces lilacinus* may be useful as a new sweetener containing ''rare'' disaccharides from a maltose or starch hydrolyte.

References

1) Tsujisaka, Y., and Fukumoto, J., Studies on maltose splitting enzymes of moulds. Part II. On the reaction mechanism of the crystalline transglucosidase of *Aspergillus niger*. *Nippon Nogeikagaku Kaishi* (in Japanese), **37**, 747–752 (1963).
2) Yamasaki, Y., Suzuki, Y., and Ozawa, J., Purification and properties of α-glucosidase from *Penicillium purpurogenum*. *Agric. Biol. Chem.*, **40**, 669–676 (1976).
3) Chiba, S., Kimura, A., Kobori, T., and Saitoh, K., Quantitative determination of disaccharides produced from soluble starch through transglucosylation of buckwheat α-glucosidase. *J. Jpn. Soc. Starch Sci.*, **32**, 213–216 (1985).
4) Konishi, Y., and Shindo, K., Production of nigerose, nigerosyl glucose, and nigerosyl maltose by *Acremonium* sp. S4G13. *Biosci. Biotechnol. Biochem.*, **61**, 439–442 (1997).
5) Yamamoto, K., Urushibata, T., Konishi, Y., and Tsujii, M., Japan Kokai Tokkyo Koho, 1891001 (June 11, 2000).
6) Murosaki, S., Muroyama, K., Yamamoto, Y., Kusaka, H., Liu, T., and Yoshikai, Y., Immunopotentiating activity of nigerooligosaccharides for the T helper 1-like immune response in mice. *Biosci. Biotechnol. Biochem.*, **63**, 373–378 (1999).
7) Murosaki, S., Muroyama, K., Yamamoto, Y., Liu, T., and Yoshikai, Y., Nigerooligosaccharides augments natural killer activity of hepatic mononuclear cells in mice. *Int. Immunopharmacol.*, **2**, 151–159 (2002).
8) Chaen, H., Yamamoto, T., Nishimoto, T., Nakada, T., Fukuda, S., Sugimoto, T., Kurimoto, M., and Tsujisaka, Y., Purification and characterization of a novel phosphorylase, kojibiose phosphorylase, from *Thermoanaerobium brockii*. *J. Appl. Glycosci.*, **46**, 423–429 (1999).
9) Shiota, M., and Yatake, T., Japan Tokkyo Koho, 3022958 (Jan. 31, 1991).
10) Chaen, H., Nishimoto, T., Nakada, T., Fukuda, S., Kurimoto, M., and Tsujisaka, Y., Enzymatic synthesis of kojioligosaccharides using kojibiose phosphorylase. *J. Biosci. Bioeng.*, **92**, 177–182 (2001).
11) Kobayashi, I., Yasuno, S., Hashimoto, H., Ikura, K., Nakano, H., and Kitahata, S., Analysis of positional isomers of α-linked glucooligosaccharides by *p*-aminobenzoic ethyl ester-conversion method. *J. Appl. Glycosci.*, **49**, 461–468 (2002).
12) Yasuno, S., Murata, T., Kokubo, K., Yamaguchi, T., and Kamei, M., Two-mode analysis by high-performance liquid chromatography of *p*-aminobenzoic ethyl ester-derivatized monosaccharides. *Biosci. Biotechnol. Biochem.*, **61**, 1944–1946 (1997).
13) Reisfeld, R. A., Lewis, U. J., and Williams, D. E., Disk electrophoresis of basic proteins and properties of polyacrylamide gels. *Nature*, **195**, 281–283 (1961).
14) Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970).
15) Lowry, O. H., Rosebrough, J. N., Farr, A. L., and Randall, R. J., Protein measurement with the Folin Phenol Reagent. *J. Biol. Chem.*, **193**, 265–275 (1951).
16) Konishi, Y., and Shindo, K., Japan Kokai Tokkyo Koho, 59559 (Mar. 7, 1995).
17) Sugawara, S., Nakamura, Y., and Shimomura, T., Substrate specificity and some properties of crystalline mold maltase. *Agric. Biol. Chem.*, **25**, 358–361 (1961).
18) Yamasaki, Y., Miyake, T., and Suzuki, Y., Properties of crystalline α-glucosidase from *Mucor javanicus*. *Agric. Biol. Chem.*, **37**, 251–259 (1972).