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Purification and Characterization of a Novel Fungal α-Glucosidase from *Mortierella alliacea* with High Starch-hydrolytic Activity

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The fungal strain *Mortierella alliacea* YN-15 is an arachidonic acid producer that assimilates soluble starch despite having undetectable α-amylase activity. Here, a α-glucosidase responsible for the starch hydrolysis was purified from the culture broth through four-step column chromatography. Maltose and other oligosaccharides were less preferentially hydrolyzed and were used as a glucosyl donor for transglucosylation by the enzyme, demonstrating distinct substrate specificity as a fungal α-glucosidase. The purified enzyme consisted of two heterosubunits of 61 and 31 kDa that were not linked by a covalent bond but stably aggregated to each other even at a high salt concentration (0.5 M), and behaved like a single 92-kDa component in gel-filtration chromatography. The hydrolytic activity on maltose reached a maximum at 55°C and in a pH range of 5.0–6.0, and in the presence of ethanol, the transglucosylation reaction to form ethyl-α-D-glucoside was optimal at pH 5.0 and a temperature range of 45–50°C.

Key words: arachidonic acid; carbohydrate hydrolase; α-glucosidase; *Mortierella alliacea*; starch

Microbial oils rich in polyunsaturated fatty acids may replace marine fish oils as a food material because of certain advantages, which include ease of processing and a stable supply. Several lower fungi, algae, and marine diatoms are commercial sources of eicosapentaenoic and docosahexaenoic acids.[1] For arachidonic acid production, as an alternative to previously reported strains such as *Mortierella alpina* and *M. elongata*,[2–4] we have isolated a filamentous fungus, *M. alliacea* YN-15.[5] The isolate uses either glucose or soluble starch even at a high concentration (~15%) for the characteristic cell growth and productivity of arachidonic acid. However, when soluble starch was used as a sole carbon source, the mycelial oil content was less than that in a glucose medium. Moreover, undigested soluble starch remained until the late phase of its culture, which was accompanied by a decline in the oil content, suggesting that the accumulated lipid was re-used as an energy source.[5] Throughout the fermentation, we could not detect even a trace of endo-type amylolytic enzyme activity, but did detect exo-type glucose-forming activity. These findings imply that the glucose-forming enzyme is responsible for the digestion of soluble starch and that the incomplete digestion would be due to the formation of the limit dextrin by the enzyme. In fact, when the starch medium was treated with α-amylase (EC 3.2.1.1) from another origin, the isolate fully used the liquefied starch and accumulated a level of lipids comparable to when glucose was used as a carbon source. Therefore, the enzyme(s) playing an essential role in releasing glucose from starch in the YN-15 culture would be a certain type of saccharogenic exo-type enzyme deficient or weak in debranching and liquefying activities.

α-Glucosidase (EC 3.2.1.20) is an exo-type carbohydrase with diverse specificity that catalyzes the release of α-D-glucose from the non-reducing terminal of α-glucosyl-linked substrates.[6] The α-glucosidases from animals, plants, and fungi have higher activity toward maltooligosaccharides than heterogeneous substrates such as sucrose and allylglucosides.[7] In particular, the fungal enzymes preferentially hydrolyze the shorter chain length of the saccharides substrate: α-glucosidases crystallized from *Aspergillus oryzae*[8] and *A. niger*[9] had slight activity toward α-glucans, but the enzymes purified from *Mucor javanicus*,[10] *Mu. racemosus*,[11] *Paecilomyces varioti*,[12] and *Penicillium* spp.[13,14] degraded soluble starch to some extent. Their substrate specificities are in contrast to those of fungal glucoamylases (EC 3.2.1.3), the other exo-type en-

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Abbreviations: α-EG, ethyl α-glucoside; PNPG, *p*-nitrophenyl α-D-glucopyranoside; PNPM, *p*-nitrophenyl α-D-maltoside

zymes that act more effectively on α-glucans such as starch and glycogen.[15–18] In this paper, we describe the purification and characterization of an exo-type carbohydrate hydrolase from *M. alliacea* with unusual substrate specificity.

Materials and Methods

Microorganism and culture conditions. The *M. alliacea* strain YN-15 was maintained on an agar medium of 5% corn meal agar and 1.5% bacto agar at 15°C. The first culture was prepared by incubation of the strain at 28°C for 3 d in a medium (5 ml in a 15-ml test tube) containing 20 g soluble starch, 0.2 g yeast extract, 5 g $NaNO_3$, 5 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 20 mg $FeSO_4 \cdot 7H_2O$, 10 mg $CaCl_2$, 10 mg $ZnSO_4 \cdot 7H_2O$, 2 mg $MnCl_2 \cdot 4H_2O$, 0.2 mg $CuSO_4 \cdot 5H_2O$, 0.2 mg KI, 0.2 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 0.2 mg H_3BO_3 per liter, pH 7.0. Sixty 500-ml shaking flasks each containing 100 ml of the same medium, except that $NaNO_3$ was replaced by $(NH_4)_2HPO_4$, were inoculated with the first culture, and these cultures were continued at 28°C for 7 d with reciprocal shaking.

Enzyme purification. An exo-type starch hydrolytic enzyme was purified from the supernatant of the *M. alliacea* YN-15 culture pursuant to the following procedures at 4°C, unless otherwise stated, by measurement of *p*-nitrophenyl α-D-glucopyranoside (PNPG) hydrolytic activity as described below.

(i) Step 1: Ethanol precipitation. The culture broth (6 l) was filtered to remove mycelia. After the addition of 11.2 l of cold ethanol, the suspension was kept in a refrigerator for 1 h. The resulting precipitate was collected by centrifugation at 12,000 $\times g$ for 15 min and dissolved in a 0.05 M sodium phosphate buffer (pH 7.0) containing 2 M ammonium sulfate. The insoluble materials were removed by further centrifugation at 20,000 $\times g$ for 30 min.

(ii) Step 2: Hydrophobic interaction chromatography. The supernatant (1 l) was put on a HiLoad Phenyl Sepharose column (2.6 × 10 cm; Amersham Bioscience, Uppsala, Sweden) equilibrated with a 0.05 M sodium phosphate buffer (pH 7.0) containing 2 M ammonium sulfate. After an extensive wash of the column with the buffer, the adsorbed proteins were eluted with ammonium sulfate solution (600 ml) by a linear gradient from 2 to 0 M at a flow rate of 10 ml/min, and enzyme activity fractions (120 ml) were collected.

(iii) Step 3: First cation exchange chromatography. The pooled fraction was dialyzed against a 0.02 M sodium acetate buffer (pH 5.0) and put on a Resource S column (6 ml; Amersham Bioscience) equilibrated with the same buffer. After removal of the non-adsorbed protein, the elution was done by a 120-ml linear gradient from 0 to 0.25 M NaCl at a flow rate of 3 ml/min. Enzyme activity fractions (24 ml) were collected.

(iv) Step 4: Second cation exchange chromatography. In a similar manner to that described in Step 3, except that the flow rate was 2 ml/min, the pooled fraction was put on, and resolved again, on a Resource S column, and fractions (15 ml) containing enzyme activity were collected.

(v) Step 5: Gel filtration chromatography. The resulting enzyme solution was concentrated to 1 ml and gel-filtered on a HiLoad Superdex 200 column (2.6 × 60 cm; Amersham Bioscience) equilibrated with a 0.05 M sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl at a flow rate of 0.5 ml/min. Enzyme activity fractions (21 ml) were pooled.

Anomeric analysis. The enzyme solution was dialyzed against a 0.01 M sodium acetate buffer (pH 5.0). A reaction mixture containing 5 mM *p*-nitrophenyl α-D-maltoside (PNPM; Nacalai Tesque, Kyoto, Japan), 0.005% (w/v) arabitol (as an internal standard), and the enzyme solution was incubated at 25°C for up to 6 min. Portions were immediately frozen by immersion of the tube in liquid N_2 and lyophilized. The freeze-dried sample was converted into trimethylsilyl derivatives by incubation with 100 μl of a trimethylsilylation reagent, TMSI-H (GL Science, Tokyo, Japan), at 80°C for 10 min, and was analyzed quantitatively on a gas-liquid chromatograph (GC-8A; Shimadzu, Kyoto, Japan) equipped with a glass column (0.26 × 200 cm) packed with 3% Silicone SE-30 on Chromosorb WAW-DMCS (60–80 mesh; GL Science) at 170°C with N_2 gas as a carrier.[19]

Enzyme assays. α-Glucosidase activity was measured by the release of *p*-nitrophenol from the substrate PNPG. An assay mixture (0.12 ml) consisting of a 0.1 M phosphate buffer (pH 5.0), 5 mM PNPG, and enzyme solution was incubated at 37°C for 15 min. The reaction was stopped by the addition of Na_2CO_3 at a concentration of 0.1 M, and absorbance of the reaction mixture was measured at 400 nm. One unit of the enzyme activity was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per minute ($\varepsilon = 18{,}400$ l/mol/cm).

The purified enzyme was examined for its hydrolysis and transglucosylation activities toward various substrates: maltose, trehalose, isomaltose, maltitol, amylose EX-I (DP = 18), pullulan (kindly provided by Hayashibara Biochemical Laboratories, Okayama, Japan), kojibiose, ethyl α-glucoside (α-EG), nigerose (Wako Pure Chemical, Osaka, Japan), sucrose, soluble starch (Katayama Chemical, Osaka, Japan), PNPG (Sigma, St. Louis, MO), glycogen, amylopectin (Nacalai Tesque), and dextran (Seikagaku Kogyo, Tokyo, Japan). For the hydrolysis analysis, a reaction mixture (100 μl) composed of

8.2 μg/ml enzyme, 0.4% (w/v) substrate, and 40 mM sodium acetate buffer (pH 5.0) was incubated at 37°C for 30 min. The reaction was stopped by the addition of two volumes of 2 M Tris-HCl buffer (pH 7.0),[20] and the release of glucose was measured by a Glucose C-II Test Kit Wako (Wako Pure Chemical). The hydrolytic activity was represented as the release of glucose only from the non-reducing terminal of the substrate, where the amount of glucose from the reducing terminal was excluded. For the transglucosylation assay, a reaction mixture (100 μl) composed of 8.2 μg/ml enzyme, 40% ethanol, 0.4% (w/v) substrate, and 40 mM sodium acetate buffer (pH 5.0) was incubated at 45°C for 30 min. To stop the reaction, the sample was heated in a boiling-water bath for 10 min. After the sample (100 μl) was mixed with rhamnitol as an internal standard and dried, the acetylation was done by the addition of 200 μl each of acetic anhydride and pyridine then heated at 100°C for 1 h. The solvent was evaporated, and the sample was dissolved in ethanol. The acetylated α-EG was analyzed on a gas-liquid chromatograph (GC-8A) equipped with a glass column (0.32 × 100 cm) packed with 10% LAC 4R-886 on Chromosorb WAW (100–120 mesh; GL Science) at 200°C with N_2 gas as a carrier.

Subsite analysis. Maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose (>96% purity) were kindly provided by Hayashibara Biochemical Laboratories and used as substrates for measurement of the kinetic parameters for their hydrolysis. The Michaelis constant, K_m, the maximum velocity, V, and the molecular activity, k_0 (V/e_0), were calculated from Lineweaver-Burk plots and the molecular concentration of the enzyme, e_0. The subsite affinities, A_i (i, subsite number), and the intrinsic rate constant, k_{int}, were evaluated from the parameters by the theory of Hiromi *et al.*[21]

Measurement of physical properties. Glucose and α-EG generated in the following reactions were measured as described above.

(i) pH stability. Enzyme solutions (8.2 μg/ml) in a Britton-Robinson buffer (5 mM citrate, 5 mM KH_2PO_4, 5 mM H_3BO_3, 5 mM diethylbarbituric acid), which has a wide pH range, were adjusted to various pH and kept at 4°C for 24 hr. Two volumes each of 1.0 M sodium acetate buffer (pH 5.0) and 1% (w/v) maltose solution were added, and the mixture was incubated at 37°C for 15 min.

(ii) Heat stability. After 20 μl of the enzyme solution (0.82 μg) was kept at various temperatures for 15 min, 40 μl each of 0.1 M sodium acetate buffer (pH 5.0) and 1% (w/v) maltose were added, and the mixture was incubated at 37°C for 15 min.

(iii) Effects of pH on maltose hydrolytic and α-EG forming activities. For maltose hydrolytic reactions, the mixture composed of 0.82 mg/ml enzyme and 0.4% (w/v) maltose in the Britton-Robinson buffer adjusted at the indicated pH was incubated at 37°C for 15 min. For α-EG-forming reactions, 20 μl of enzyme (0.82 μg), 40 μl of ethanol, and 40 μl of 1% (w/v) maltose in a 0.1 M citrate-phosphate buffer adjusted at the indicated pH was incubated at 45°C for 30 min.

(iv) Effects of temperature on maltose hydrolytic and α-EG-forming activities. For maltose hydrolytic reactions, a mixture composed of 0.04 M sodium acetate (pH 5.0), 0.4% (w/v) maltose, and enzyme (0.82 mg/ml) was incubated at various temperatures for 15 min. For the α-EG-forming reaction, 8.2 μg/ml enzyme was used and the reaction time was 30 min. Ethanol was added at 40% when the α-EG-forming activity was measured.

Protein analysis. Protein concentration was measured with a DC Protein Assay Kit (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Electrophoresis on a 7.5% non-denaturing polyacrylamide slab gel (Native PAGE) at pH 8.8 was done as described by Davis.[22] SDS-PAGE was done as described by Laemmli[23] in the presence or absence of β-mercaptoethanol. For two-dimensional (2D)-PAGE, an Immobiline Dry Strip 4–7 (Amersham Bioscience) was used to resolve proteins in the first dimension, and SDS-12.5%-PAGE was done in the second dimension. The proteins in the gels were stained by 2D-Silver Stain-II "Daiichi" (Daiichi Kagaku, Tokyo, Japan) according to the manufacturer's instructions.

Amino-terminal peptide sequencing. The amino-terminal sequence of the enzyme that was separated by SDS-PAGE and transfered to a polyvinylidene fluoride membrane was analyzed by Edman degradation on a protein sequencer (Model 492; Applied Biosystems, Tokyo, Japan).

Results

Purification of enzyme

In a preliminary study, the PNPG hydrolytic activity reached its maximum at 7 d in a 9-d cultivation of *M. alliacea* on a medium containing 2% soluble starch. The activity was purified from the culture supernatant through ethanol precipitation and four-step column chromatography at 33-fold with a yield of 2.3%, and the specific activity of the purified enzyme was 3.2 U/mg (Table 1). The gel filtration chromatography demonstrated that the purified enzyme showed a single peak with a substantial PNPG-hydrolytic activity (Fig. 1(A)).

Anomeric configuration of hydrolyzed product

The purified enzyme released glucose, but not mal-

Table 1. Summary of Purification of α-Glucosidase from *M. alliacea* YN-15

Purification step	Volume (ml)	Protein (mg)	PNPG hydrolytic activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	5600	1200	120	0.10	100	1.0
Ethanol fractionation	1000	140	29	0.21	24	2.1
Phenyl Sepharose	120	18	10	0.56	8.3	5.5
1st Resource S	24	5.7	7.0	1.2	5.8	12
2nd Resource S	15	4.3	6.4	1.5	5.3	15
Superdex 200	21	0.88	2.8	3.2	2.3	33

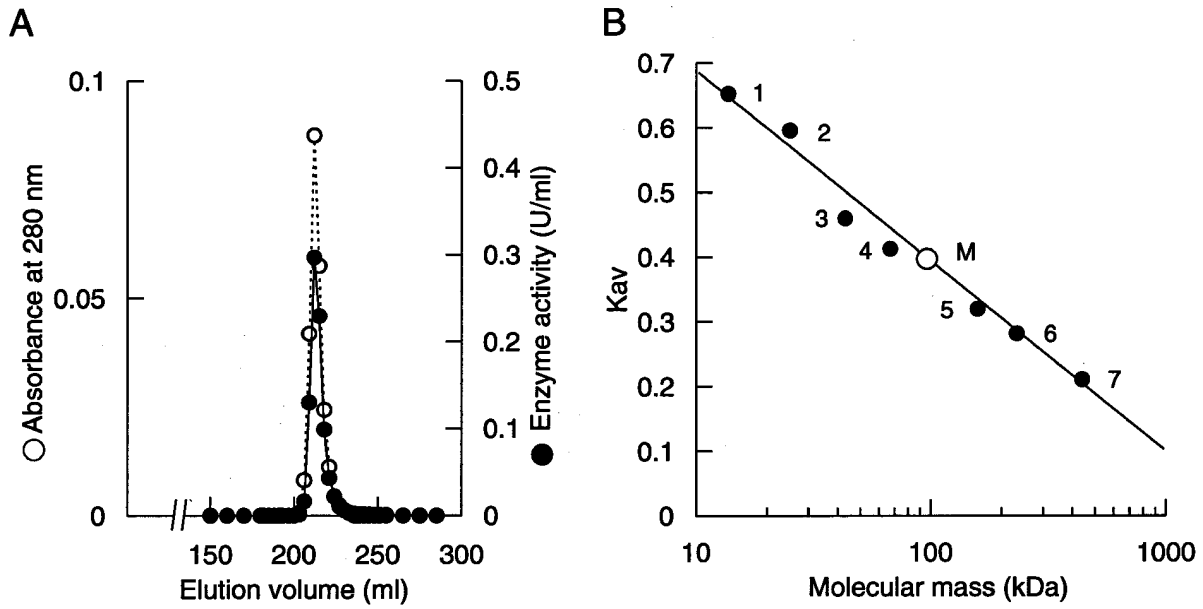

Fig. 1. Purification of α-Glucosidase from *M. alliacea* YN-15.

(A) The PNPG hydrolytic activity eluted from the 2nd Resource S column was put on a Superdex 200 column (2.6 × 60 cm) equilibrated with 0.05 M phosphate-buffered saline. Fractions containing high activity (elution volume 206–227 ml) were collected. (B) The relative mass was estimated by gel filtration on a Superdex 200 PC 3.2/30 column (Amersham Bioscience) equilibrated with 0.05 M sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl. The K_{av} was defined as $(V_R - V_0)/(V_C - V_0)$, where V_0 is the void volume of the column, V_R is the elution volume of the protein, and V_C is the geometric bed volume. Calibration standards used were as follows: 1, ribonuclease A (13.7 kDa); 2, chymotrypsinogen A (25 kDa); 3, ovalbumin (43 kDa); 4, bovine serum albumin (67 kDa); 5, aldolase (158 kDa); 6, catalase (232 kDa); and 7, ferritin (440 kDa). M, α-glucosidase from *M. alliacea* YN-15 (estimated at 92 kDa).

tooligosaccharides, as the sole product during digestion of soluble starch (not shown), suggesting that the action pattern of the enzyme is of the exo-type. α-Glucosidase and glucoamylase are typical exo-type carbohydrases and distinguished in the stereochemical course of the hydrolysis reaction that occurs with retention and inversion, respectively, of the anomeric configuration.[24] Accordingly, the change in the configuration at the anomeric carbon of released glucose was examined in the initial reaction of the purified enzyme to PNPM. As shown in Fig. 2, α-glucose quickly increased with a minor level of β-glucose unavoidably generated through a spontaneous mutarotation of some of the liberated α-glucose.[19] Therefore, the purified enzyme could be defined as α-glucosidase.

Substrate specificity

The hydrolytic activity of the enzyme toward various substrates is summarized in Table 2. Homogeneous substrates such as maltose and soluble starch were acted on more than heterogeneous ones such as sucrose and PNPG. Among the former substrates, the enzyme acted preferentially on the glucosyl linkage with an anomeric configuration of α-1,4 (maltose, starch, amylose, amylopectin, and glycogen), α-1,3 (nigerose), and α-1,2 (kojibiose) to some extent but scarcely on α-1,6 (isomaltose, pullulan, and dextran). Above all, a higher activity for hydrolysis was obtained on soluble starch rather than maltose. This property is unique among fungal α-glucosidases, most of which have a tendency to hydrolyze maltooligosaccharides more rapidly than polysaccharides.[7]

Although fungal glucoamylases also hydrolyze polysaccharides preferentially to maltooligosaccharides, these enzymes are rationally not capable of catalyzing transglucosylation reactions.[6] To test this

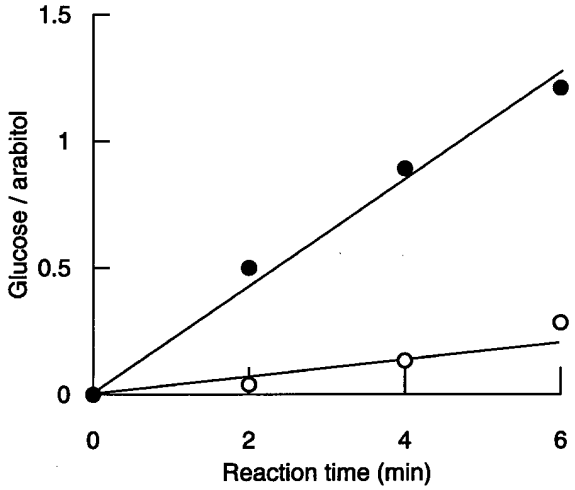

Fig. 2. Changes with Time in α- and β-Glucose Produced through PNPM Hydrolysis by *M. alliacea* α-Glucosidase.
The amount of glucose relative to arabitol was calculated from their peak areas on the chromatogram. *Closed circles*, α-glucose; *open circles*, β-glucose.

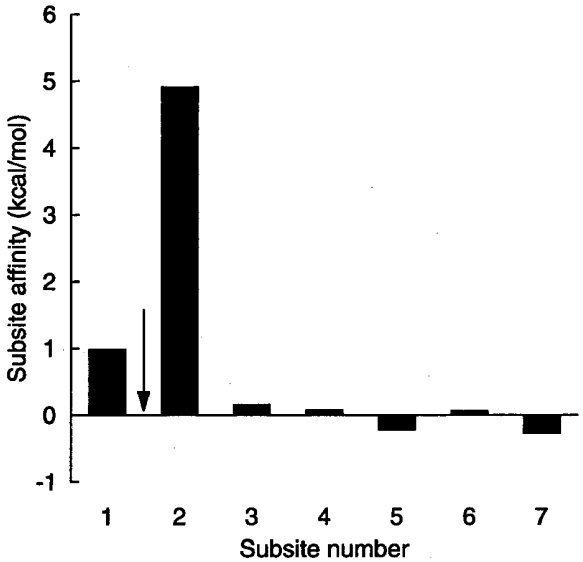

Fig. 3. Subsite Affinity of *M. alliacea* α-Glucosidase.
An intrinsic rate constant (k_{int}) of 65 s^{-1} was found. The arrow indicates the position of the catalytic site.

on the α-glucosidase from *M. alliacea*, a series of hydrolysis reactions on the various substrates was done in the presence of ethanol. Our enzyme could use glycogen and soluble starch rather than maltose to effectively transfer a glycosyl residue to ethanol (Table 2). The substrate specificity on the transglucosylation reaction indicated that the purified α-glucosidase is responsible at least for effective hydrolysis of soluble starch and glycogen, demonstrating a novel property for a fungal α-glucosidase.

Hydrolysis rate parameters and subsite structure

To further clarify the difference in substrate specificity of this enzyme, the rate parameters for the hydrolysis of maltooligosaccharides with various chain lengths were measured. The molecular activity (k_0) was independent of the polymerization degree of the substrate (Table 3). This property is essentially similar to those of other α-glucosidases and clearly distinct from any reported glucoamylases.[6] On the other hand, log ($1/K_m$) values, which can be regarded as the affinity of the enzyme for each substrate, were at a maximum at maltotetraose ($n=4$) and decreased with an increase of the polymerization degree of the substrate. Thus, a measure of the effectiveness of the enzymic reaction, log (k_0/K_m), was the highest for this substrate.

Based on these rate parameters, a subsite analysis was done by the method of Hiromi *et al.*[21] As shown in Fig. 3, the second subsite (A_2) had the largest affinity (4.91 kcal/mol; the same value was obtained by methods I and II[21]), and the affinity of the first subsite (A_1; 0.98 kcal/mol) was greater than that of the

Table 2. Substrate Specificity of *M. alliacea* α-Glucosidase

Substate	Hydrolysis[a,b] (μmol of glucose from non-reducing end/mg protein/min)	α-EG formation[b] (mg α-EG/mg protein/min)
Maltose (α-1,4)	29.8±1.7	0.91±0.05
Trehalose (α, α-1,1)	0.3±0.1	ND[c]
Kojibiose (α-1,2)	7.0±0.3	0.16±0.03
Nigerose (α-1,3)	26.1±0.7	0.28±0.04
Isomaltose (α-1,6)	1.0±0.1	ND[c]
Sucrose (α-1, β-2)	0.9±0.1	ND[c]
Maltitol	5.3±0.1	0.06±0.01
PNPG	3.4±0.2	0.02±0.01
α-EG	0.3±0.1	—
Soluble starch	42.9±5.2	1.19±0.08
Amylose (DP=18)	8.3±0.4	—
Amylopectin	34.3±1.9	—
Glycogen	30.2±2.9	1.56±0.06
Pullulan	0.9±0.1	ND[c]
Dextran	0.2±0.1	ND[c]

[a] Glucose from reducing terminal of substrate was not taken into account.
[b] Values are from at least three independent experiments and represented as the average±SE.
[c] Not detected (less than 0.0005 mg α-EG/mg-protein/min).

Table 3. Rate Parameters of α-Glucosidase from *M. alliacea* YN-15[a]

Substrate	K_m (mM)	V_{max}[b]	k_0 (s^{-1})	k_0/K_m ($mM^{-1}\cdot s^{-1}$)
Maltose	3.5	6.88	58.6	16.6
Maltotriose	2.7	6.66	56.8	21.4
Maltotetraose	2.4	6.68	56.9	23.9
Maltopentaose	2.8	5.56	47.3	16.7
Maltohexaose	2.9	6.19	52.7	18.3
Maltoheptaose	4.9	6.70	57.1	11.8

[a] Reaction, 37°C, pH 5.0; Molecular mass, 92,000.
[b] Glucose (mg) liberated from the non-reducing terminal of substrate/mg-protein/min.

Table 4. Comparison between Observed and Calculated Rate Parameters for *M. alliacea* α-Glucosidase

Substrate	n[a]	K_m (mM)		k_0 (s^{-1})		k_0/K_m ($mM^{-1}\cdot s^{-1}$)	
		Calcd.[b]	O/C[c]	Calcd.[b]	O/C[c]	Calcd.[b]	O/C[c]
Maltose	2	3.1	1.13	51.5	1.14	16.6	1.00
Maltotriose	3	2.5	1.08	52.9	1.07	21.2	1.01
Maltotetraose	4	2.4	1.00	56.9	1.00	23.7	1.01
Maltopentaose	5	3.2	0.88	53.1	0.89	16.6	1.01
Maltohexaose	6	3.1	0.94	57.5	0.92	18.5	0.99

[a] Degree of polymerization of substrate in glucose units.
[b] Calculated by using the values of A_i and k_{int} obtained by method of Hiromi *et al.*[21]
[c] The ratio of observed values in Table 3 to calculated ones.

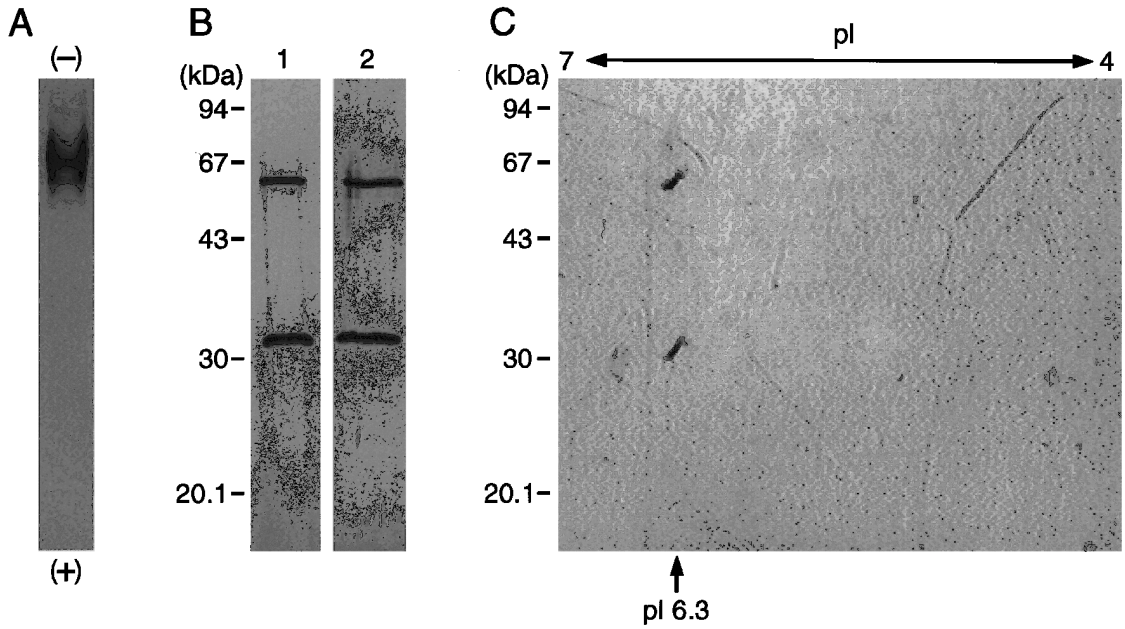

Fig. 4. Native PAGE (A), SDS-PAGE (B), and 2D-PAGE Analyses (C) of α-Glucosidase from *M. alliacea* YN-15.
Native PAGE was done on a 7.5% polyacrylamide gel at pH 8.8. Lanes 1 and 2 in panel B indicate the absence and presence of β-mercaptoethanol in the sample buffer, respectively. Amounts of analyzed protein were 0.42 μg (A and B) and 2.4 μg (C). Molecular mass markers: phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase *b* (30 kDa), and trypsin inhibitor (20.1 kDa).

third one (A_3; 0.16 kcal/mol). When the rate parameters, K_m and k_0, were calculated from the A_i and k_{int} values, there seemed to be good agreement between the calculated values and the experimental ones (in Table 3) with a maximum deviation of about 14%, indicating the validity of the assessment (Table 4). The subsite pattern ($A_1 > A_3$) was similar to those of other fungal and plant α-glucosidases[6] but was the reverse of glucoamylases of which A_3 is greater than A_1.[21] Furthermore, the reported subsite affinities at position 2 or more of glucoamylases have been positive numbers,[21] but those of α-glucosidases including ours were not necessarily so, reflecting the difference in mode of the enzyme-substrate complex. These results do not contradict the identification of our enzyme as an α-glucosidase.

Physical properties

The molecular mass of the enzyme was estimated at about 92 kDa on gel chromatography with the running buffer containing 0.5 M NaCl (Fig. 1(B)). Interestingly, the enzyme that gave a single band on the native PAGE (Fig. 4(A)) had been separated into two components of 61 kDa and 31 kDa on the SDS-PAGE, independent of the reduction by β-mercaptoethanol (Fig. 4(B)). The two bands were also resolved on the 2D-PAGE at the column of pI 6.3 (Fig. 4(C)). The amino-terminal peptide sequences of the 61- and 31-kDa subunits were DDMXSNVQTRKDXGYL and ASTSIKYSINNAGRQAPL, respectively, which had little similarity to each other and to the sequences of any other proteins presented in databases. These results indicate that the α-glucosidase from *M. alliacea* is composed of heterodimers that are not covalently linked but stably ag-

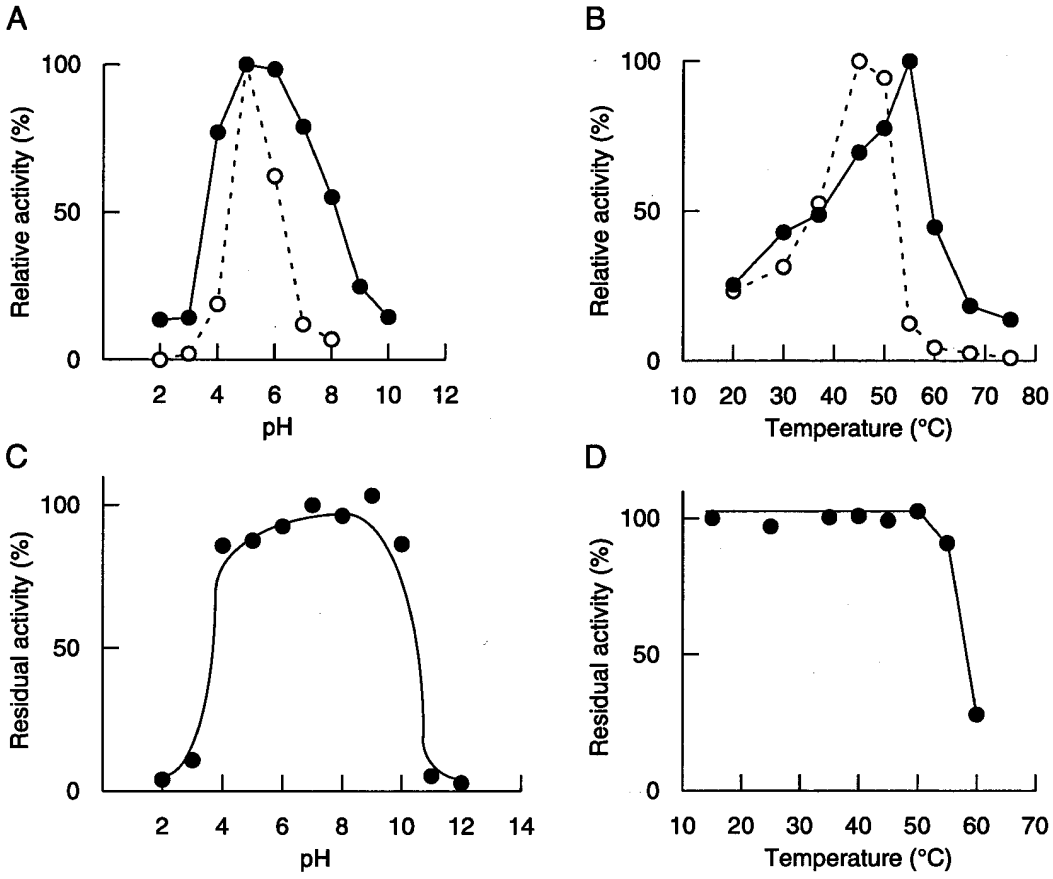

Fig. 5. Physical Properties of *M. alliacea* α-Glucosidase.

(A) Effects of pH on maltose hydrolytic and α-EG forming activities. Maltose hydrolytic and α-EG forming activities at pH 5.0 were 93 μmol glucose/mg-protein/min and 1.2 mg of α-EG/mg-protein/min, respectively. *Closed circles*, maltose hydrolytic activity; *open circles*, α-EG forming activity. (B) Effects of temperature. Maltose hydrolytic activity at 55°C was 153 μmol glucose/mg-protein/min. α-EG forming activity at 45°C was 0.67 mg of α-EG/mg-protein/min. Symbols are the same as in panel *A*. (C) pH stability. Hydrolytic activity at pH 7.0 was 73 μmol glucose/mg-protein/min. (D) Heat stability. Hydrolytic activity at 40°C was 57 μmol glucose/mg-protein/min.

gregated to each other with a salt-resistant nature.

The optimum pH and temperature were the pH range of 5.0–6.0 and 55°C, respectively, for maltose hydrolysis, and pH 5.0 and the range of 45–50°C for transglucosylation forming α-EG (Figs. 5(A) and 5(B)). The enzyme stability at various pH or temperature was measured by its maltose hydrolytic activity. The enzyme was stable after a 24-h incubation in buffer with pH from 4.0 to 10.0 (Fig. 5(C)). As for temperature, retention of the enzyme at 50°C for 15 min had practically no effect on the activity, while heating at 60°C resulted in an ~80% reduction (Fig. 5(D)).

Discussion

Glucoamylases from the fungi *A. niger*, *A. oryzae*, *Penicillium oxalicum*, and *Mu. rouxianus* can hydrolyze soluble starch much faster than maltose at the initial velocity ratio of 4–7:1.[15–18] In contrast, fungal α-glucosidases show lower activity toward soluble starch. For example, the hydrolytic activity of α-glucosidase from *Mu. javanicus* on soluble starch was less than half of that on maltose.[10] In this regard, the *M. alliacea* α-glucosidase, which degrades polysaccharides better than oligosaccharides (Table 2), could be extraordinary. Primarily, α-glucosidase and glucoamylase, as the typical exo-type carbohydrate hydrolases, are distinguished from each other by the anomeric configuration of their products: α-glucosidase gives α-glucose and glucoamylase provides β-glucose.[19,24] The study on the configuration of the anomeric carbon on the hydrolyzed product conclusively demonstrated that the purified enzyme was the former one (Fig. 2). The high specificity of the enzyme for soluble starch and glycogen was also observed in the transglucosylation reaction in the presence of ethanol (Table 2). This could further rule out the possibility that the enzyme is a glucoamylase that lacks transglucosylation activity.[19] Moreover, the independence of the molecular activity (k_0) on the polymerization degree of the maltooligosaccharide substrates supported the identification of the enzyme as α-glucosidase (Table 3).

Indeed, the k_0 values for glucoamylases show strong dependence on the saccharide chain length.[21,25] Therefore, it could be concluded that the *M. alliacea* enzyme is a member of a novel fungal α-glucosidase group.

α-Glucosidases from various origins have been classified into two groups in terms of their conserved amino acid sequences and substrate specificities.[6] The first group contains bacterial and insect enzymes that have a high hydrolytic activity toward heterogeneous substrates. On the other hand, plant and fungal enzymes included in the second group hydrolyze homogeneous substrates such as maltooligosaccharides, soluble starch, and glycogen more rapidly. However, for the latter group, the enzymes acting on oligosaccharides and the ones on polysaccharides should be discriminated based on the fine structure of the enzyme and/or other criteria. Our current data could not explain the difference between *M. alliacea* and plant α-glucosidases, both of which showed high specificities for polysaccharides. For instance, on kinetic parameters, the K_m of our enzyme (Table 3) and that of barley seed α-glucosidase[26] on oligosaccharides (DP ≥ 4) increased with an increase of the polymerization degree, but those of α-glucosidases from sugar beet and rice decreased.[26] Since amylopectin was preferentially hydrolyzed by the *M. alliacea* enzyme rather than amylose (Table 2), the branched structure of the substrate might be responsible for rapid hydrolysis, and, therefore, the oligosaccharides with a straight chain structure, especially of a high polymerization degree, were not effectively hydrolyzed. The elucidation of the higher order structure of our enzyme and its relation to the substrate recognition will be necessary to illustrate in depth the differences from other enzymes.

The *M. alliacea* α-glucosidase was expressed by arachidonic acid fermentation in a starch medium and purified from the culture supernatant. We do not believe that this enzyme was leaked from cells by the disruption of mycelia or by release from cell membranes during the cultivation and/or purification process because the cell extracts (with 4 M urea) did not contain significant levels of corresponding activity (data not shown). This is in contrast to that of the α-glucosidases from other *Zygomycetes* fungi including *Mu. javanicus*[27] and *Mu. racemosus*,[28] which are cell-bound types that could be recovered by the urea treatment. Moreover, these *Mucor* enzymes consist of single polypeptides. This characteristic was also distinct from ours, which is composed of two subunits of 61 kDa and 31 kDa (Fig. 4). In *Ascomycetes* fungi, such heterodimeric types of α-glucosidases were reported to be purified from a *koji* culture of *A. niger*.[9,29] This enzyme, however, was thought to be expressed as a single polypeptide and then processed through a limited proteolysis by unknown protease(s), although the physiological significance of the post-translational modification remains unclear.[29,30] We have obtained preliminary data indicating that the two subunits of the *M. alliacea* enzyme are encoded by one gene. It is therefore interesting to examine whether or not an unprocessed version of the enzyme, over-expressed in *Escherichia coli* for instance, retains the substrate binding and catalytic activities, and to elucidate the meaning of the polypeptide processing.

The original aim of our study was to investigate a mechanism underlying the partial starch degradation as a possible limiting step in arachidonic acid fermentation by the strain YN-15. Since no activity of the endo-type enzyme could be detected in the culture broth when soluble starch was used as a carbon source, it is feasible that the α-glucosidase of interest plays an indispensable role in assimilation of the starch medium. As far as debranching activity is absent in the culture, it is naturally expected that the limit dextrin is accumulated in accordance with depletion of the non-reducing terminal of the substrate accessible by α-glucosidase. This idea is supported at least in part by the fact that the purified enzyme did not effectively attack the glucosyl linkage of α-1,6 (Table 2), comprising the branched structure of polysaccharides. Treatment of starch with α-amylase/debranching enzymes (*e.g.* pullulanase) or introduction of isoamylase genes may be pragmatic as a countermeasure.

Finally, the *M. alliacea* α-glucosidase with a strong activity degrading polysaccharides might be useful for other industrial applications. The transglucosylation reaction in the presence of ethanol as an acceptor (Table 2) resulted in the formation of α-EG, a sweetening and flavoring agent that has attracted interest owing to its skin barrier effect[31] and noncariogenicity.[32] The α-EG has been produced on an industrial scale by a transglucosylation reaction using maltose as a glucosyl donor.[33] In case that glycogen or soluble starch is used as a raw material, ordinary α-glucosidase must supply maltose with the help of isoamylase and β-amylase or α-amylase. The improvement of debranching activity on the α-glucosidase reported in this study could provide an advantage in the development of a one-step reaction system for mass production of α-EG from polysaccharides.

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