Effects of Temperature on 6-*O*- α -Maltosyl Cyclodextrin Production from α -Maltosylfluoride and Cyclodextrins

Yoshimichi YOSHIMURA,*[†] Sumio KITAHATA and Shigetaka OKADA

*Osaka Municipal Technical Research Institute,
1-6-50, Morinomiya, Jyoto-ku, Osaka 536, Japan*

**Fujisawa Research Laboratory, Tokuyama Soda Co., Ltd.,
2051 Endo, Fujisawa 252, Japan*

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The effects of temperature on 6-*O*- α -maltosyl cyclodextrins (G_2 -CDs) production from α -maltosylfluoride (α - G_2 F) and cyclodextrins (CDs) by the transfer action of debranching enzymes such as pullulanase and isoamylase were studied.

The amounts of 6-*O*- α -maltosyl α -cyclodextrin (G_2 - α -CD) production by purified pullulanase from *Aerobacter aerogenes* (A-pullulanase) and from *Bacillus acidopullulyticus* (B-pullulanase) increased with a rise in temperature, e.g., the amounts at 60°C were about 1.5 times higher than those at 30°C. Initial transfer ratios (G_2 - α -CD formed/ α - G_2 F consumed) of A-pullulanase and B-pullulanase were about 62% and 25% (at 40°C), and about 50% and 15% (at 20°C), respectively. The transfer ratios of both A-pullulanase and B-pullulanase in the reaction using β -CD or γ -CD as acceptor also increased with a rise in temperature.

The transfer ratios were little affected by any change in temperature or any kind of acceptor CDs, in the case of isoamylase, and were about 60%.

Branched cyclodextrins are the homogeneous oligosaccharides having maltooligosaccharides such as glucose, maltose, and maltotriose linked by α -1,6 glucosidic linkages to CDs.¹⁾ Recently, they have become of interest because of their high solubility, compared with CDs.^{2,3)}

We have reported that G_2 -CDs were produced efficiently and specifically from α - G_2 F and CDs by the transfer action of debranching enzymes such as pullulanase and isoamylase.^{4,5)}

Branched CDs are also formed by the condensation reaction of maltose or maltotriose with CDs,^{6~8)} and by the action of cyclodextrin glucanotransferase on amylopectin, following digestion by glucoamylase.^{1,9)}

As previously reported,⁵⁾ in the reaction of α - G_2 F and CDs, the yields of G_2 -CDs varied

according to the origin of the enzymes and kinds of CDs used as acceptors. It was suggested that the difference in the amounts of G_2 -CD production by these enzymes was based on the difference in the mode of action on α - G_2 F used as substrate, either transfer or hydrolytic action.

In our previous paper,⁴⁾ we reported that the amount of G_2 - α -CD produced by crude B-pullulanase increased with a rise of reaction temperature.

This paper describes the details of an effect of temperature on G_2 -CDs formation by three debranching enzymes.

MATERIALS AND METHODS

Materials. Hepta-*O*-acetyl- α -maltosylfluoride, mp 173.5~177°C, $[\alpha]_D^{25} + 111.8^\circ$ ($c=0.8$, CHCl_3), was synthe-

[†] To whom correspondence should be sent.

Abbreviations: G_2 -CD, 6-*O*- α -maltosyl cyclodextrin; (G_2)₂-CD, dimaltosyl cyclodextrin; α - G_2 F, α -maltosylfluoride; A-pullulanase, pullulanase from *Aerobacter aerogenes*; B-pullulanase, pullulanase from *Bacillus acidopullulyticus*.

sized by treating maltose octaacetate with cold, anhydrous hydrogen fluoride, by the procedures previously described.^{10,11} α -G₂F was generated by deacetylating the heptaacetate at 0°C with fresh sodium methoxide in dry methanol. Solutions, of known concentration in dry methanol, were kept in a desiccator at -20°C.

Purified pullulanase from *Aerobacter aerogenes* and purified isoamylase from *Pseudomonas amyloclavata* were purchased from Seikagaku Kogyo Co., Ltd. Purified pullulanase from *Bacillus acidipullulyticus*¹² was donated by Novo Industri Japan Ltd. In this study, the enzyme reaction was done in the buffer at pH 5.6 (A-pullulanase), 5.0 (B-pullulanase), and 4.0 (isoamylase), respectively. α -CD, β -CD, γ -CD and pullulan were supplied by Hayashibara Biochemical Laboratories, Inc. Amylopectin from potatoes was purchased from Sigma Chemical Company. Other chemicals used were of the highest grade commercially available.

Assay of pullulanase and isoamylase activity. For the assay of pullulanase activity, a reaction mixture containing 0.3 ml of 4% pullulan in 0.1 M acetate buffer (pH 5.6 for A-pullulanase or pH 5.0 for B-pullulanase), and 0.3 ml of enzyme solution was incubated at 40°C. In the case of isoamylase, 0.3 ml of 4% amylopectin in 0.1 M acetate buffer (pH 4.0) was incubated with 0.3 ml of enzyme solution. After 30 min, the reaction was stopped by adding 0.9 ml of 0.5 M carbonate buffer (pH 10.0), and the reducing sugars released were measured in 0.5-ml samples by the Somogyi-Nelson method,^{13,14} with D-glucose as the standard. One unit of the activity was defined as the amount which liberated one μ mol of reducing sugars as D-glucose per minute under the aforementioned conditions.

Measurement of G₂-CDs and (G₂)₂-CDs by HPLC. After the reaction was stopped by adding 1 N sodium hydroxide to the reaction mixture, the G₂-CDs and (G₂)₂-CDs were measured by HPLC. A high performance liquid chromatograph (Hitachi 635A) was fitted with a Polygosyl 10-NH₂ column (4.0 $\times$ 300 mm) and a refractometer (Shodex RE-11) as a detector. Elution was done with acetonitrile-water (65:35, v/v) at a flow rate of 2 ml/min.

Fluoride anion measurement. Fluoride anion concentrations were measured¹⁵ in the presence of TISAB buffer [1 M acetate buffer, pH 5.2; 1 M sodium chloride; 0.4% 1,4-cyclohexanebis(dinitrilotetraacetic acid)monohydrate] with a specific fluoride-ion probe (Iwaki specific ion meter, Model 225, and an Orion combination fluoride electrode, Model 96-09).

Calculation of the transfer ratio. Values of the transfer ratios were calculated as follows:

$$\text{Ratio (\%)} = \frac{[\text{G}_2\text{-}\alpha\text{-CD formed}]}{[\text{\alpha-G}_2\text{F consumed}]} \times 100$$

Amounts of α -G₂F consumed were measured by measuring amounts of fluoride anion released.

RESULTS

Non-enzymatic degradation of α -G₂F

To examine the rate of non-enzymatic degradation of α -G₂F, solutions containing 20 mM α -G₂F and 100 mM acetate buffer (pH 5.0) in a total volume of 350 μ l were incubated at various temperatures. At intervals, 50 μ l of each solution was withdrawn and the fluoride anions released were measured. The degradation rates at 20, 40, 50, and 60°C were 1.2, 2.3, 6.5, and 21.0% per an hour, respectively.

Effects of temperature on G₂- α -CD and (G₂)₂- α -CD formation

Reaction mixtures containing 40 mM α -G₂F, 90 mM α -CD, 100 mM acetate buffer and a debranching enzyme (2.7 μ l/ml) in a total volume of 150 μ l were incubated for 1 hr at various temperatures. After the reaction was stopped by adding 50 μ l of 1 N sodium hydroxide to the reaction mixture, the amounts of G₂- α -CD and (G₂)₂- α -CD were measured by HPLC. (Table I)

The amounts of G₂- α -CD formed by A-

TABLE I. EFFECTS OF TEMPERATURE ON (G₂)₂- α -CD FORMATION

Reaction mixtures containing 40 mM α -G₂F, 90 mM α -CD and a debranching enzyme (2.7 u/ml) were incubated for 1 hr.

	G ₂ - α -CD (mM)	(G ₂) ₂ - α -CD (mM)	(G ₂) ₂ - α -CD/ G ₂ - α -CD
A-Pullulanase			
30°C	20.8	2.6	0.13
45°C	30.0	4.4	0.15
60°C	30.1	5.4	0.18
B-Pullulanase			
30°C	8.1	0.9	0.11
45°C	9.7	0.6	0.06
60°C	12.4	0.4	0.03
Isoamylase			
30°C	32.4	3.1	0.10
45°C	33.6	3.6	0.11
60°C	30.1	2.8	0.09

pullulanase and by B-pullulanase increased with a rise in temperature. The amounts at 60°C were 1.5 times higher than those at 30°C.

The amount of $(G_2)_2\text{-}\alpha\text{-CD}$ formed by A-pullulanase increased with a rise in temperature, but in the reaction of B-pullulanase, the amount of $(G_2)_2\text{-}\alpha\text{-CD}$ production decreased.

$G_2\text{-}\alpha\text{-CD}$ and $(G_2)_2\text{-}\alpha\text{-CD}$ formations by isoamylase were not affected by temperature changes.

Under these conditions, the main transfer product of the reaction was $G_2\text{-}\alpha\text{-CD}$; the $(G_2)_2\text{-}\alpha\text{-CD}/G_2\text{-}\alpha\text{-CD}$ ratios of A-pullulanase, B-pullulanase, and isoamylase were 0.13, 0.11, and 0.10 (at 30°C), and 0.18, 0.03, and 0.09 (at 60°C), respectively. So, to avoid a complicating factor, we do not take into account the production of $(G_2)_2\text{-CDs}$ in the analysis of further experimental results.

Effects of temperature on the transfer ratios in the $G_2\text{-}\alpha\text{-CD}$ forming reaction

Since the results shown in Table I imply $G_2\text{-}\alpha\text{-CD}$ accumulated for 1 hr of incubation, the results are not always reflected in the initial transfer ratios. To examine the initial transfer ratios of A-pullulanase and B-pullulanase, reduced amounts of both enzymes were incubated with $\alpha\text{-G}_2\text{F}$ and $\alpha\text{-CD}$ at 20 and 40°C. At intervals, 100 μl of each reaction mixture was withdrawn and the reaction was stopped by the addition of 30 μl of 1N sodium hydroxide, and more 50- μl samples were removed to measure the amounts of fluoride anion released.

As shown in Fig. 1A and B, the use of $\alpha\text{-G}_2\text{F}$ by A-pullulanase, which was indicated by release of fluoride anion, was almost complete within 60 min at 40°C, while $\alpha\text{-G}_2\text{F}$ consumption was about 50% at 20°C. The G_2 transfer ratio at 40°C was about 62%; significantly higher than that at 20°C (48 to 55%). B-Pullulanase also had a higher transfer ratio at higher temperatures: the transfer ratio was about 15% at 20°C and about 25% at 40°C (Figs. 1C, D). Thus, the ratio of the hydrolysis reaction of $\alpha\text{-G}_2\text{F}$ over the transfer reaction of G_2 residues from $\alpha\text{-G}_2\text{F}$ to $\alpha\text{-CD}$ is surely

FIG. 1. Effects of Temperature on the Transfer Ratios in the $G_2\text{-}\alpha\text{-CD}$ Forming Reaction.

Reaction mixtures containing 40 mM $\alpha\text{-G}_2\text{F}$, 90 mM $\alpha\text{-CD}$, 100 mM acetate buffer (pH 5.6), and A-pullulanase (1.5 units) in a total volume of 1.50 ml were incubated at (A) 20°C and (B) 40°C. Conditions of incubation for B-pullulanase (2.1 units) were the same as those for A-pullulanase, except for the use of 100 mM acetate buffer (pH 5.0): C, 20°C; D, 40°C. (○—○), fluoride anion released; (●—●), $G_2\text{-}\alpha\text{-CD}$ formed; (△—△), the transfer ratio [$G_2\text{-}\alpha\text{-CD}$ formed/ $\alpha\text{-G}_2\text{F}$ consumed].

changed by alterations in temperature, even in the early stage of reaction.

Effects of temperature on $G_2\text{-}\beta\text{-CD}$ and $G_2\text{-}\gamma\text{-CD}$ formation by three debranching enzymes

The transfer ratios of three debranching enzymes in the reactions using $\beta\text{-CD}$ and $\gamma\text{-CD}$ as acceptors were also examined. Owing to the poor solubility of $\beta\text{-CD}$, 20 mM $\alpha\text{-G}_2\text{F}$ and 40 mM $\beta\text{-CD}$ were used for the $G_2\text{-}\beta\text{-CD}$ forming reaction. The results are shown in Fig. 2. In the reaction with $\beta\text{-CD}$, the transfer ratios of both A-pullulanase and B-pullulanase increased with a rise in temperature.

But in the reaction of $G_2\text{-}\gamma\text{-CD}$ formation, there was anomalous behavior: the transfer ratio of B-pullulanase rose at 60°C in spite of a decrease of the ratio with a rise of temperature

FIG. 2. Transfer Ratios of Debranching Enzymes on G₂-CDs Formation.

Reaction conditions are similar to those described in Fig. 1. G₂-CDs formed were: A, G₂-α-CD; B, G₂-β-CD; C, G₂-γ-CD. Debranching enzymes used were: (○—○), A-pullulanase; (△—△), B-pullulanase; (●—●), isoamylase.

FIG. 3. Effects of Temperature on G₂-γ-CD Formation by B-Pullulanase.

Reaction mixture containing 40 mM α-G₂F, 90 mM γ-CD, 100 mM acetate buffer (pH 5.0), and B-pullulanase (1.4 units) in a total volume of 1.5 ml were incubated at (A) 20°C, (B) 40°C, and (C) 60°C. (○—○), fluoride anion released; (●—●), G₂-γ-CD formed; (△—△), the transfer ratio (G₂-γ-CD/α-G₂F).

up to 50°C (Fig. 2C).

The ratios were little affected by any change in temperature or any kind of acceptor CDs, in the case of isoamylase, and were about 60%.

To discover the cause of the above anomalous behavior, the initial transfer ratios of B-pullulanase were examined (Fig. 3). The amount of G₂-γ-CD production began to decrease after 20 min of incubation at 40°C. The amounts of maltose and γ-CD increased with decreasing amounts of G₂-γ-CD production

(data not shown). It was therefore found that G₂-γ-CD was hydrolyzed by B-pullulanase.

The maximum transfer ratios at 20, 40, and 60°C were 32, 37, and 43%, respectively. Thus Fig. 3 shows that, even in the case of G₂-γ-CD formation by B-pullulanase, rising temperature tends to increase the efficiency of G₂-transfer reaction.

DISCUSSION

In the experiments on G₂-α-CD production, it is not necessary to consider an inactivation of three debranching enzymes and a spontaneous degradation of α-G₂F. Though A-pullulanase and isoamylase was unstable at 60°C, the amounts of enzymes used in the experiments was enough to consume the α-G₂F completely (data not shown). As the G₂-α-CD forming reaction at 60°C was completed within 10 min, losses of α-G₂F due to non-enzymatic degradation at 60°C were negligibly small (less than 3%).

It was found that the anomalous behavior shown in Fig. 2C was due to hydrolysis of G₂-γ-CD by B-pullulanase. The reason why G₂-γ-CD was little hydrolyzed at 60°C (Fig. 3) was that B-pullulanase became inactive after 20 min of incubation.

It has consequently become apparent that the ratio of transfer reaction from α-G₂F to α-, β- and γ-CD increased with a rise in temperature in the case of A-pullulanase and B-pullulanase. This effect of temperature on the G₂-α-CD production was independent of the purity of debranching enzymes. Some changes in the protein structure of the enzyme caused by a higher temperature may help binding of CD into an acceptor site of pullulanase.

On the other hand, the action of isoamylase on α-G₂F was little affected by temperature changes.

It is already known that the lower the temperature of enzyme reactions, the more synthesis of dextran by dextranucrase¹⁶⁾ and of levan by levansucrase¹⁷⁾ happens. In other word, the ratio of transfer reaction over hydrolysis reaction decreases with increases in

FIG. 4. Proposed Mechanisms for the Action of Debranching Enzyme on $\alpha\text{-G}_2\text{F}$.

G-G-F is a $\alpha\text{-G}_2\text{F}$, (E) represents a debranching enzyme, G represents a glucosyl units, — is an $\alpha\text{-1,4}$ glucosidic linkage, and $\downarrow$ is an $\alpha\text{-1,6}$ glucosidic linkage.

temperature.

Further work is needed to explain the difference in the response of these enzymes to reaction temperatures.

The reaction of debranching enzymes on $\alpha\text{-G}_2\text{F}$ may occur as shown in Fig. 4. An irreversible cleavage of the C-F glycosylic bond of $\alpha\text{-G}_2\text{F}$ is first catalyzed by a debranching enzyme, and an enzyme-substrate complex ((E)-G-G) is formed. Next the reaction proceeds by transferring the maltosyl residue to an appropriate acceptor.

If water is chosen as an acceptor, the product is maltose (Fig. 4 (2)). If $\alpha\text{-G}_2\text{F}$ is also used as the acceptor, branched oligosaccharides such as 6¹-O- α -maltosylmaltosylfluoride and 6²-O- α -maltosylmaltosylfluoride (B_4F) are produced. In the reaction on $\alpha\text{-G}_2\text{F}$ without an addition of any acceptor such as $\alpha\text{-CD}$, the transfer reaction (1) competes with the hydrolysis reaction (2). To clarify the effects of temperature on the ratio of reaction (1) over reaction (2), we tried to measure the amount of maltose produced. In the reaction of A-pullulanase and B-pullulanase, it seemed likely that the maltose-producing rate at 20°C was larger than those at 40°C. But maltose could not be measured because of the small amount of maltose production

in the initial period of the reaction and of additional maltose production by hydrolysis of the transfer products such as B_4F .

In the presence of excess amounts of $\alpha\text{-CD}$, branched oligosaccharides such as B_4F were not found, and only branched $\alpha\text{-CD}$ was produced. This suggests that $\alpha\text{-CD}$ is used as the acceptor in preference to $\alpha\text{-G}_2\text{F}$, and the hydrolysis reaction (2) competes with the transfer reaction (3). Since the hydrolysis reaction of $\text{G}_2\text{-}\alpha\text{-CD}$ by pullulanase is inhibited by residual $\alpha\text{-CD}$,⁵⁾ the ratio of reaction (3) over reaction (2) was accurately measured.

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