Food Sci. Biotechnol. Vol. 18, No. 2, pp. 570 ~ 573 (2009)

RESEARCH NOTE

Food Science and Biotechnology

ⓒ The Korean Society of Food Science and Technology

Enzymatic Preparation of Maltooctaose-rich Mixture from Starch Using a Debranching Enzyme of *Nostoc punctiforme*

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Abstract The debranching enzyme of *Nostoc punctiforme* (NPDE) is a novel enzyme that catalyzes the hydrolysis of α-1,6-glycosidic linkages in starch, followed by the sequential hydrolysis of α-1,4-glycosidic linkages. The debranching activity of NPDE is highly specific for branched chains with a degree of polymerization (DP)>8. Moreover, the rate of hydrolysis of α-1,4-linkages by NPDE is greatly enhanced for maltooligosaccharides (MOs) with a DP>8. An analysis of reaction mixtures containing various starches revealed the accumulation of maltooctaose (G8) with glucose and maltose. Based on the novel enzymatic properties of NPDE, an MO mixture containing more than 60% G8 with yield of 18 g G8 for 100 g starch was prepared by the reaction of NPDE with soluble starch, followed by ethanol precipitation and gel permeation chromatography (GPC). The yield of the G8-rich mixture was significantly improved by the addition of isoamylase. In summary, a 4-step process for the production of a G8-rich mixture was developed involving the enzymatic hydrolysis of starch by NPDE.

Keywords: *Nostoc punctiforme*, cyanobacteria, debranching enzyme, maltooctaose-rich mixture, maltooligosaccharide

Introduction

Maltooligosaccharides (MOs) are used in various food industries due to their beneficial characteristics, including the modulation of hygroscopicity, viscosity, sweetness, food stability, soft sensory taste, and water activity (1,2). MOs have traditionally been produced using α-amylases with starch as the substrate; however, the products usually contain MOs with various degrees of polymerization (DPs), and the composition of the products is inconsistent due to difficulty in controlling the progress of the reaction. Therefore, the physicochemical properties of the products are likely to change according to the batch. The methods used to produce MOs with specific DPs for use as food ingredients generate products with a consistent composition and physicochemical properties (1). Several novel enzymes that can produce MOs with a narrow DP range have been developed, including maltotetraose-, maltopentaose-, and maltohexaose-forming amylases from several bacterial sources (3-7). To the best of our knowledge, however, no amylase forming an MO longer than G6 from starch has been reported. Oguma *et al.* (8) and Uchida *et al.* (9) developed a novel method for the production of maltohexaose (G6), maltoheptaose (G7), and maltooctaose (G8) via the enzymatic hydrolysis of cyclodextrinase (CDase) from *Bacillus sphaericus* with α-, β-, and γ-cyclodextrin (CD) as the substrate, respectively. However, a considerable amount of smaller MOs was produced; thus, further purification was needed. Recently, the enzymatic preparation of G6, G7, and G8 from α-, β-, and γ-CD, respectively, using a thermostable amylase from *Pyrococcus furiosus* was reported (10). The yield achieved using this process exceeded 90% due to the strong preference of the enzyme for CDs over MOs. However, CDs, particularly γ-CD, are relatively expensive substrates compared with starch.

Recently, we reported a novel debranching enzyme from *Nostoc punctiforme* PCC73102 (NPDE) having a dual hydrolysis activity toward both α-1,6- and α-1,4- glycosidic linkages of the substrate (11). NPDE hydrolyzed α-1,6-glycosidic linkage of starch, followed by sequential hydrolysis of α-1,4-glycosidic linkage. G8 has been found to be an intermediate during the hydrolysis of polysaccharides such as soluble starch, amylopectin, and amylose by the enzyme due to its high specificity toward large MO with DP>8.

Here, we described a novel method for the preparation of a G8-rich mixture using the debranching enzyme of *N. punctiforme* (NPDE) with a dual hydrolysis activity for starch substrate.

Materials and Methods

Purification of recombinant NPDE Recombinant *Escherichia coli* MC1061 cells [F^-, *araD*139, *recA*13, D(*araABC-leu*)7696, *gal*U, *gal*K, *lac*X74, *rpsL*, *thi*, *hsdR*2, and *mcrB*] carrying pTKNPDE6xH were cultured overnight in 1 L of Luria-Bertani broth [1%(w/v) Bacto-

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Received October 8, 2008; Revised November 21, 2008; Accepted November 25, 2008

tryptone, 0.5%(w/v) yeast extract, and 0.5%(w/v) NaCl] supplemented with 20 mg/mL kanamycin (11). The recombinant NPDE was purified using nickel-nitrilotriacetic acid affinity chromatography (Qiagen, Hilden, Germany) as previously described (12). The eluted target proteins were dialyzed against 50 mM sodium phosphate buffer (pH 7.5). The protein concentration was determined according to the Bradford method (13) using bovine serum albumin as the standard.

Enzyme assay The hydrolytic activity of NPDE was assayed at 40°C in 50 mM sodium phosphate buffer (pH 7.5) using 0.5%(w/v) soluble starch (Showa Chemical Co., Ltd., Osaka, Japan) as the substrate and 3,5-dinitrosalicylic acid as described by Miller (14). One unit of enzymatic activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars/min.

Thin layer chromatography (TLC) To examine the activity pattern of NPDE, 0.5 mL of purified enzyme was incubated with 0.5 mL of 1%(w/v) substrate in 50 mM sodium phosphate buffer (pH 7.5) at 40°C for 18 hr. Soluble starch (Showa Chemical Co.), amylopectin from potato, corn starch, and rice starch (Sigma-Aldrich, St. Louis, MO, USA) were used as substrates. The reaction products were analyzed by TLC on Whatman K5F silica gel plates (Whatman, Maidstone, UK) with a developing solvent of 1-propyl alcohol-ethyl acetate-water (6:2:3, v/v/v). The TLC plate was visualized as described previously (10).

Preparation of a G8-rich mixture from starch The method used to prepare the G8-rich mixture is shown in Fig. 1. Soluble starch (5%, w/v) in 50 mM sodium phosphate buffer (pH 7.5) was incubated with 0.05 U of NPDE at 40°C for 18 hr. The reaction was stopped by boiling for 5 min. MOs longer than G8 and all unreacted substrate were removed by ethanol precipitation. Four volumes of ethanol were then added to the reaction mixture and the precipitate was discarded after centrifugation at 13,000×g for 20 min. The supernatant was then concentrated using a Savant AES 1010 SpeedVac (GMI Inc., Ramsey, MN, USA). To remove all short MOs, the concentrated supernatant was subjected to preparative high performance liquid chromatography (HPLC) (LC-918; JAI Ltd., Tokyo, Japan) using a preparative gel permeation chromatography (GPC) column (JAIGEL W-251, 2×50 cm; JAI Ltd.) and a refractive index detector (RI detector-50; JAI Ltd.).

Preparation of G8 from isoamylase-treated starch Soluble starch (0.5%, w/v) in 50 mM sodium acetate buffer (pH 4.3) was reacted with isoamylase (0.36 U/mg) at 60°C for 60 hr. The reaction was stopped by boiling for 10 min. Next, the pH of the mixture was raised to 7.5 (the optimum pH for NPDE) by the addition of 500 mM disodium phosphate solution. The isoamylase-treated starch was then incubated with 0.025 U of NPDE at 40°C for 32 hr. Ethanol precipitation followed by HPLC was subsequently performed for further purification.

High performance anion exchange chromatography (HPAEC) The composition of the reaction products was assayed by HPAEC using a pulsed amperometric detector (ED40; Dionex Co., Sunnyvale, CA, USA). The system was equipped with a CarboPac PA-1 column (0.4×25 cm, 10 μm particle diameter, Dionex Co.) and run with a linear gradient of 0-0.6 M sodium acetate in 0.15 M NaOH with a flow rate of 1 mL/min.

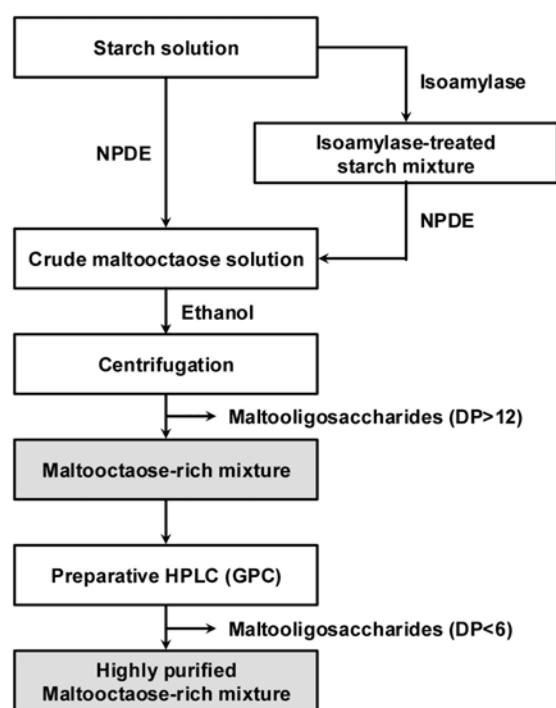

Fig. 1. Method used for the enzymatic preparation of a maltooctaose-rich mixture using NPDE.

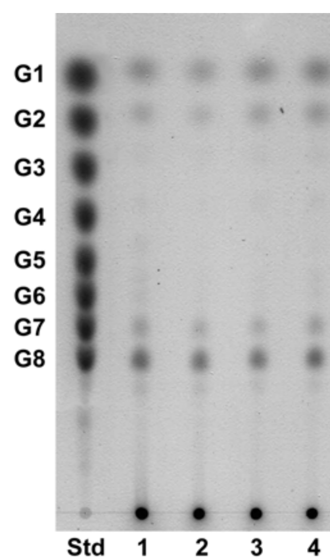

Fig. 2. Analysis of the products generated from various starches by NPDE using TLC. Each substrate (0.5%, w/v) was incubated with NPDE at 40°C for 18 hr. Std, maltooligosaccharide standards (from glucose [G1] to maltooctaose [G8]); lane 1, amylopectin from potato; lane 2, corn starch; lane 3, rice starch; lane 4, soluble starch.

Results and Discussion

Preparation of a G8-rich mixture from starch with NPDE Previously, we reported that NPDE catalyzed the hydrolysis of α-1,4- and α-1,6-glycosidic linkages in such polysaccharides as glycogen, amylopectin, and starch (11), and that the hydrolytic profile for starch was unique. An analysis of the reaction mixture by TLC revealed the accumulation of a significant amount of G8 with glucose (G1) and maltose (G2), whereas G7 and maltononaose (G9) were produced in trace amounts in reaction mixtures containing NPDE with various starches as the substrate (Fig. 2). In addition, except for G1 and G2, only trace amounts of MOs smaller than G7 were detected. Thus, this reaction may be used to prepare a G8-rich mixture via the hydrolysis of starch, G1, and G2 by NPDE. MOs longer than G13 were successfully removed from the reaction mixture by ethanol precipitation (Table 1, Fig. 3). For

Table 1. Composition (%) of the maltooctaose (G8)-rich mixture prepared by the process shown in Fig. 1

Maltooligo-saccharide	Reaction of NPDE with starch			Reaction of NPDE with isoamylase-treated starch		
	Reaction mixture	Ethanol precipitation	Prep. HPLC	Reaction mixture	Ethanol precipitation	Prep. HPLC
G1	14	23	ND[1)]	10	16	ND
G2	10	15	ND	5	10	ND
G3	1	3	ND	1	3	ND
G4	2	3	ND	2	4	ND
G5	1	1	ND	2	3	ND
G6	1	2	1	3	4	1
G7	7	8	17	6	12	16
G8	**30**	**35**	**68**	**32**	**40**	**60**
G9	5	5	11	11	5	15
G10	2	2	3	3	1	5
G11	1	1	Trace	3	1	2
G12	2	1	Trace	3	1	1
G13-G20	24	1	Trace	19	Trace	Trace
Yield (g G8/100 g starch)	**5.4**	**4.3**	**4.1**	**26**	**20**	**18**

[1)]Not detected.

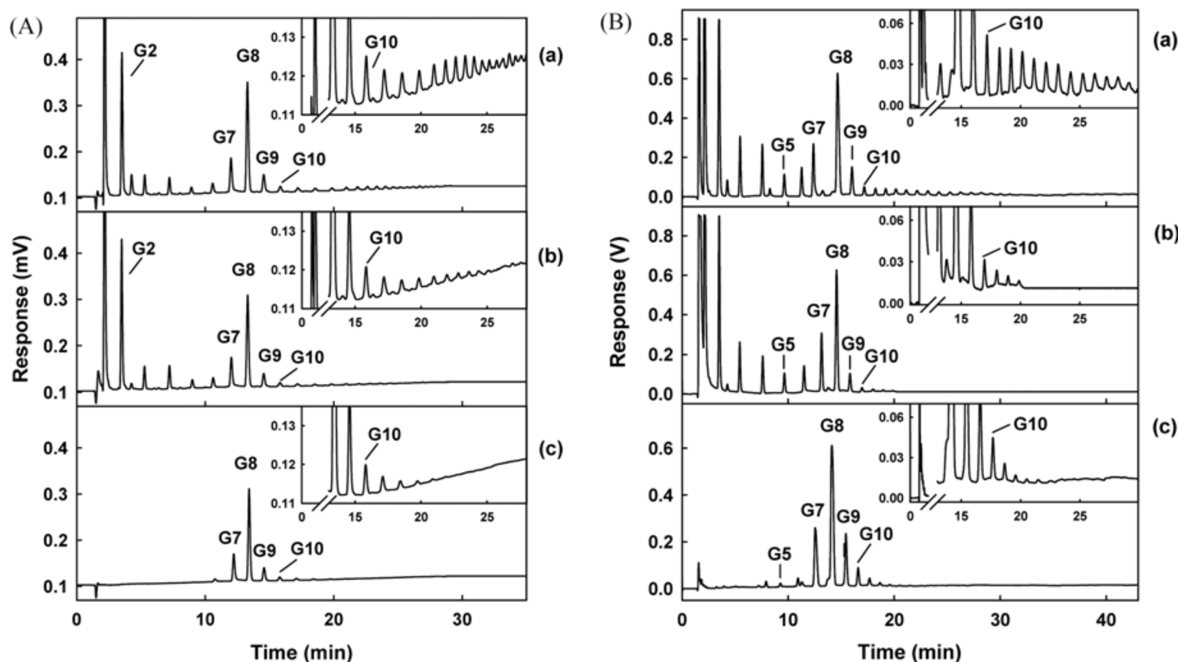

Fig. 3. HPAEC of a maltooctaose-rich mixture prepared from soluble starch (A) and an isoamylase-treated soluble starch mixture (B) using NPDE. The reaction mixture (a in panels A and B) was purified by ethanol precipitation (b in panels A and B) followed by gel permeation chromatography (c in panels A and B). For each reaction, 5%(w/v) soluble starch or isoamylase-treated soluble starch was incubated with NPDE at 40°C for 18 and 32 hr, respectively. The inner panels enlarged Y axis to make the base line of the larger panels clear.

further purification, preparative HPLC using a GPC column was performed. An analysis of the products by HPAEC revealed that all MOs shorter than G6 were removed, while the G8 content was increased from 35 to 68% by ethanol precipitation and GPC, respectively (Table 1, Fig. 3A, b and c).

Preparation of a G8-rich mixture from isoamylase-treated starch with NPDE Despite the high level of G8 in the final product, the yield was only 6% of the initial amount of substrate. As reported previously, NPDE catalyzed the debranching of the α-1,6-glycosidic linkages in the starch first, followed by the hydrolysis of the resulting MOs. During the debranching process, NPDE was shown to have a strong preference for MOs longer than G8 (11). Furthermore, the k_{cat}/K_m values for NPDE toward α-1,4-glycosidic linkages were found to be higher than those for MOs smaller than G8. As a result, the branched chains may be hydrolyzed such that chains with a DP>8 are reduced faster than those with a DP<8 (11). However, the strong dependence of NPDE on branch chain length may limit the production of MOs from starch. To enhance the yield, isoamylase was added to aid in debranching. Using isoamylase-treated starch as the substrate, the amount of G8 was significantly increased whereas that of unreacted substrate was dramatically decreased (Table 1). After ethanol fractionation and GPC, the G8 content was enhanced up to 60% of the total MOs in the final product (Table 1, Fig. 3B, b and c). The G8 content was slightly less than that in the previous reaction because the proportion of MOs longer than G8 was increased in the final product (Table 1). However, the production yield of the G8-rich mixture from isoamylase-treated starch was improved (approximately 30%).

In conclusion, the accumulation of G8 in the reaction mixture was due to both the debranching and hydrolytic activities of NPDE and the enzyme's substrate length preference.

Acknowledgments

This work was supported by a Grant from ARPC (Agricultural R&D Promotion Center), Ministry for Food, Agriculture, Forestry, and Fisheries, Korea. Choi J-H was supported by a scholarship from the Brain Korea 21 Project.

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