A Way of Enhancing the Inhibitory Effect of Phosphoryl Oligosaccharides on the Formation of a Calcium Phosphate Precipitate Using the Coupling Reaction of Cyclomaltodextrin Glucanotransferase

Hiroshi KAMASAKA,* Kenji TO-O, Kaname KUSAKA, Takashi KURIKI, Takashi KOMETANI and Shigetaka OKADA

Biochemical Research Laboratory, Ezaki Glico Co. Ltd. (4-6-5, Utajima, Nishiyodogawa-ku, Osaka 555, Japan)

In a previous study, we prepared phosphoryl oligosaccharides (POs) from potato starch hydrolysate and investigated their inhibitory effect on the formation of a calcium phosphate precipitate. In this study, we investigated the enhancement of the inhibitory effect using the coupling reaction of cyclomaltodextrin glucanotransferase (CGTase) (EC 2.4.1.19). In this treatment, the effect of the POs was obviously improved and conversion of the PO structure was observed. By the coupling reaction of CGTase, the fraction of POs with one phosphoryl group decreased, and the fraction with at least two phosphoryl groups increased to 33% from 10%. Phosphoryl maltopentaose was the main substrate for the coupling reaction of the enzyme. The products (CGT-POs) obtained after CGTase reaction were fractionated by ion-exchange chromatography into three fractions, CGT-PO1, CGT-PO2a, and CGT-PO2b. The fractions of CGT-PO2a and CGT-PO2b were the main components of the CGT-POs having an inhibitory effect on calcium phosphate formation. The fraction CGT-PO2a was maltoligosaccharide with an average degree of polymerization (DP) of 7.89, to which two phosphoryl groups were attached. The fraction CGT-PO2b was maltoligosaccharide with an average DP of 11.06, to which three phosphoryl groups were attached. In addition, the fraction CGT-PO2b was more effective than fraction CGT-PO2a since the threshold value of the concentration of CGT-PO2b to exhibit the inhibitory effect was 7-fold lower than that of CGT-PO2a.

Potato starch is known to contain a small amount of covalently-bound phosphoryl groups

* To whom correspondence should be addressed.

Abbreviations: POs, phosphoryl oligosaccharides; CGTase, cyclomaltodextrin glucanotransferase; CGT-POs, POs treated by CGTase; BLA, bacterial liquefying α-amylase; GA, glucoamylase; BSA, bacterial saccharifying α-amylase; DP, degree of polymerization; PO-1, phosphoryl oligosaccharide-1 fractionated from POs; PO-2, phosphoryl oligosaccharide-2 fractionated from POs; Glc-6-P, D-glucose 6-phosphate; Glc-1,6-diP, D-glucose 1,6-diphosphate; Fru-6-P, D-fructose 6-phosphate; fru-1,6-di-P, D-fructose 1,6-diphosphate CD, cyclodextrin; HPAEC, high-performance anion-exchange chromatography; PAD, pulsed amperometric detector; HPLC, high-performance liquid chromatography; 6-phosphoryl residue, glucosyl residues attached to the ester phosphoryl group at C-6; 3-phosphoryl residue, glucosyl residues attached to the ester phosphoryl group at C-3; 6α-phosphoryl maltose, O-6-phosphoryl-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranose; 6′-phosphoryl maltotriose, O-6-phosphoryl-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranose; 6″-phosphoryl maltotetraose, O-6-phosphoryl-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranose; 6‴-phosphoryl maltopentaose, O-6-phosphoryl-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranose; 6‴′-phosphoryl maltotetraose, O-6-phosphoryl-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranose; 6‴‴-phosphoryl maltopentaose, O-6-phosphoryl-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranose; 6‴‴′-phosphoryl maltotetraose, O-6-phosphoryl-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranose; 6‴‴‴-phosphoryl maltopentaose, O-6-phosphoryl-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranose; 6‴‴′′-phosphoryl maltotetraose, O-6-phosphoryl-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranose; 6‴‴‴′-phosphoryl maltopentaose, O-6-phosphoryl-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranose;
in its components, one of 200 to 500 glucose residues on average being phosphorylated.\textsuperscript{1,2)} We prepared POs from potato starch hydrolysate using BLA (EC 3.2.1.1), glucoamylase (GA) (EC 3.2.1.3), and pullulanase (EC 3.2.1.41), and found that the POs inhibited the formation of a calcium phosphate precipitate.\textsuperscript{3,4)} It is widely recognized that phosphate salt tends to form an insoluble complex with calcium under the slightly alkaline conditions of the human intestine, and that the formation of a calcium phosphate precipitate causes a reduction of calcium absorption from the intestine.\textsuperscript{5,6)} On the other hand, it has been reported that oligosaccharides improve intestinal bacterial flora, prevent carcinogenesis, and decrease the calorie effect of food.\textsuperscript{7-9)} We have reported a new function of the oligosaccharides, POs; inhibition of the formation of calcium phosphate.\textsuperscript{3)}

The POs were fractionated into two fractions; PO-1 and PO-2. We have also described an investigation of the PO structures.\textsuperscript{3,10)} Fraction PO-1 was composed of phosphoryl maltotriose, maltotetraose, and maltopentaose having one phosphoryl group. Fraction PO-2 was predominantly composed of phosphoryl maltopentaose and maltohexaose having two phosphoryl groups, and fraction PO-2 was newly found in a potato starch hydrolysate. Although both PO-1 and PO-2 fractions had a ability to form complexes with calcium, fraction PO-2 had the stronger inhibitory effect and made more stable and soluble complexes with the calcium ion.\textsuperscript{3)} This capacity of PO-2 was equal to that of casein phosphopeptides.\textsuperscript{11,12)} The capacity of the PO-1 fraction was inferior to that of the PO-2 fraction, while it was main part of the POs. From the results of the inhibitory effect and the structural analyses, it was concluded that these abilities were dependent upon the amount of ester phosphoryl groups in the molecule.\textsuperscript{3)}

CGTase catalyzes intramolecular transglycosylation to form cyclodextrin (CD) from starch (cyclization reaction). In the presence of a suitable acceptor such as glucose, the enzyme catalyzes intermolecular transglycosylation (coupling reaction).\textsuperscript{13)} In this study, we describe away of enhancing the inhibitory effect of POs on the formation of a calcium phosphate precipitate using the coupling reaction of CGTase and analyze the structures of the effective fractions. The relationship between the calcium solubilizing ability and the number of phosphoryl groups incorporated is also described.

**MATERIALS AND METHODS**

**Enzymes.** CGTase from *Bacillus macerans* was purchased from Amano Pharmaceutical Co. (Nagoya, Japan). Alkaline phosphatase from *Escherichia coli* was from Sigma Chemicals (St. Louis, MO, USA). BLA from *Bacillus amyloliquefaciens* was a product from Ueda Chemicals Co. (Osaka, Japan), and GA from *Rhizopus* sp. was from Toyobo Co. (Osaka, Japan). Pullulanase from *Aerobacter aerogenes* was a product of Hayashibara Biochemical Lab. (Okayama, Japan). BSA from *Bacillus subtilis* was from Nagase Biochemicals Co. (Fukuchiyama, Japan) and Glc-6-P dehydrogenase was purchased from Oriental Yeast Co. (Tokyo, Japan). The glycerol measurement kit and sorbitol dehydrogenase from sheep liver were purchased from Boehringer Mannheim GmbH (Mannheim, Germany).

**Other materials.** Chitopearl BCW-2501 (Fuji Spinning Co., Tokyo, Japan) was used for anion-exchange column chromatography. Daisopak SP-120-5-ODS-BP (Daiso Co., Osaka, Japan) was used for ODS column chromatography, and the Sephadex G-10 was from Pharmacia Fine Chemicals Co. (Uppsala, Sweden). CarboPac PA-100 (4ϕ × 250 mm) and its preparative column (22ϕ × 250 mm) were purchased from Dionex Corp. (Sunnyvale, CA, USA). Authentic maltooligosaccharides with a DP of 1-6 were purchased from Sigma Chemicals. Glc-6-P and Glc-1, 6-diP were purchased from Boehringer Mannheim GmbH and Nacalai Tesque (Kyoto, Japan). All other chemicals and materials used were of analytical or commercial grade.

**Preparation of CGT:POs.** The POs prepared from the potato starch hydrolysate\textsuperscript{3)} (2.0 g)
were dissolved in 20 ml of a 50 mM acetate buffer (pH 5.5) and incubated with 20 units of CGTase at 50°C for 48 h. The reaction was stopped by heat treatment in a boiling-water bath for 5 min. After the precipitate had been centrifuged off, the products were fractionated by ion-exchange chromatography using a method mentioned in a previous paper.10 The products were analyzed by HPAEC. The chromatographic conditions of HPAEC with a Dionex DX-300 (Dionex Corp., Sunnyvale, CA, USA) gradient chromatography system were as mentioned previously.3

Analytical method. The total amount of carbohydrates was measured by the phenol-sulfuric acid method.14 The structures were then analyzed as follow:

1. Chemical analyses. Determination of the contents of the reducing-terminal residue was conducted using borohydride methods. The sample (60 µl) was reduced by the addition of 5 µl of 3% NaBH₄ dissolved in a 0.01 N NaOH solution at 40°C for 1 h. The reduced sample was hydrolyzed with 0.7 N HCl at 100°C for 4 h. After acid hydrolysis, the reducing end was measured as sorbitol by the method of MANNERS et al.,15 and glucose was measured by the method of MIWA et al.16 The phosphate group at C-6 of the glucosyl residue was also measured as Glc-6-P using Glc-6-P dehydrogenase after acid hydrolysis.2 DP values were also calculated as \([\text{glucose (mol)} + \text{sorbitol (mol)} + \text{Glc-6-P (mol)}]/[\text{sorbitol (mol)}]\). The organic phosphate of each PO after acid hydrolysis was measured as inorganic phosphate17 after dephosphorylating with an alkaline phosphatase treatment.3

2. Enzymatic analyses. After treatment with CGTase, the fractionated products (1 mg) of CGT-POs were solubilized in 1 ml of a 20 mM acetate buffer (pH 5.5). Five units of BSA or GA were added to the solution and incubated at 50°C or 15 h. The solution was heated in a boiling-water bath for 5 min to stop the reaction. The products obtained from each reaction were analyzed by HPAEC using PAD. In this treatment, 3β-phosphoryl maltotriose (S1) and 6β-phosphoryl maltotriose (S3) were obtained, and their contents were calculated from the PAD response using the following response factors: S1, 0.93; S3, 1.0.

Inhibitory effect on the formation of calcium phosphate. To examine the inhibitory effect, a modification of the method of YAMAMOTO et al.,5 which mimics the in vivo condition, was adopted. The test solution (100 µl) was mixed well with 500 µl of a 20 mM phosphate buffer (pH 7.4) containing 5 mM NaN₃ and 80 mM KCl. The initial pH level of this solution was precisely adjusted to pH 7.4 with NaOH or HCl before 400 µl of a solution containing 10 mM CaCl₂ and 5 mM NaN₃ was added. The final concentrations of calcium and phosphate were 4 mM and 10 mM, respectively. The mixture was then incubated in a 5436 Thermomixer (Eppendorf Netheler Hinz, Hamburg, Germany) by shaking at 30 ± 0.5°C for appropriate periods, after which the solution was taken out and centrifuged at 10,000 × g for 1 min. The calcium concentration of the supernatant was measured by the calcium C-Test (Wako Pure Chemical Industries), and the percentage of soluble calcium in the supernatant per total calcium initially added was calculated.

Detection of the transglycosylation reaction of CGTase in each substrate of PO-1 fraction. Each substrate (J-N in Fig. 1a) of the PO-1 fraction was purified using HPAEC with a preparative column.16 The substrates (1 µg) were dissolved in 100 µl of a 50 mM acetate buffer (pH 5.5) and incubated with 20 units of CGTase at 50°C for 48 h. The reaction was stopped by heat treatment in a boiling-water bath for 5 min. The products were analyzed by thin-layer chromatography (TLC). One micro-liter of a 1% sample solution was spotted on a TLC plate. TLC was carried out by the ascending method with silica gel (Merck, Darmstadt, Germany) and a solvent solutions of ethanol–water–acetic acid (35: 15: 1, v/v). Spots were visualized by spraying H₂SO₄-methanol (1:1, v/v) and then heating at 130°C.
RESULTS

Preparation of POs treated with CGTase.

The POs prepared from the potato starch hydrolysate were analyzed using HPAEC, and the results are shown in Fig. 1a. The POs were clearly distinguishable by this system according to the number and positions of the phosphate groups linked to each molecule as described in a previous paper.3) After CGTase reaction on the POs, the products (CGT-POs) were also analyzed using HPAEC as shown in Fig. 1b. The peaks like the PO-2 fraction increased, and the peaks of K, M and N decreased with CGTase treatment. These facts suggest that transglycosylation of the PO-1 fraction obviously occurred due to the action of CGTase, and that coupling-reaction products (CGT-PO2) like the PO-2 fraction were obtained. Additionally, CDs were hardly detected in Fig. 1b. The amounts of CGT-PO2 produced at various PO concentrations were analyzed as shown in Fig. 2. The content of CGT-PO2 became maximum at 10–40% of POs in the reaction mixture, and the final content of CGT-PO2 was from 10% to about 33% as shown by HPAEC analysis. The reaction was hindered by evolution of the PO viscosity when a PO concentration over of 40% was attained. The optimum conditions for the synthesis of the coupling-reaction products were 50°C and pH 5.5 when 20 U/ml of the enzyme was used.

Inhibitory effect of CGT-POs on the formation of a calcium phosphate precipitate.

Figure 3 shows the effect of CGT-POs on the formation of a calcium phosphate precipitate.
In comparison with the control, calcium remained soluble for the first hour after the addition of POs. After 2 h, the amount of soluble calcium gradually decreased. On the other hand, calcium remained soluble for 2 h after the addition of CGT-POs. As a result, the inhibitory effect of POs could be improved with CGTase treatment. This indicates that the amount of the effective fraction in the POs increased after the CGTase reaction.

**Fractionation of the CGT-POs.**

A CGT-PO solution (1%, 300 ml) was prepared with 10 mM acetate buffer (pH 4.5). The solution was applied on a Chitopearl BCW-2501 column equilibrated with the same buffer. The adsorbed CGT-POs were eluted from the column with the same buffer containing 0.1 M NaCl (for the fraction of CGT-PO1), 0.2 M NaCl (for the fraction of CGT-PO2a), and 0.5 M NaCl (for the fraction of CGT-PO2b), respectively. Each fraction was concentrated and then applied on a Sephadex G-10 column equilibrated with deionized water in order to remove the NaCl. The eluted saccharides were collected and freeze-dried. The fractions of CGT-PO1 (900 mg), CGT-PO2a (680 mg), and CGT-PO2b (200 mg) were obtained as powder, and the HPAEC chromatograms of the CGT-PO2a and CGT-PO2b fractions are shown in Fig. 4a and 4c, respectively.

**Structural analyses of the fractions CGT-PO1, CGT-PO2a, and CGT-PO2b.**

1. **Chemical analyses.**

The results of chemical analyses of CGT-PO1, CGT-PO2a, and CGT-PO2b are shown in Table 1. The average \( \bar{D}_P \) values were obtained from the contents of sorbitol, glucose, and Glc-6-P after acid hydrolysis. The covalent bond between the phosphoryl groups and glucose in Glc-6-P was stable under the condition of acid hydrolysis, however other phosphoryl bonds were not stable and tended to release inorganic phosphate.\(^{2,10}\) The average \( \bar{D}_P \) values of CGT-PO2a and CGT-PO2b were extended to 7.89 and 11.06, respectively, from 5.65 of PO-2. The average \( \bar{D}_P \) of fraction CGT-PO1 decreased to 3.12 from 4.02 of PO-1. These results indicate that CGT-PO2a and CGT-PO2b have two and three phosphoryl groups in a molecule, respectively, which were produced from fraction PO-1 by the transglycosylation reaction of CGTase.
Table 2. Contents of phosphoryl groups attached to C-3 and C-6 of glucosyl residues in POs before or after CGTase reaction.

<table>
<thead>
<tr>
<th>Sample</th>
<th>C-3(^a)</th>
<th>C-6(^a)</th>
<th>C-6(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO-1</td>
<td>29.0</td>
<td>71.0</td>
<td>71.0</td>
</tr>
<tr>
<td>CGT-PO1</td>
<td>13.8</td>
<td>86.2</td>
<td>86.0</td>
</tr>
<tr>
<td>PO-2</td>
<td>—</td>
<td>—</td>
<td>44.0</td>
</tr>
<tr>
<td>CGT-PO2a</td>
<td>28.7</td>
<td>71.3</td>
<td>78.4</td>
</tr>
<tr>
<td>CGT-PO2b</td>
<td>44.4</td>
<td>55.6</td>
<td>59.8</td>
</tr>
</tbody>
</table>

\(^a\)Calculated from HPAEC-PAD response.
\(^b\)Calculated from Glc-6-P dehydrogenase methods.

2. Enzymatic analyses.

Each CGT-PO fraction was individually treated with BSA and GA. The products were analyzed using HPAEC. Although most of the PO-2 fraction was indigestible and remained after BSA hydrolysis, CGT-PO2a and CGT-PO2b could be hydrolyzed (Fig. 4b and d) and produced 3\(^2\)-phosphoryl maltotriose (S1), 6\(^2\)-phosphoryl maltose (S2), and 6\(^3\)-phosphoryl maltotriose (S3). On the other hand, a small amount of indigestible CGT-PO2a and CGT-PO2b remained (Fig. 4b and d). These indigestible fragments were the PO-2 fraction. The contents of the ester phosphoryl groups attached to C-6 and C-3 of the glucosyl residues could be measured as the contents of 3\(^2\)-phosphoryl maltotriose (S1) and 6\(^2\)-phosphoryl maltotriose (S3) except for a small amount of indigestible fragments like the PO-2 fraction (Table 2). As the amount of S2 produced was very small, it could not be analyzed further. Furthermore, the contents of the 6-phosphoryl residues were also measured by the enzymatic methods. A small difference in values was observed because of the indigestible fragments like the PO-2 fraction. In the CGT-PO1 fraction, the contents of the phosphoryl groups at C-3 of the glucosyl residue decreased and that of the phosphoryl groups at C-6 increased due to the action of CGTase. The ratio of C-3 to C-6 phosphoryl groups of CGT-PO2a was similar to that of the PO-1 fraction. The ratio of C-3 phosphoryl group in CGT-PO2b was relatively higher than the other fractions (Table 2). This indicated that both C-3 and C-6 phosphoryl substrates of the PO-1 fraction were used for producing the CGT-PO2a and that more C-3 phosphoryl substrates of the PO-1 fraction were used for producing the fraction of CGT-PO2b.

Fig. 5. Dependence of inhibitory effects on the formation of a calcium phosphate precipitate upon the concentration of CGT-PO2a and CGT-PO2b.

The final concentrations of CGT-PO2a and CGT-PO2b are represented as the horizontal axis, and those of calcium and phosphate were 4 mM and 10 mM, respectively. The mixtures were incubated by shaking at 30°C for 6 h. Symbols: □, CGT-PO2a; ■, CGT-PO2b.

Inhibitory effects of fractions CGT-PO2a and CGT-PO2b on the formation of a calcium phosphate precipitate.

Fractions CGT-PO2a and CGT-PO2b were examined for their ability to inhibit the formation of a calcium phosphate precipitate under the conditions shown in Fig. 3. The sufficient inhibitory effects were found after 4 h in both fractions, such as the PO-2 fraction.\(^3\) The conclusion was that CGT-PO2a and CGT-PO2b were effective fractions in CGT-POs. Then, the dependence of the inhibitory effect upon the concentration of CGT-PO2a and CGT-PO2b was examined. A sigmoidal curve was observed as shown in Fig. 5. In the case of the PO-1 fraction, the sigmoidal curve was not observed under these conditions. It has also been reported that the effective substrates such as alginate and CPP exhibited the sigmoidal curve, and that the concentration had a threshold value to exhibit the inhibitory effect.\(^5,12\) The effect was obtained at a concentration over the point of inflection of the curve (the threshold value). The fraction CGT-PO2b was more effective than the fraction CGT-PO2a since the threshold value of the concentration of CGT-PO2b (0.11–0.21 mM) for the inhibitory effect was about 1/7 of that of CGT-PO2a (0.68–1.36 mM).
Enhancement of POs on Inhibition of Ca-Pi Formation

Fig. 6. Thin-layer chromatogram of CGT-POs.

1, 6\(^{a}\)-phosphoryl maltotriose treated with CGTase; 2, 6\(^{b}\)-phosphoryl maltotriose treated with CGTase; 3, 6\(^{c}\)-phosphoryl maltotetraose treated with CGTase; 4, 6\(^{d}\)-phosphoryl maltopentaose treated with CGTase; 5, 3\(^{a}\)-phosphoryl maltotriose treated with CGTase; 6, 3\(^{a}\)-phosphoryl maltotetraose treated with CGTase; 7, 3\(^{b}\)-phosphoryl maltopentaose treated with CGTase; 8, CGT-POs; 9, POs; M\(_{1}\), glucose, authentic maltooligosaccharides (G2-G6), PO-1 fraction, and PO-2 fraction; M\(_{2}\), Glc-6-P and Glc-1, 6-diP.

Detection of the transglycosylation reaction of CGTase in each substrate of PO-1 fraction.

In order to confirm which compounds in the PO-1 fraction could be the substrate for the CGTase reaction, each purified compound from the PO-1 fraction was individually used for the reaction. The transglycosylation reaction was clearly observed when 3\(^{b}\)-phosphoryl maltopentaose and 6\(^{d}\)-phosphoryl maltopentaose were used as a substrate, respectively (Fig. 6). The reaction was slightly observed when 6\(^{a}\)-phosphoryl maltotetraose was used. However, the transglycosylation reaction could not be detected when the other substrates were used under these conditions.

DISCUSSION

The transglycosylation of the PO-1 fraction was accomplished and an increase in the PO-2 fraction was observed as shown in Fig. 1. As a result, the inhibitory effect on the formation of a calcium phosphate precipitate of POs was obviously improved (Fig. 3). A structural analysis of the CGT-PO2 fraction shows an extension of chain length of glucosyl residues due to the transglycosylation reaction of CGTase. The CGT-PO2 was fractionated to CGT-PO2a and CGT-PO2b fractions from the number of attached phosphoryl groups by ion-exchange chromatography. The fraction CGT-PO2b was more effective than the fraction CGT-PO2a, since the threshold value of the concentration of CGT-PO2b for the inhibitory effect was lower than that of CGT-PO2a (Fig. 5). Additionally, though a significant effect and the threshold value could not be found for the monophosphoryl saccharides such as Glc-6-P, Frc-6-P, PO-1 and CGT-PO1, these properties could be found in the diphosphoryl saccharides such as Glc-1, 6-diP, Frc-1, 6-diP, PO-2 and CGT-PO2a. The threshold values were almost the same as the CGT-PO2a fraction (0.68–1.36 mm) under these conditions. The threshold value of CGT-PO2b (0.11–0.21 mm) was about 1/7 of that of CGT-PO2a. The CGT-PO2b fraction had three phosphoryl groups in the molecule (Table 1). The results support the belief that the inhibitory effect on the formation of a calcium phosphate precipitate depends upon the amount of phosphoryl groups in the POs.

The transglycosylation reaction could be clearly observed when 3\(^{a}\)-phosphoryl maltopentaose and 6\(^{d}\)-phosphoryl maltopentaose were used as a substrate, respectively (Fig. 6). We could not detect the reaction of CGTase when the other substrates were used under these conditions. This TLC system was useful to confirm the transglycosylation of POs since the substances linked to one or at least two phosphoryl groups of each molecule showed different Rf values, and were clearly distinguished from neutral oligosaccharides. The peaks of 3\(^{a}\)-phosphoryl maltopentaose (K) and 6\(^{a}\)-phosphoryl maltopentaose (N) also obviously decreased as shown in Fig. 1b. These results indicate that 3\(^{a}\)-phosphoryl maltopentaose and 6\(^{a}\)-phosphoryl maltopentaose are important substrates in transglycosylation catalyzed by CGTase. In this reaction, released glucose was also detected by HPAEC and TLC as shown in Figs. 1b and 6, respectively, although CDs could not be observed. Hydrolysis occurred while transglycosylation was in progressed. In addition, transglycosylation was slightly observed when 6\(^{a}\)-phosphoryl maltotetraose was used, and the peak of 6\(^{a}\)-
phosphoryl maltotetraose (M) was also decreased as shown in Fig. 1b. From these results, it is estimated that 6'-phosphoryl maltotetraose tends to work as an acceptor molecule rather than as a donor molecule. Further studies are being contemplated to investigate the preparation of a large quantity of each PO-1 fraction for the determination of the precise mechanism of PO transglycosylation.

The structure of the CGT-PO2 fraction was estimated as follow. The CGT-PO2 fraction was easily hydrolyzed to 3'-phosphoryl maltotriose (S1) and 6'-phosphoryl maltotriose (S3) by the action of GA and BSA (Fig. 4). They had also been obtained from fraction PO-1 by the action of BSA and GA as described in a previous study. These facts indicated that the CGT-PO2 fraction was obviously produced from the PO-1 fraction by the transglycosylation reaction of CGTase. From the enzymatic and chemical analyses, the possible structures of the CGT-PO2a fraction are as shown in Fig. 7. Most of the glucosyl residues in the reducing site of CGT-PO2a would be lacking as the result of hydrolysis, for its average DP was about 8. In this context, the structure of the CGT-PO2b fraction would be the products of the transglycosylation of the CGT-PO2a fraction and 3-phosphoryl components according to the results in Tables 1 and 2. However, further study is needed to determine their precise structures.

Additionally, the structure of the PO-2 fraction, which has been newly found in potato starch, was estimated as follows. The PO-2 fraction contained two phosphoryl groups attached to the molecule and the average degree of DP was 5.69 as shown in Table 1. 6'-phosphoryl maltotriose (S3) or 3'-phosphoryl maltotriose (S1) could not be produced from the PO-2 fraction by the action of BSA and GA. However, a small amount of glucose was produced. This indicates that the glucosyl residues attached to the phosphoryl groups of the PO-2 fraction would be located nearby. The possible structures of the PO-2 fraction are as shown in Fig. 7.

In this research, we proposed a method for the enhancement of the inhibitory effect of POs on the formation of a calcium phosphate precipitate. Further studies on calcium absorption in vivo under the existence of CGT-POs are in progress.

REFERENCES

CGTase の転移反応によるリン酸化オリゴ糖のカルシウムリン酸沈澱阻害能の増強

堤阪 宽, 戸尾健二, 日下 要
栗木 隆, 米谷 俊, 岡田茂孝

江崎グリコ株式会社生物化学研究所
(555 大阪市西淀川区曙島 4-6-5)

著者らは馬鈴薯澱粉の加水分解物よりリン酸化オリゴ糖を調製し, これにカルシウムリン酸沈澱阻害効果を見いだした。今回, cyclomaltodextrin glucanotransferase (CGTase) のリン酸化オリゴ糖への作用について調べた。酵素反応後のカルシウムリン酸沈澱形成阻害能は向上した。そして, 酵素の転移反応によるリン酸化オリゴ糖の構造の変化が観察された。反応後の生成物は, イオン交換樹脂で CGT-PO1, CGT-PO2a, および CGT-PO2b の 3 画分に分画された。これらのうち, CGT-PO2a と CGT-PO2b の 2 画分に効果が観察された。CGT-PO2a 画分は平均重合度が 7.89 であり, リン酸基が 2 個結合した構造をしていた。また, CGT-PO2b 画分は平均重合度が 11.06 であり, リン酸基が 3 個結合した構造をしていた。さらに, CGT-PO2b 画分の効果は, CGT-PO2a の効果より顕著に高いくことも明らかとなった。

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