Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Research 341 (2006) 420–424
Carbohydrate RESEARCH

Note

Enzymatic preparation of maltohexaose, maltoheptaose, and maltooctaose by the preferential cyclomaltooligosaccharide (cyclodextrin) ring-opening reaction of *Pyrococcus furiosus* thermostable amylase

Sung-Jae Yang,[a] Hee-Seob Lee,[a] Jung-Woo Kim,[a] Myoung-Hee Lee,[a] Joong-Hyuck Auh,[a] Byong-Hoon Lee[b] and Kwan-Hwa Park[a,*]

[a]*Center for Agricultural Biomaterials and Department of Food and Animal Biotechnology, School of Agricultural Biotechnology, Seoul National University, Shillim-dong, Kwanak-gu, Seoul 151-921, Republic of Korea*
[b]*Department of Food Science and Agricultural Chemistry, McGill University, 21,111 Lakeshore Road, Ste-Anne-de-Bellevue, Quebec, Canada H9X 3V9*

Received 11 July 2005; accepted 14 November 2005
Available online 20 December 2005

Abstract—Specific-length maltooligosaccharides, particularly maltohexaose, maltoheptaose, and maltooctaose, were prepared from cyclomaltooligosaccharides (cyclodextrins, CDs) by the preferential cyclodextrin ring-opening reaction of an amylolytic enzyme from *Pyrococcus furiosus*. The enzyme primarily produces maltohexaose, maltoheptaose, and maltooctaose by hydrolyzing α-, β-, and γ-CD, respectively. This study aims to develop a high-efficiency synthesis of specific maltooligosaccharides at high-purity.

α-/β-/γ-cyclodextrins $\xrightarrow[\text{ring-opening reaction}]{\text{enzymatic}}$ maltohexaose/maltoheptaose/maltooctaose

The thermostable amylase from *Pyrococcus furiosus* (PFTA) acts mainly by opening cyclomaltooligosaccharides (cyclodextrins, CDs) to produce corresponding maltooligosaccharides, but it does not have substantial hydrolyzing activity on the resulting open-chain maltooligosaccharides. Using PFTA oligosaccharides such as maltohexaose (G6), maltoheptaose (G7), and maltooctaose (G8) and a mixture rich in them can be readily prepared in the laboratory or on an industrial scale.

In recent years, amylases for specifically producing maltooligosaccharides have been employed on an industrial scale and widely used in foods and pharmaceuticals.[1,2] Maltooligosaccharides and their derivatives with defined structures are used in clinical laboratories to measure alpha-amylase activity in human serum or urine. The substrate for determining alpha-amylase activity is preferably a maltooligosaccharide composed of seven glucose units with modified reducing- and non-reducing-terminal glucoses.[3–5] The demand for maltooligosaccharides such as maltohexaose, maltoheptaose, and maltooctaose has therefore increased. However, the production of these maltooligosaccharides is relatively tedious and expensive.[6]

In the enzymatic process for starch conversion, some amylases can produce maltodextrins with a high content of specific maltooligosaccharides. Amylases, which mainly produce G6 can be either *exo*- or *endo*-type enzymes. The enzymes act on starch to produce G6 in a relatively large quantity, and the G6 is subsequently

* Corresponding author. Tel.: +82 2 880 4852; fax: +82 2 873 5095; e-mail: parkkh@plaza.snu.ac.kr

0008-6215/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2005.11.031

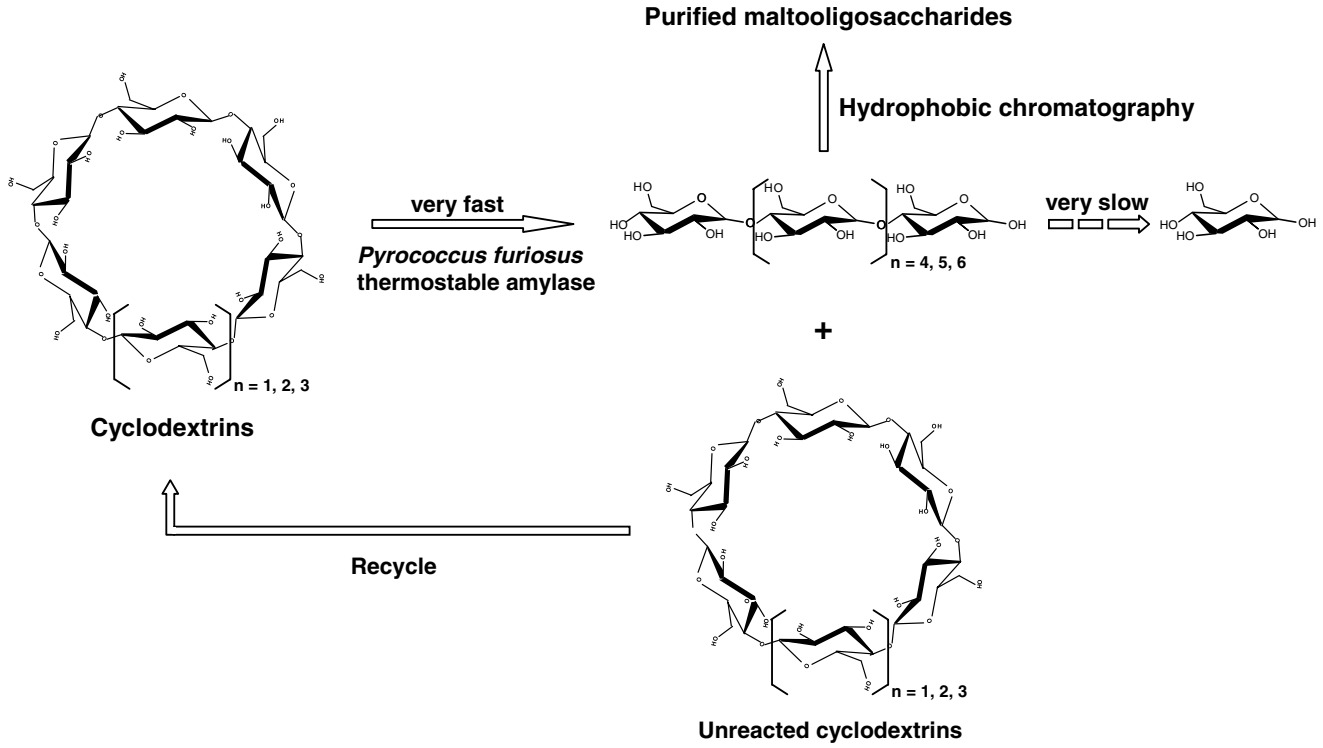

Figure 1. A simplified scheme for the enzymatic preparation of G6, G7, and G8.

separated from the mixture containing other maltooligosaccharides.[7] Using this process, it is difficult to achieve a high purity of specific maltooligosaccharides.

The preparation of maltoheptaose and maltooctaose from β-cyclodextrin (β-CD) and γ-cyclodextrin (γ-CD) was investigated using cyclomaltodextrinase.[8] The enzyme did not hydrolyze β-CD more effectively than linear oligosaccharides, thereby producing a significant amount of smaller maltoooligosaccharides. Uchida et al.[9] developed a synthetic process for producing G8 and maltononaose (G9) from CDs by the coupling reaction of cyclomaltodextrinase. However, the simultaneous preparation of G6, G7, and/or G8 by hydrolysis or a coupling reaction was accompanied by the production of a substantial amount of other maltooligosaccharides such as glucose (G1), maltose (G2), and maltotriose (G3) that had to be removed by subsequent purification.

We previously reported that a novel amylolytic enzyme from *P. furiosus* was able to open the CD-ring with extremely high efficiency and that the hydrolysis rate of the resulting linear maltooligosaccharide was far slower than that seen with cyclodextrins.[10] The enzyme could primarily produce maltoheptaose by hydrolyzing β-CD, resulting in the accumulation of G7.

In this paper, we describe an efficient enzymatic preparation of maltooligosaccharides including G6, G7, and G8 from CDs by the preferential CD-ring-opening reaction of the enzyme and subsequent purification. A simplified version of the process for the enzymatic preparation of G6, G7, and G8 is shown in Figure 1.

Table 1. Kinetic parameters for the hydrolysis of various substrates

Substrate	k_{cat}[a] (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
α-Cyclodextrin	605 ± 17	4.42 ± 0.42	136.9 ± 13.6
β-Cyclodextrin	501 ± 11	4.68 ± 0.39	107.1 ± 9.2
γ-Cyclodextrin	481 ± 13	6.82 ± 0.54	70.5 ± 5.9
Maltohexaose	255 ± 6	28.43 ± 2.78	9.0 ± 0.9
Maltoheptaose	140 ± 3	12.31 ± 1.55	11.4 ± 1.5
Maltooctaose	107 ± 4	15.62 ± 1.92	6.9 ± 0.9

[a] Kinetic parameters were determined in 50 mM NaOAc buffer (pH 4.5) at 85 °C.

The ring-opening rates of CDs were compared with the further hydrolysis rate of the resulting maltooligosaccharides (Table 1). The K_m values for linear maltooligosaccharides such as G6, G7, and G8 were higher than those for corresponding CDs (Table 1). The results indicated that linearized maltooligosaccharides do not efficiently bind to the catalytic site of the enzyme. The k_{cat}/K_m value for β-CD ring opening was 10 times higher than that for hydrolysis of G7. The relevant value for β-CD by *Bacillus sphaericus* cyclodextrinase was three times larger than that for G7.[8] Likewise the values of k_{cat}/K_m for α- and γ-CD were 15- and 10-fold higher, respectively, than those of the corresponding linear maltooligosaccharides, G6 and G8.

TLC analysis of the reaction products showed that G6, G7, and G8 were predominant initial products (Fig. 2). The G7 content was about 90% after a 10 min reaction, and smaller maltooligosaccharides such as G1, G2, G3, maltotetraose (G4), and maltopentaose

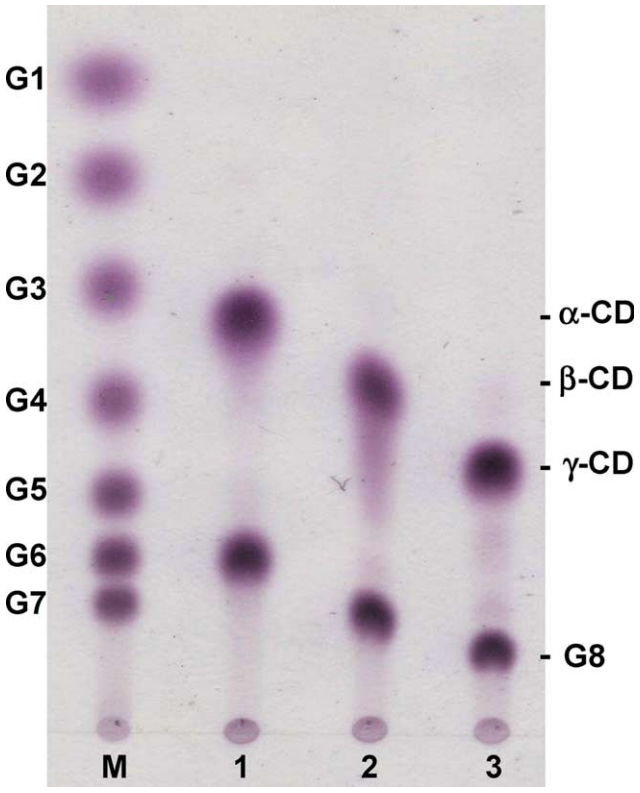

Figure 2. TLC analysis of the reaction products formed from various cyclodextrins by PFTA. Lane M, maltooligosaccharide standards (glucose to maltoheptaose); lane 1, α-CD; lane 2, β-CD; lane 3, γ-CD. PFTA (0.5 U) was reacted with various cyclodextrins (CDs) at a concentration of 1% (w/v) in 50 mM NaOAc buffer (pH 4.5) at 85 °C for 10 min.

(G5) formed in trace amounts. The remaining β-CD could easily be removed either by hydrophobic chromatography or by precipitation with an organic solvent such as toluene. When the reaction mixture was purified by hydrophobic chromatography, the purity of G7 was greater than 90%. Of the other products, G2, G3, G4, G5, and G6 were found to be present in amounts less than 10% by high-performance anion-exchange chromatography (HPAEC) analysis (Fig. 3). Similarly, our enzymatic preparation method for G7 from β-CD was also useful for the preparation of G6 and G8 with a purity of higher than 90% from α-CD and γ-CD, respectively (Fig. 1).

The time course of the reaction using PFTA and β-CD showed that the yield of G7 was influenced by the reaction time. (See Supplementary data section.) Thus, to achieve the high purity and yield of G7, it is necessary to optimize reaction conditions such as the reaction time and enzyme concentration for preventing further hydrolysis of G7.

In conclusion, PFTA is a very special enzyme that can easily open up the certain cyclodextrin molecules to produce specific-length maltooligosaccharides, particularly maltohexaose, maltoheptaose, and maltooctaose. Those maltooligosaccharides are in demand with claims of specific applications in food and pharmaceutical industries. The discovery of novel enzymes, for example, PFTA, producing new types of oligosaccharides would enable to provide a variety of functional carbohydrate compounds.

1. Experimental

1.1. General methods

Cyclodextrins (α-, β-, and γ-cyclodextrin), maltohexaose, and maltoheptaose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thin-layer chromatogra-

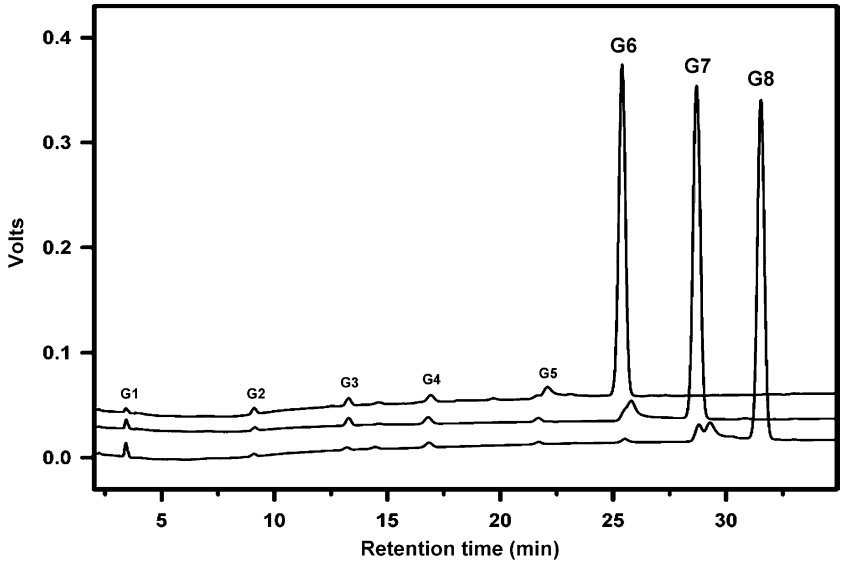

Figure 3. HPAEC chromatogram of G7-rich maltooligosaccharide mixture produced by the ring-opening reaction of PFTA. PFTA was reacted with 1% (w/v) β-CD at 85 °C; unreacted β-CD in the reaction mixture was removed by hydrophobic chromatography.

phy (TLC) was performed on Whatman K5F silica gel plates (Whatman, Maidstone, United Kingdom) with 3:1:1 2-PrOH–EtOAc–H_2O as the solvent system. After irrigating twice, the TLC plate was dried and visualized by dipping it into a solution containing 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in MeOH and then heating it for 10 min at 110 °C.[11] High-performance anion-exchange chromatography (HPAEC) was performed on a Dionex (Sunnyvale, CA, USA) DX-500 system with a pulsed amperometric detector (Dionex). The system was equipped with a CarboPac PA-100 column (4 × 250 mm, Dionex) and run with a gradient of 0–0.6 M NaOAc in 0.15 M NaOH. The protein concentration was determined according to the Bradford method,[12] with bovine serum albumin as a standard.

1.2. Enzyme assay

PFTA was purified from a broth culture of an *E. coli* BL21(DE3) transformant harboring pETPFTA-6h, as described previously.[10] The activity of PFTA was assayed at 85 °C in 50 mM NaOAc buffer (pH 4.5) using 3,5-dinitrosalicylic acid (DNS), as described by Miller.[13] The reaction mixture was composed of 150 μL of 1% (w/v) β-cyclodextrin (β-CD) solution as a substrate and 150 μL of enzyme solution. The mixture was incubated at 85 °C for 10 min to facilitate the enzymatic reaction, and the reaction was terminated by quenching on ice. One unit (U) of enzyme activity was defined as the amount of enzyme that split 1 mmol equivalent of glycosidic bonds in the substrate per min under the reaction conditions.

1.3. Preparation of maltoheptaose from β-cyclodextrin reaction mixture

PFTA (0.5 U) was added to a solution of β-CD (10 mg/mL) in 50 mM NaOAc buffer (pH 4.5). The reaction mixture was incubated at 85 °C for 10 min, and the reaction was stopped by boiling for 1 h. To absorb the unreacted β-CD in the reaction solution, butyl-Sepharose (Pharmacia) was used as an adsorbent for hydrophobic chromatography. The reaction concentrate was passed through a butyl-Sepharose column (2.5 cm × 7 cm with 15 mL packed resin) equilibrated with H_2O. Afterwards, the linear maltooligosaccharides were eluted from the column using H_2O. The eluted fraction containing maltoheptaose as the major hydrolysis product of β-CD was collected and concentrated by rotary evaporation.

1.4. Measurement of kinetic parameters

Kinetic parameters of PFTA for cyclodextrins (α-, β-, and γ-cyclodextrin), maltohexaose, maltoheptaose, and maltooctaose were determined. Maltooctaose was prepared from γ-cyclodextrin by the ring-opening reaction with PFTA. To further purify maltooctaose, paper chromatography was carried out in the descending mode on Whatman 3MM paper (20 × 20 cm). Initially, the paper was irrigated with 65:35 PrOH–H_2O as the solvent system for 24 h. After air-drying the paper, a second irrigation was performed with 3:1:1 2-PrOH–EtOAc–H_2O for 24 h. Spots on the paper were located using an $AgNO_3$ reagent to verify the separation of maltooctaose. The paper was sectioned and eluted with deionized H_2O, and the purified maltooctaose was concentrated by rotary evaporation and used for evaluating kinetic parameters. The substrate concentrations for the kinetic analysis ranged from 0.4 to 14 mM for cyclodextrins and 1.0 to 25 mM for linear maltooligosaccharides. The reaction mixture was composed of 300 μL enzyme and 300 μL of substrate in 50 mM NaOAc buffer (pH 4.5). The enzyme was 4.5 nM for cyclodextrins and 0.2 μM for linear maltooligosaccharides. Samples (50 μL) at 85 °C were taken at intervals of 90 s, and the reaction was immediately stopped by adding an equal volume of 0.1 M HCl on ice. After neutralizing by adding an equal volume of 0.1 N NaOH, the amount of hydrolyzed substrate was quantitatively analyzed by HPAEC. Kinetic data were transformed to Lineweaver–Burk plots with the SigmaPlot program (version 5.0; SPSS Inc., Chicago, IL). The K_m values were calculated from the slopes of the curves, and the catalytic turnover values (k_{cat}) were calculated by dividing the maximal reaction velocities by the total amount of enzyme in the reaction mixture.

Acknowledgments

This study was supported partly by the Korean Ministry of Science and Technology through the 21st Century Frontier R&D Programs in Microbial Genomics & Applications Center (Grant No. MG05-0301-4-0) and partly by Korea Research Foundation Grant (KRF-2004-005-F00054). We acknowledge the financial support of the Brain Korea 21 Project in the form of a scholarship to S.-J. Yang.

Supplementary data

Supplementary data (time-course data for the PFTA reactions) associated with this article can be found in the online version at doi:10.1016/j.carres.2005.11.031.

References

1. Nakano, M.; Chaen, H.; Sugimoto, T.; Miyake, T. U.S. Patent 5,739,024, 1998.

2. Nakakuki, T. *Trends Glycosci. Glycotechnol.* **2003**, *15*, 57–64.
3. Ogawa, K.; Murata, T.; Usui, T. *Carbohydr. Res.* **1991**, *212*, 289–294.
4. Rauscher, E.; Schaich, E.; Neumann, U.; Wahlefeld, A.; Gruber, W.; Empl, B. U.S. Patent 4,818,692, 1989.
5. Rauscher, E.; Neumann, U.; Wahlefeld, A. W.; Hagen, A.; Gruber, W.; Ziegenhorn, J.; Schaich, E.; Deneke, U.; Michal, G.; Weimann, G. U.S. Patent 5,108,913, 1992.
6. Hashim, S. O.; Delgado, O. D.; Martínez, M. A.; Hatti-Kaul, R.; Mulaa, F. J.; Mattiasson, B. *Enzyme Microb. Technol.* **2005**, *36*, 139–146.
7. Tsukamoto, A.; Kimura, K.; Ishii, Y.; Takano, T.; Yamane, K. *Biochem. Biophys. Res. Commun.* **1998**, *151*, 25–31.
8. Oguma, T.; Kikuchi, M.; Mizusawa, K. *Biochim. Biophys. Acta* **1990**, *1036*, 1–5.
9. Uchida, R.; Nasu, A.; Tobe, K.; Oguma, T.; Yamaji, N. *Carbohydr. Res.* **1996**, *287*, 271–274.
10. Yang, S. J.; Lee, H. S.; Park, C. S.; Kim, Y. R.; Moon, T. W.; Park, K. H. *Appl. Environ. Microbiol.* **2004**, *70*, 5988–5995.
11. Robyt, J. F.; Mukerjea, R. *Carbohydr. Res.* **1994**, *251*, 187–202.
12. Bradford, M. *Anal. Biochem.* **1976**, *72*, 248–254.
13. Miller, G. L. *Anal. Chem.* **1959**, *31*, 426–428.