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Action of α-D-glucosidase from *Aspergillus niger* towards dextrin and starch

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ABSTRACT

The transglucosidation reactions of α-D-glucosidase with dextrin and starch from *Aspergillus niger* were investigated. When a mixture of dextrin and [U-^{13}C]maltose was incubated with α-D-glucosidase at 25 °C for 5 d, [U-^{13}C]glucosyl groups were linked to dextrin with a degree of polymerization (DP) of up to 14, which was detected by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS). When a mixture of starch and [U-^{13}C]maltose was incubated with the α-D-glucosidase, no [U-^{13}C]glucosyl residues were transferred to the starch. Methylation analysis revealed that the chain length of the α-D-glucosidase-treated starch was shortened and the proportion of α-(1 → 6) linkages did not increase. These results suggested that α-D-glucosidase catalyzed the transglucosidation reaction to dextrin with DP up to 13, but this enzyme catalyzed only the hydrolysis of starch.

1. Introduction

α-D-Glucosidase [EC 3.2.1.20, α-D-glucoside glucohydrolase], various types of which have been purified and characterized, is an exo-type glycosidase that releases α-D-glucose from the non-reducing end of substrates and is widely distributed in microorganisms, plants, insects, and mammals (Chiba, 1997; Chiba et al., 2003). Several α-D-glucosidases catalyze the transfer reaction to maltooligosaccharides and have been applied for the synthesis of oligosaccharides (Kimura, Yoshida-Kitahara, & Chiba, 1987; Malá, Dvořáková, Hraval, & Králová, 1999; Nakao et al. 1994; Watanabe et al., 2005). α-D-Glucosidase produces α-anomer by retaining anomeric configuration. Catalysis by retaining glucosidase proceeds via a double-displacement mechanism in which a covalent glucosyl-enzyme intermediate is formed and hydrolyzed via an oxocarbenium ion intermediate (Chiba, 1997; Withers, 2001). Transglucosidation reaction is assumed to occur via a glucosyl-enzyme intermediate that undergoes nucleophilic attack by a hydroxyl group of another substrate molecule. α-D-Glucosidases from different sources show diverse substrate specificity and regioselectivity. *Bacillus stearothermophilus* α-D-glucosidase synthesizes maltotriose from maltose, whereas Brewer's yeast α-D-glucosidase synthesizes oligomers consisting of α-(1 → 3), α-(1 → 4), and α-(1 → 6) linkages (Malá et al., 1999). *Aspergillus nidulans* α-D-glucosidase has high activity for the synthesis of isomaltose and isomaltotriose from maltose with a yield of 50% (Kato et al., 2002). Although most α-D-glucosidases transfer α-D-glucose onto short oligosaccharides, several α-D-glucosidases from mammalian tissues transfer [U-^{14}C]glucosyl residues to glycogen (Hers, 1963; Jeffrey, Brown, & Brown, 1970; Palmer, 1971; Torres & Olavarria, 1964). Rabbit muscle's acid α-D-glucosidase incorporated 1% of [U-^{14}C]glucosyl residues from [U-^{14}C]maltose into glycogen using α-(1 → 4) and α-(1 → 6) linkages (Palmer, 1971). In contrast with mammalian α-D-glucosidase, all reported microbial α-D-glucosidases transfer α-D-glucose onto short oligomers but high-molecular α-glucans such as starch or glycogen.

Aspergillus niger α-D-glucosidase synthesizes oligosaccharides from long maltooligosaccharides such as maltoheptaose as well as from short oligomers such as maltose and maltotriose, and several products had higher molecular weights than that of maltoheptaose (Ota, Okamoto, & Wakabayashi, 2009; Pazur, Cepure, Okada, & Forsberg, 1977). *A. niger* α-D-glucosidase also catalyzes the transfer of glucosyl residues from starch to glucose (Pazur & Ando, 1961) as well as the hydrolysis of α-(1 → 4) linkages at the non-reducing end of starch (Chiba, 1997). However, no study dealing with the transglucosidation to starch has been reported. Recently, Ao et al. reported that maize starch digested with maltogenic α-amylase and *A. niger* α-D-glucosidase contained a considerable proportion of α-(1 → 6) linkages as detected by ^{1}H NMR (Ao et al., 2007). It was suggested that this result was due to the transglucosidation reaction to starch based on the study of Pazur et al. (1977). However, they obtained the enzyme-treated starch by precipitation with no further purification. Thus, the product might be contaminated by low-molecular weight isomaltooligosaccharides produced from the starch by the α-D-glucosidase. The aim of the

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present study was to confirm *A. niger* α-D-glucosidase will transfer glucosyl residues to high-molecular weight α-glucans such as dextrin and starch.

Matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used for the characterization of neutral carbohydrates (Broberg, Koch, Andersson, & Kenne, 2000; Laštovičková & Chmelík, 2006; Losso & Nakai, 1997; Wang, Jiang, Vasanthan, & Sporns, 1999). The transfer of [U-^{13}C]glucosyl residues from [U-^{13}C]maltose to α-glucans was observed in the incubation mixture with MALDI-TOF MS. This report examines the action of *A. niger* α-D-glucosidase towards dextrin and starch using these techniques.

2. Experimental

2.1. Materials

2.1.1. Chemicals

[U-^{13}C]Maltose was purchased from Omicron Co. (USA). Dextrin (Sandec 180, average molecular weight – 7800) was a gift from Sanwa Cornstarch Co., Ltd. (Japan). Starch from waxy corn was purchased from Sigma Co. (USA). All chemicals were used without further purification.

2.1.2. Enzymes

Isoamylase (derived from *Pseudomonas amyloderamosa*) was purchased from Hayashibara Biochemical Labs. (Japan). α-D-Glucosidase ("Transglucosidase L", derived from *A. niger*) was a gift from Amano Enzyme Co. (Japan) and purified to be free from glucoamylase as described below.

2.2. Methods

2.2.1. Purification of α-D-glucosidase

The crude α-D-glucosidase (5 g) was dissolved in 25 mL of 0.02 M phosphate buffer (pH 7.2) and dialyzed against the same buffer. The enzyme solution was subjected to purification using a DEAE–Sepharose Fast Flow column (volume 500 mL; GE Healthcare Bio-Sciences Co., USA) equilibrated with 0.02 M phosphate buffer (pH 7.2) with monitoring at 280 nm. The enzyme was eluted using a NaCl (0–1 M) linear gradient. The active fractions were collected and concentrated by ultrafiltration (Hydrosart 10 kDa cut off, Sartorius AG, Germany). The enzyme activity was measured by the method reported by Tanimura et al. with slight modifications (Tanimura, Kitamura, Fukuda, & Kikuchi, 1979). Each fraction (0.5 mL) was mixed with 0.5 mL of 7.5 mM *p*-nitrophenyl-glucoside in 10 mM sodium acetate buffer (pH 5.0). After incubation for 10 min at 40 °C, the mixture was cooled on ice and 0.5 mL of 10% Na_2CO_3 was added. The amount of *p*-nitrophenol released was measured using a UV detector at 420 nm. One unit of transglucosidase activity was defined as the amount of enzyme that hydrolyzed 1 μmol of *p*-nitrophenyl-glucoside per min under the above conditions. The purified enzyme solution was used for the following experiments.

2.2.2. MALDI-TOF MS

MALDI-TOF mass spectra were obtained on an AXIMA-TOF^2 (Shimadzu Co., Japan) mass spectrometer using 2,4,6-trihydroxyacetophenone (THAP) as a matrix. Each sample (0.5 μL) was mixed with 0.5 μL of THAP in acetonitrile and dried at room temperature. Calibration was performed by using a mixture of maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), and maltoheptaose (G7) as standards. The molar masses were analyzed in the positive reflectron mode. For each acquisition, 200 laser shots were fired and the resulting spectra were averaged.

2.2.3. GC–MS analysis

GC–MS analysis was performed using an HP 5890 (Hewlett-Packard) gas chromatograph coupled to an HP 5972 (Hewlett-Packard) mass spectrometer with an NB-1 column (0.25 mm × 60 m × 0.4 μm; GL Sciences Inc., Japan). After sample injection with split mode, the oven temperature program was started and the temperature was maintained for 2 min at 140 °C, then raised at 2 °C/min from 140 °C to 200 °C, 10 °C/min from 200 °C to 250 °C, and maintained for 18 min at 250 °C. Mass spectra in the electron impact mode (EI) were generated at 70 eV, ion source temperature at 220 °C, and at a scan range from *m/z* 40 to 550 Da.

2.2.4. SEC-ELSD

The analytical and preparative HPLC system (Waters Co. USA) consisted of a 600S gradient pump system combined with an ELSD 2000 (Alltech, USA) and a SEC column (TSK-gel G3000PW, 7.8 × 300 mm, 7 μm; Tosoh, Japan); flow rate, 0.5 mL/min; solvent, water.

2.2.5. Preparative ODS-HPLC

The HPLC system (Waters Co. USA) consisted of a 600S gradient pump system combined with an ELSD and a ODS column (ODS-AQ, 4.6 × 250 mm, 5 μm, YMC Co., Ltd. Japan); flow rate, 0.6 mL/min; solvent, water:CH_3CN (92:8 v/v).

2.2.6. MALDI-TOF-MS analysis of α-D-glucosidase action on a mixture of dextrin and [U-^{13}C]maltose

Dextrin (20 mg) and [U-^{13}C]maltose (5 mg, 14 μmol) were dissolved in 40 μL of water. To the solution, 9 μL of α-D-glucosidase

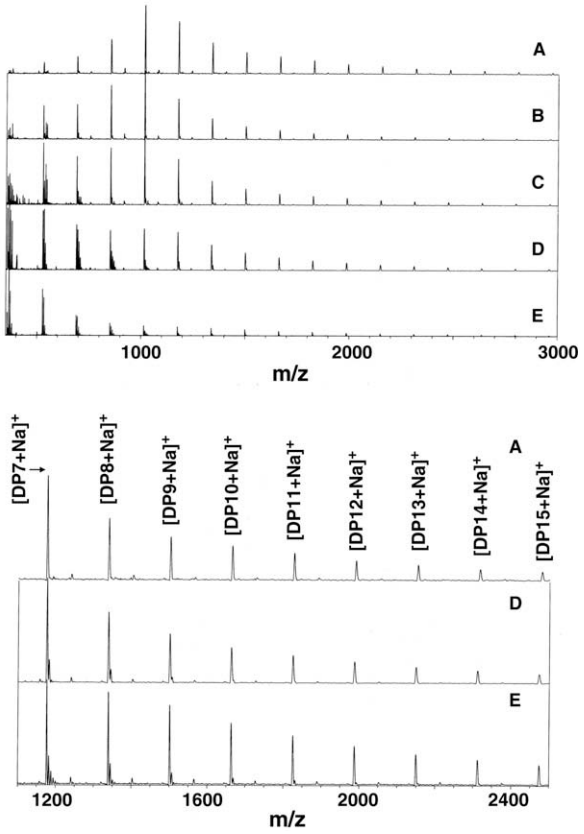

Fig. 1. MALDI-TOF MS analysis of digests of dextrin and [U-^{13}C]maltose with *A. niger* α-D-glucosidase. (Matrix; THAP: reflectron: positive mode, top; *m/z* 350–3000 Da, bottom; *m/z* 1100–2500 Da) digestion for (A) 0 h, (B) 0.5 h, (C) 2 h, (D) 1 d, and (E) 5 d.

solution (0.19 U) was added, and the reaction mixture was stirred at room temperature (25 °C). Aliquots (1 μL) of the mixture were withdrawn at different time intervals. Each aliquot was mixed with 20 μL of water, and 0.5 μL of the mixture was subjected to analysis. Their molar ratios of oligosaccharides containing [U-^{13}C]glucosyl residues were estimated from the peak height of their cluster ion $[M+Na]^+$. The molar ratio shown in Fig. 2 was defined as a sum of peak heights of oligosaccharides that contained at least one [U-^{13}C]glucosyl residue over the peak height of oligosaccharide consisting of only [U-^{12}C]glucosyl residues.

2.2.7. MALDI-TOF MS analysis of α-D-glucosidase action on a mixture of starch and [U-^{13}C]maltose

Starch from waxy corn (20 mg) was suspended in 40 μL of water and [U-^{13}C]maltose (5 mg, 14 μmol) was added. To the solution, 9 μL of α-D-glucosidase solution (0.19 U) was added, and the reaction mixture was stirred at room temperature (25 °C). The reaction was performed for various time periods (1 h, 1 d, and 7 d). When the reaction was terminated, 0.4 mL of water was added to the reaction mixture, the mixture was centrifuged (12,000*g*), and the supernatant was discarded. These procedures were repeated four times. The precipitate was resuspended in 200 μL of 0.1 M NaOH, and heated to 100 °C for 10 min to induce gelatinization. The gelatin was cooled to room temperature and 200 μL of 0.1 M HCl was added. Debranching of the sample was performed using isoamylase (Harada, Misaki, Akai, Yokobayashi, & Sugimoto, 1972). An aliquot of the solution (150 μL) was withdrawn and mixed with 850 μL of 0.1 mol/L sodium acetate (pH 4.0). To the solution, 10 μL of isoamylase (590 U) was added, and then the mixture was incubated for 24 h at 40 °C and heated to 100 °C for 10 min to end the reaction. The reaction mixture was filtered and subjected to an SEC (solvent; water). The fraction with t_R 14.0–20.7 min (19.9 mg) was lyophilized, and subjected to the MALDI-TOF MS.

2.2.8. HPLC preparation of oligosaccharides from the enzymatic digests of dextrin and [U-^{13}C]maltose

Dextrin (20 mg) and [U-^{13}C]maltose (5 mg, 14 μmol) were digested using α-D-glucosidase for 2 d as described above. The reaction mixture was put into 1 mL of boiled water immediately and heated at 100 °C for 10 min, cooled to room temperature, and 0.1 mL of the mixture was subjected to an SEC-ELSD. This procedure was repeated 10 times. The fraction with t_R 16.0–18.0 min was combined and lyophilized (2.1 mg). The fraction was dissolved in 0.8 mL of water, and 0.1 mL of the solution was subjected to the ODS-HPLC separation for further purification. This procedure was repeated eight times. The fractions obtained were lyophilized, subjected to the MALDI-TOF-MS to estimate their DP, and subjected to the methylation analyses.

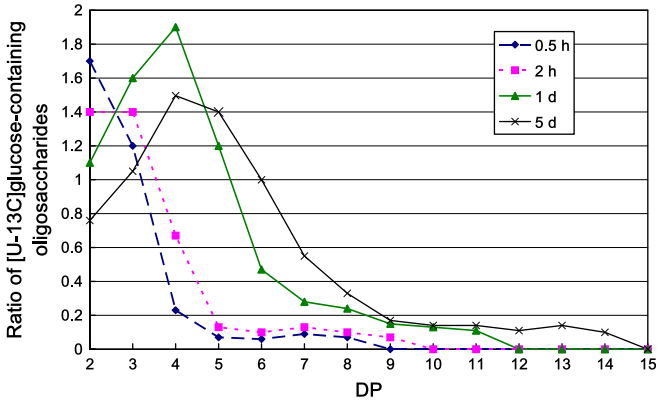

Fig. 2. Molar ratios of oligosaccharides containing at least one [U-^{13}C]glucosyl residue over oligosaccharides consisting of only [U-^{12}C]glucosyl residues during the digestion of dextrin and [U-^{13}C]maltose with *A. niger* α-D-glucosidase, estimated from the peak height of their cluster ion $[M+Na]^+$ detected with MALDI-TOF MS.

2.2.9. Methylation analysis of oligosaccharides isolated from the enzymatic digests of dextrin and [U-^{13}C]maltose

Methylation was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide according to procedure of Ciucanu and Kerek (1984), modified as reported by Needs and Selvendran (1993), except that the reaction was performed using microwave reaction apparatus (Discover, CEM, USA) at 30 °C for 0.5 h after sonication at room temperature for 10 min (Singh & Tiwari, 2008). The methylated products were hydrolyzed using 0.5 M HCl/aqueous 80% AcOH at 70 °C for 0.5 h, followed by reduction and acetylation to obtain partially methylated alditol acetates (PMAAs). PMAAs were identified by their fragment ions in EI-MS and by their relative retention times in GC. Their molar ratios were estimated from the peak area of total ion chromatogram of GC–MS. The relative molar ratios of [U-^{13}C]PMAA and [U-^{12}C]PMAA ([U-^{13}C]PMAA/[U-^{12}C]PMAA) were estimated from the peak areas of their characteristic fragment ions (*m*/*z*) as follows:

1,5-diacetyl-2,3,4,6-tetramethylglucitol (non-reducing end): *m*/*z* 209/205 Da,
1,4,5-triacetyl-2,3,6-trimethylglucitol (1,4-linked glucose): *m*/*z* 237/233 Da,
1,5,6-triacetylated-2,3,4-trimethylglucitol (1,6-linked glucose): *m*/*z* 192/189 Da.

2.2.10. Preparation of the α-D-glucosidase-treated starch for methylation analysis

Starch from waxy corn (5 mg) was suspended in 50 μL of water, and α-D-glucosidase solution (0.05 U) was added, and the reaction mixture was stirred for 15 h at 10 °C. When the reaction was complete, 1 mL of water was added to the reaction mixture, the mixture was centrifuged (12,000*g*) for 15 min, and the supernatant was discarded. These procedures were repeated four times, and the precipitate was lyophilized. The white powder of the enzyme-treated starch was obtained (1.6 mg) and subjected to methylation analysis.

2.2.11. Methylation analysis of starch

The sample (0.5 mg) and tetrabutylammonium bromide (5 mg) were suspended in 0.34 mL of 8% NaOH/DMSO and sonicated at 25 °C for 10 min. To the reaction mixture, 60 μL of CH_3I was added, and reacted using a microwave reaction apparatus at 30 °C for 2 h and released for 2 d at room temperature. The methylated products were dialyzed (MW 8000 cut-off, BioDesign Inc., USA) and lyophilized. These procedures were repeated once for complete methylation. The sample was hydrolyzed with 0.5 M HCl/aqueous 80% AcOH at 70 °C for 0.5 h, followed by reduction with $NaBH_4$, and acetylation with Ac_2O/pyridine to obtain PMAA. PMAAs were identified by their fragment ions in EI-MS and relative retention times in GC. Their molar ratios were estimated from the peak areas of the total ion chromatogram from GC–MS. Methylation analyses were performed three times for each sample, and the resulting spectra were averaged.

3. Results and discussion

Incubation of the mixture of dextrin and [U-^{13}C]maltose with *A. niger* α-D-glucosidase led to the formation of [U-^{13}C]glucosyl residue-containing oligosaccharides, which were detected using MAL-

DI-TOF MS as a cluster ion $[M+Na]^+$. As shown in Fig. 1, the *m/z* values of $[M+Na]^+$ of oligosaccharides containing $n \times$ {[U-^{13}C]glucosyl} residues could be observed, which were $6 \times n$ larger than that of [U-^{12}C]dextrin ($n = 1, 2\ldots$). The ratio of [U-^{13}C]glucosyl residue-containing oligosaccharides was shown in Fig. 2. At 0.5 h of incubation, [U-^{13}C]glucosyl residues were linked to dextrin with DP up to 8 (Figs. 1B and 2). As the reaction progressed for 5 d, oligomers with DP up to 14 contained [U-^{13}C]glucosyl residues were obtained (Figs. 1E and 2). When the incubation was performed for 7 d, [U-^{13}C]glucose-containing oligomer larger than DP14 was not detected (data not shown). It is suggested that the α-D-glucosidase could transfer α-D-glucose onto oligomers with DP up to 13. Oligomers were obtained from the reaction mixture of dextrin and [U-^{13}C]maltose digested for 2 d with size exclusion chromatography–evaporative laser scattering detector (SEC–ELSD) followed by ODS-HPLC, which were then subjected to methylation analysis. It was revealed that the major products up to DP6 were isomaltooligosaccharides, whereas the molar ratio of 1,5-diacetyl-2,3,4,6-tetramethylglucitol (non-reducing end), 1,4,5-triacetyl-2,3,6-trimethylglucitol {α-(1 → 4) linkage}, and 1,5,6-triacetylated-2,3,4-trimethylglucitol {α-(1 → 6) linkage} in DP7 was 1:5:1, the result suggesting that the enzyme transferred a glucosyl residue to the non-reducing end of maltooligomers with DP6 (Fig. 3). Thus, the main chain of the oligomers with DP larger than 6 consisted of α-(1 → 4) linkages.

The incubation of starch and [U-^{13}C]maltose with the enzyme was also examined, and the resulting starch was debranched with isoamylase followed by detecting with MALDI-TOF MS. In contrast with dextrin, no oligomer contained [U-^{13}C]glucosyl residues (data not shown); the result suggested that the enzyme did not transfer glucosyl residues to starch. Incubation of starch without [U-^{13}C]maltose with α-D-glucosidase was performed, and methylation analysis revealed that the ratio of the peak area of non-reducing ends over that of α-(1 → 4) linkages increased from 4.9% to 5.8% after the enzyme treatment, and 1,5,6-triacetylated-2,3,4-trimethylglucitol was not detected (data not shown). This result indicated that *A. niger* α-D-glucosidase did not transfer any glucosyl residues to starch but hydrolyzed it, and in this case, the enzyme did not hydrolyze α-(1 → 4) linkages of α-(1 → 4,6) branch points.

Chiba mentioned that *A. niger* α-D-glucosidase hydrolyzes soluble starch much slower than maltose, and the substrate specificity depends upon the subsite affinities (Chiba, 1997). *A. niger* α-D-glucosidase has eight subsites in the active site, and three of these subsites are effective for the binding of maltooligosaccharides. Rabbit muscle α-D-glucosidase effectively hydrolyzes α-glucans such as glycogen and soluble starch as well as maltose (Matsui, Sasaki, Takemasa, Kaneta, & Chiba, 1984). Thus, it was assumed that the differences in the activities of the transglucosidation reactions with α-glucan between *A. niger* α-D-glucosidase and rabbit muscle α-glucosidase are due to their difference in subsite affinities, and the non-reducing end of starch could hardly access the covalent β-glucosyl-enzyme intermediate (Withers, 2001) in the active site of *A. niger* α-D-glucosidase. This mechanism explains our results that *A. niger* α-D-glucosidase transferred [U-^{13}C]glucosyl residues to short dextrin with DP up to 13, and it catalyzed only the hydrolysis of high molecular α-glucans such as starch.

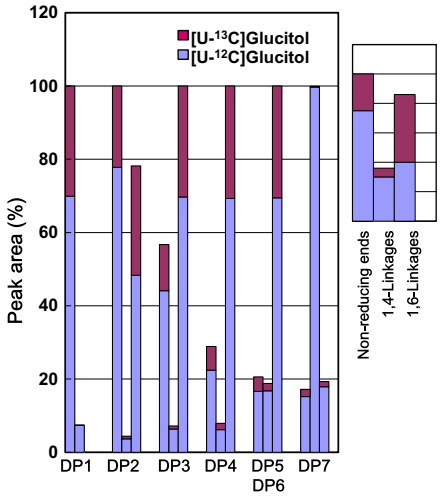

Fig. 3. Methylation analyses of the oligosaccharides obtained by preparative ODS-HPLC from the digests of dextrin and [U-^{13}C]maltose with *A. niger* α-D-glucosidase for 2 d.

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