Volume 290, number 1,2, 13–16 FEBS 10154 September 1991

ADONIS 001457939100865Z

Hydrolysis of branched cyclodextrins by a cyclodextrin-hydrolyzing enzyme from *Bacillus sphaericus* E-244

Tetsuya Oguma[1], Mamoru Kikuchi[2] and Kiyoshi Mizusawa[2]

[1]*Noda Institute for Scientific Research, 399 Noda, Noda City, Chiba Pref. 278, Japan* and [2]*Research and Development Division, Kikkoman Corporation, 399 Noda, Noda City, Chiba Pref. 278, Japan*

Received 15 May 1991; revised version received 13 July 1991

The action of a cyclodextrin-hydrolyzing enzyme from *Bacillus sphaericus* E-244 on branched α- and β-cyclodextrins was investigated. Glucosyl-α-cyclodextrin (6-*O*-α-D-glucosylcyclomaltohexaose) and maltosyl-α-cyclodextrin (6-*O*-α-D-maltosylcyclomaltohexaose) were hydrolyzed to 6^3-*O*-α-D-glucosylmaltohexaose and 6^3-*O*-α-D-maltosylmaltohexaose, respectively. Glucosyl-β-cyclodextrin (6-*O*-α-D-glucosylcyclomaltoheptaose) and maltosyl-β-cyclodextrin (6-*O*-α-D-maltosylcyclomaltoheptaose) were also transformed mainly to 6^4-*O*-α-D-glucosylmaltoheptaose and 6^4-*O*-α-D-maltosylmaltoheptaose, respectively. These results suggest that the cyclodextrin-hydrolyzing enzyme cleaves branched α- and β-cyclodextrins at an α-1,4 linkage which is located furthest from the branching point on the cyclodextrin ring.

Branch; Cyclodextrin; Cyclomaltodextrinase; Hydrolysis; Maltooligosaccharide; *Bacillus sphaericus*

1. INTRODUCTION

Cyclo/dextrins (CDs) are cyclic oligosaccharides composed of 6, 7, 8 or more glucose residues linked to one another by α-1,4 linkages [1], which are resistant to hydrolysis by α-amylase [2]. We have reported that a CD-hydrolyzing enzyme from *Bacillus sphaericus* E-244 can hydrolyze β-CD most rapidly among α-glucans [3], and shows different substrate specificity, optimum pH and molecular weight from known cyclomaltodextrinases (EC 3.2.1.54) (CDases) of *Bacillus macerans* [4] and *B. coagulans* [5].

Recently, we noticed that this enzyme can hydrolyze not only ordinary CDs but also branched CDs such as those having a stub of a glucose or maltose residue at the C-6 position in one of the α-1,4 linked glucose units of the CD rings. Although with regard to Taka-amylase A from *Aspergillus oryzae* [6], α-amylase from *Thermoactinomyces vulgaris* [6], and CD glucanotransferase (EC 2.4.1.19) (CGTase) from *Bacillus macerans* [7], the sites of branched CD cleaved by the actions of these enzymes have been investigated, there has been no report on those of known CDases.

In this study, we analyzed the branched oligosaccharides formed from branched CDs by CD-hydrolyzing enzyme, using the actions of exo-type amylases, and deduced the main sites of branched CDs cleaved by the hydrolase.

Abbreviations: CDs, cyclodextrins; CDases, cyclomaltodextrinases; CGTase, cyclodextrin glucanotransferase; G_2, G_3, G_4, G_5, G_6, and G_7; maltose, maltotriose, maltotetraoase, maltopentaose, maltohexaose, and maltoheptaose; G-, M-α-CD; glucosyl-, maltosyl-α-cyclodextrin; G-, M-β-CD; glucosyl-, maltosyl-β-cyclodextrin; 6^n-α-G-G_3, -G_4, -G_5, -G_6, and -G_7; 6^n-*O*-α-D-glucosyl-maltotriose, -maltotetraose, -maltopentaose, -maltohexaose, and -maltoheptaose; 6^n-α-M-G_3, -G_4, -G_5, -G_6 and -G_7; 6^n-*O*-α-D-maltosyl-maltotriose, -maltotetraose, -maltopentaose, -maltohexaose, and -maltoheptaose

Correspondence address: T. Oguma, Noda Institute for Scientific Research, 399 Noda, Noda City, Chiba Pref. 278, Japan. Fax: (81) (471) 23 5959.

2. MATERIALS AND METHODS

2.1. *Carbohydrates*

Branched CDs were kindly provided by Dr K. Hara of Ensuiko Sugar Refining Co., Ltd., Japan. Panose (6^2-*O*-α-D-glucosylmaltose) and isomaltose were purchased from Tokyo Kasei, Japan. D-Glucose and a series of maltooligosaccharides (from G_2 to G_7) used as standard for HPLC analysis were purchased from Wako Chemicals, Japan, and Seishin Pharmaceuticals, Japan, respectively.

2.2. *Enzymes*

CD-hydrolyzing enzyme was prepared and purified as described previously [3]. G_3-forming amylase was kindly provided by Dr K. Wako of Nikken Kagaku, Japan. The action of the amylase on α-glucans proceeds from the non-reducing ends of the substrates to form the α anomer of G_3, but does not skip the branching points of branched substrates [8,9]. Isopullulanase (EC. 3.2.1.57, pullulan 4-glucanohydrolase) was kindly donated by Associate Professor Y. Sakano of Tokyo Noko University. This enzyme hydrolyzes pullulan to produce isopanose (6-*O*-α-D-maltosylglucose) and is useful for analyzing the structure of glucosyl branched oligosaccharides [10]. β-Amylase, CGTase and pullulanase (EC 3.2.1.41) were purchased from Sigma, Amano Pharmaceuticals, and Boehringer Mannheim, respectively.

2.3. *Standard branched maltooligosaccharides*

The standard branched maltooligosaccharides were prepared from 20 mM branched α-CDs and 200 mM glucose by the coupling reaction of CGTase (CGTase 1.2 U, pH 5.8, temperature 40°C, incubation time 2 h) [7] and analyzed by HPLC using a TSK gel amide 80 column

following the analytical conditions described previously [3]. The branched sugars produced by the action of CD-hydrolyzing enzyme or various exo-type amylases were identified by their retention times on HPLC analysis.

2.4. *Time courses of hydrolysis of branched CDs by CD-hydrolyzing enzyme from* B. sphaericus

Four milliliters of a 2.5% solution of each branched CD, 0.5 ml of 100 mM phosphate buffer (pH 7.5), 0.4 ml of distilled water and 0.1 ml of 50 U/ml enzyme solution were mixed and incubated for 1 h for branched β-CDs or 4 h for branched α-CDs at 40°C. Two hundred microliter aliquots of the reaction mixture were withdrawn and boiled for 10 min. After centrifugation at 8000 × *g* for 10 min, the supernatant was analyzed by HPLC.

2.5. *Purification of the main products formed from branched CDs by the action of* B. sphaericus *CD-hydrolyzing enzyme*

The reaction conditions for the CDase on branched CDs were as follows; 200 mg of each branched CD, 1.0 ml of 100 mM phosphate buffer (pH 7.5), 8.0 ml of distilled water and 1.0 ml of 0.43 U/ml enzyme solution were mixed and incubated for 3 h for branched β-CDs or 20 h for branched α-CDs at 40°C, respectively. The reaction was stopped by boiling for 10 min and the denatured protein was removed by centrifugation at 8000 × *g* for 10 min. The supernatant was used as the branched sugar solution.

The purification of the main hydrolytic products from the branched sugar solution was carried out by HPLC using a preparative YMC PA 43 column (20 mm i.d. × 250 mm; Yamamura Kagaku, Japan). The operation conditions for HPLC were as follows: solvent, water/acetonitrile (45:55, v/v); flow rate, 5 ml/min; fraction size, 5 ml/fraction; column temperature, 25°C; injection volume, 0.50 ml. The saccharide fractions were pooled and dried using a rotary evaporator.

2.6. *Determination of the structures of the main products*

The structures of the main products were determined by the enzymatic analytical methods of Sakano et al. [6] and Kobayashi et al. [7] with minor modification. Ten microliters of β-amylase solution (252 U/ml), 260 μl of about a 0.35% solution of each branched sugar and 30 μl of 100 mM acetate buffer (pH 5.8) were mixed and incubated for 90 min at 40°C. One hundred microliter aliquots of the reaction mixture removed at appropriate times were boiled for 10 min, and the supernatant, after centrifugation, was analyzed by HPLC. In the same way, 10 μl of G_3-forming amylase (6.22 U/ml) or pullulanase solution (100 U/ml) and 30 μl of 100 mM acetate buffer (pH 5.8) were added to 260 μl of an approx. 0.35% solution of branched sugar and incubated for 90 min at 40°C. The reaction mixture was analyzed by HPLC after boiling and centrifugation. Twenty microliters of isopullulanase solution (1.0 U/ml) and 20 μl of 100 mM acetate buffer (pH 3.5) were added to 60 μl of the boiled solution of the G_3-forming amylase reaction mixture after incubation for 3 h at pH 3.5, and then incubated for 18 h at 35°C. The reaction was stopped by boiling for 10 min and the reaction mixture was analyzed by HPLC.

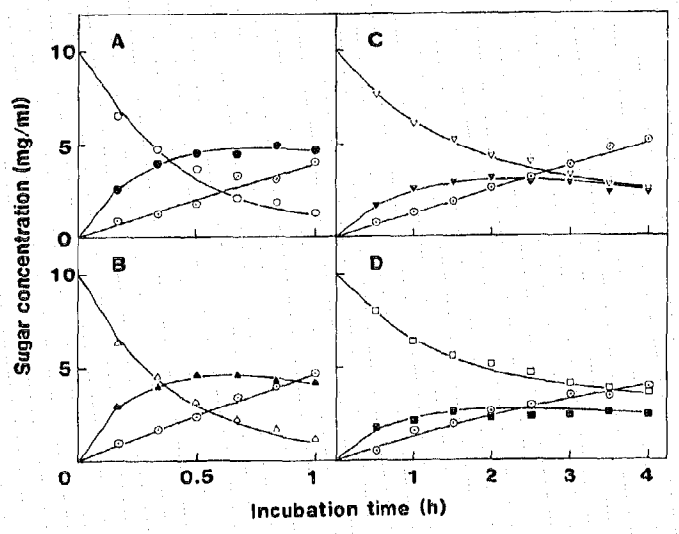

Fig. 1. Time courses of hydrolytic action on branched CDs by CD-hydrolyzing enzyme from *Bacillus sphaericus*. Reaction conditions are described in the text. Symbols: (A) G-β-CD (○), BI (●) and other sugars (⊙). (B) M-β-CD (△), BII (▲) and other sugars (⊙). (C) G-α-CD (▽), BIII (▼) and other sugars (⊙). (D) M-α-CD (□), BIV (■) and other sugars (⊙).

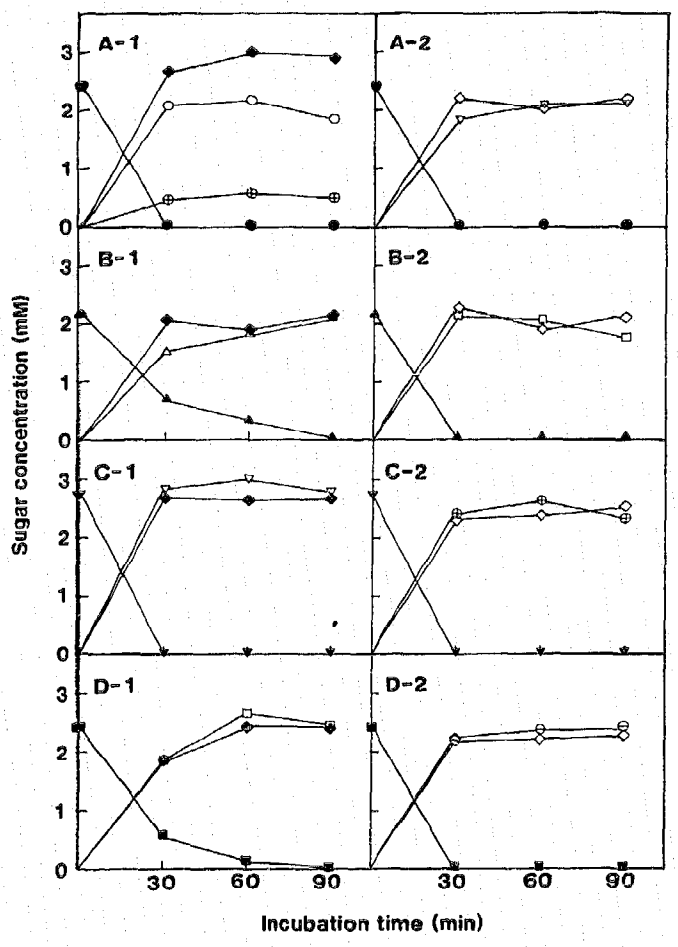

Fig. 2. Structural analyses of branched maltooligosaccharides using time courses of hydrolytic actions of β-amylase and G_3-forming amylase. Details of the enzyme reaction and anaytical conditions are described in the text. A-1, B-1, C-1 and D-1 are time courses of β-amylase action and A-2, B-2, C-2 and D-2 are those of G_3-forming amylase. Symbols: (A-1,2): BI (●), 6^n-α-G-G_5 (○), 6^n-α-G-G_4 (▽), 6^n-α-G-G_3 (⊕), G_3 (◇) and G_2 (◆). (B-1,2): BII (▲), 6^n-α-M-G_5 (△), 6^n-α-M-G_4 (□), G_3 (◇), and G_2 (◆). (C-1,2): BIII (▼), 6^n-α-G-G_4 (▽), 6^n-α-G-G_3 (⊕), G_3 (◇), and G_2 (◆). (D-1,2): BIV (■), 6^n-α-M-G_4 (□), 6^n-α-M-G_3 (⊖), G_3 (◇), and G_2 (◆).

3. RESULTS AND DISCUSSION

Time courses of the hydrolytic action of CD-hydrolyzing enzyme on branched CDs are shown in Fig. 1. In the initial stage of the enzyme reaction, G-β-CD, M-β-CD, G-α-CD and M-α-CD were hydrolyzed to

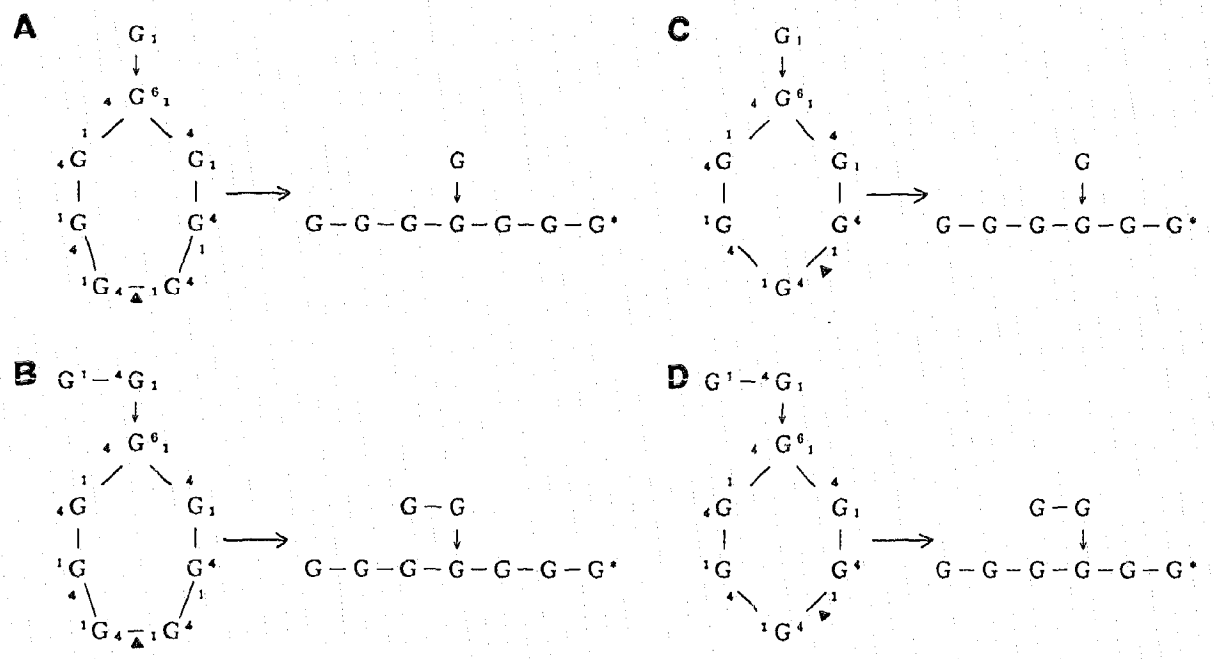

Fig. 3. Main sites of various branched CDs cleaved by CD-hydrolyzing enzyme from *Bacillus sphaericus*. (A) G-β-CD; (B) M-β-CD; (C) G-α-CD; (D) M-α-CD. Symbols: G, glucose residue; G*, reducing end of branched maltooligosaccharide; —, α-1,4-glucosidic linkage; ↓, α-1,6-glucosidic linkage; ▲, cleavage point.

give main products of 6^n-α-G-G_7 (BI), 6^n-α-M-G_7 (BII), 6^n-α-G-G_6 (BIII) and 6^n-α-M-G_6 (BIV), respectively. Two main products from M-α-CD and M-β-CD were further identified as 6^n-α-M-G_6 and 6^n-α-M-G_7, since pullulanase digestion of the main products gave G_2 and G_6 in the case of M-α-CD, and G_2 and G_7 for M-β-CD [11]. Other sugar components shown in Fig. 1 might have been produced from linear or branched oligosaccharides transformed by the action of the enzyme on branched CD, since it has been shown that G_7 produced from β-CD is gradually hydrolyzed further to smaller oligosaccharides by the same enzyme action [3].

BI was hydrolyzed mainly to G_2 and 6^n-α-G-G_5, and to G_3 and 6^n-α-G-G_4 by the actions of β-amylase and G_3-forming amylase, respectively (Fig. 2, A-1,2). β-Amylase and G_3-forming amylase never skipped the branching points, and the structure of the non-reducing end sides after the action of β-amylase on glucosyl-stabbed amylose consisted of only two types, 6^n-*O*-α-D-glucosyl-G_n and 6^{n-1}-*O*-α-D-glucosyl-G_n [12]. As the structure of 6^n-α-G-G_5 was thought to be either 6^5-α-G-G_5, BI was considered to be 6^5-α-G-G_7 or 6^4-α-G-G_7. Meanwhile BI was also considered to be $6^{1\sim4}$-α-G-G_7 from the result of the G_3-forming amylase action (Fig. 2, A-2). Thus the main structure of BI was determined as 6^4-α-G-G_7. It seemed that 6^3-α-G-G_7 and 6^2-α-G-G_7 were also contained as minor components (as shown in Fig. 2, A-1).

In the same way, since BIII was almost stoichiometrically hydrolyzed to G_2 and 6^n-α-G-G_4, and to G_3 and 6^n-α-G-G_3, by the actions of the respective amylases, the structure of BIII was determined to be 6^3-α-G-G_6 (Fig. 2, C-1,2).

On the other hand BII was stoichiometrically hydrolyzed to G_2 and 6^n-α-M-G_5, and to G_3 and 6^n-α-M-G_4 by the actions of the respective amylases (Fig. 2, B-1,2). As the non-reducing ends of the remaining β-limit dextrins after the thorough action of β-amylase on amylopectin are thought to be of only four types, 6^{n-1}-*O*-α-D-maltosyl-G_n, 6^{n-2}-*O*-α-D-maltosyl-G_n, 6^{n-1}-*O*-α-D-maltotriosyl-G_n or 6^{n-2}-*O*-α-D-maltotriosyl-G_n [13], 6^n-α-M-G_5 was considered to be either 6^3-α-M-G_5 or 6^4-α-M-G_5. Therefore, BII was considered to be 6^3-α-M-G_7 or 6^4-α-M-G_7. Furthermore, BII was also considered to be $6^{1\sim4}$-α-M-G_7 from the result of the reaction of G_3-forming amylase. 6^n-α-M-G_4 was completely hydrolyzed to G_3 and isopanose by the action of isopullulanase. Because isopullulanase has never been shown to hydrolyze any of $6^{1\sim3}$-α-M-G_4, 6^n-α-M-G_4 was considered to be 6^4-α-M-G_4 [14]. These results indicate that the structure of BII is 6^4-α-M-G_7.

In the same way, BIV was hydrolyzed to G_2 and 6^n-α-M-G_4, and to G_3 and 6^n-α-M-G_3, by the actions of the respective amylases (Fig. 2, D-1,2). Moreover 6^n-α-M-G_3 was also completely hydrolyzed to G_2 and isopanose by the action of isopullulanase. Thus the structure of BIV was determined to be 6^3-α-M-G_6.

In conclusion, the CD-hydrolyzing enzyme from *B. sphaericus* specifically hydrolyzed branched CDs at the α-1,4 linkage of the CD ring, and the branched posi-

tions of the main products were all at the C-6 position in the fourth glucose residue from the non-reducing end (as shown in Fig. 3).

Sakano et al. investigated in detail the hydrolyzed products of M-α-CD through the actions of Taka-amylase A from *Aspergillus oryzae* and α-amylase from *thermoactinomyces vulgaris* and deduced that the initial products obtained by the actions of these enzymes were 6^3-α-M-G_6 and 6^2-α-M-G_6, respectively [6]. Kobayashi et al. also reported that CGTase from *Bacillus macerans* mainly transformed G-α-CD and glucose to 6^6-α-G-G_7 by a coupling reaction at the initial stage [7]. Thus the sites of branched CDs cleaved by the actions of amylases and CGTase are various. Although there was notable difference in that our enzyme accumulated the cleaved initial product whereas Taka-amylase A rapidly hydrolyzed it, our results agreed with those for Taka-amylase A with regard to M-α-CD. We can not suggest why the cleavage points of branched CDs were so specific. However since the CD-hydrolyzing enzyme only cleaved the specific α-1,4 linkage of the branched CD ring, it seems that its specificity is due to the steric hindrance of the branch.

Acknowledgements: We thank Associate Professor Y. Sakano of Tokyo Noko University for his donation of isopullulanase and useful advice. We also thank Professors S. Chiba and S. Sagisaka of Hokkaido University, Drs S. Sugiyama, S. Nasuno, N. Saito, F. Yoshida, and S. Ishii for their encouragement.

REFERENCES

[1] Tilden, E.B. and Hudson, C.S. (1942) J. Bacteriol. 43, 527–544.
[2] Ohnishi, M. (1971) J. Biochem. (Tokyo) 69, 181–189.
[3] Oguma, T., Kikuchi, M. and Mizusawa, K. (1990) Biochim. Biophys. Acta 1036, 1–5.
[4] DePinto, J.A. and Campbell, L.L. (1968) Biochemistry 7, 121–125.
[5] Kitahata, S., Taniguchi, M., Beltran, S.D., Sugimoto, T. and Okada, S. (1983) Agric. Biol. Chem. 47, 1441–1447.
[6] Sakano, Y., Sano, M. and Kobayashi, T. (1985) Agric. Biol. Chem. 49, 3391–3398.
[7] Kobayashi, S., Ashraf, H.L., Braun, P. and French, D. (1988) Carbohydr. Res. 173, 324–331.
[8] Wako, K., Takahashi, C., Hashimoto, S. and Kanaeda, J. (1978) J. Jap. Soc. Starch Sci. (in Japanese) 25, 155–161.
[9] Kainuma, K. (1988) in: Handbook of Amylase and Related Enzymes (The Amylase Research Society of Japan, ed.), pp. 51–53, Pergamon Press, Tokyo.
[10] Sakano, Y., Masuda, N. and Kobayashi, T. (1971) Agric. Biol. Chem. 35, 971–973.
[11] Kainuma, K., Kobayashi, S. and Harada, T. (1978) Carbohydr. Res. 61, 345–357.
[12] Kainuma, K. and French, D. (1970) FEBS Lett. 6, 182–186.
[13] Summer, R. and French, D. (1956) J. Biol. Chem. 222, 469–477.
[14] Yamamoto, T. (1988) in: Handbook of Amylase and Related Enzymes (The Amylase Research Society of Japan, ed.), pp. 189–194, Pergamon Press, Tokyo.