Studies on Phosphoryl Oligosaccharides from Potato Starch

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神戸大学博士論文

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（馬鈴薯澱粉由来リン酸化オリゴ糖に関する研究）

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Abstract

New phosphoryl oligosaccharides (POs) were prepared from potato starch hydrolysate. The inhibitory effect of POs on the formation of calcium phosphate precipitate was investigated to improve the bioavailability of calcium. The POs were fractionated by an ion-exchange chromatography into two fractions, PO-1 and PO-2. Both fractions PO-1 and PO-2 had the ability to form a soluble complex with calcium, and fraction PO-2 had stronger inhibitory effect on the formation of calcium phosphate precipitate and made more stable and soluble complex with the calcium ion. Fraction PO-1 was the main part of POs and composed of maltotriose, maltotetraose, and maltopentaose to which one phosphoryl group was linked. Fraction PO-2 was predominantly composed of maltopentaose and maltohexaose to which at least two phosphoryl groups were linked. On the basis of the reaction specificities of glucoamylase (GA) [EC 3.2.1.3] and bacterial saccharifying α-amylase (BSA) [EC 3.2.1.1], and spectrometrical as well as chemical analyses, the precise structures of POs were determined and estimated. From the results of the inhibitory effect and the structural analyses, it was concluded that the effect was depended upon the number of the phosphoryl groups in the molecule. The author investigated to increase the fraction in which at least two phosphoryl groups were linked in the molecule of POs by using the coupling reaction of cyclomaltodextrin glucanotransferase (CGTase) [EC 2.4.1.19] and by producing the conjugate with ovalubmin (OVA) through the Maillard reaction. The possible usage of POs for characterization and comparison of the action patterns of several α-amylases including neopullulanase [EC 3.2.1.135] are also discussed.

Abbreviations

BLA	bacterial liquefying α-amylase
BSA	bacterial saccharifying α-amylase
°C	degree Celsius
CD	cyclodextrin
CPP	casein phosphopeptide
CGTase	cyclomaltodextrin glucanotransferase
CGT-POs	POs treated by CGTase
DP	degree of polymerization
DSC	differential scanning calorimetry
Fru-6-P	D-fructose 6-phosphate
Fru-1,6-di-P	D-fructose 1,6-diphosphate
GA	glucoamylase
Glc-1,6-di-P	D-glucose 1,6-diphosphate
Glc-1-P	D-glucose 1-phosphate
Glc-6-P	D-glucose 6-phosphate
HPLC	high-performance liquid chromatography
HPAEC	high-performance anion-exchange chromatography
HSA	human saliva α-amylase
IEF	isoelectronic focusing
M_r	relative molecular mass
OVA	ovalbumin
PAD	pulsed amperometric detector
POs	phosphoryl oligosaccharides
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
TAA	Taka-amylase A
TLC	thin-layer chromatography
(v/v)	volume:volume ratio
(w/v)	weight:volume ratio
PO-1	phosphoryl oligosaccharides-1 fractionated from POs
PO-2	phosphoryl oligosaccharides-2 fractionated from POs

PPA	porcine pancreatic α-amylase
6-p-glucosyl residue	glucosyl residues attached the ester phosphoryl group at C-6
3-p-glucosyl residue	glucosyl residues attached the ester phosphoryl group at C-3
6^2-phosphoryl maltose	6-*O*-phosphoryl-*O*-α-D-glucopyranosyl-(1→4)-D-glucose
6^3-phosphoryl maltotriose	6-*O*-phosphoryl-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose
6^2-phosphoryl maltotriose	*O*-α-D-glucopyranosyl-(1→4)-(6-*O*-phosphoryl)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose
6^4-phosphoryl maltotetraose	6-*O*-phosphoryl-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose
6^3-phosphoryl maltotetraose	*O*-α-D-glucopyranosyl-(1→4)-(6-*O*-phosphoryl)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose
6^4-phosphoryl maltopentaose	*O*-α-D-glucopyranosyl-(1→4)-(6-*O*-phosphoryl)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose
3^2-phosphoryl maltotriose	*O*-α-D-glucopyranosyl-(1→4)-(3-*O*-phosphoryl)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose
3^3-phosphoryl maltotetraose	*O*-α-D-glucopyranosyl-(1→4)-(3-*O*-phosphoryl)-*O*--α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose
3^4-phosphoryl maltopentaose	*O*-α-D-glucopyranosyl-(1→4)-(3-*O*-phosphoryl)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucose

Table of Contents

Introduction

1) Problem of calcium absorption

Calcium is the only current nutrient with constant insufficiency in the Japanese (Figure I.1). Indeed, many people ingest only a marginally adequate level of calcium, and calcium deficiency sometimes causes a variety of clinical symptoms, such as osteoporosis, hypertension, and cancer. While the etiologies of all of these diseases are multifactorial and poorly understood, there are much evidence to support the hypothesis that the increase in calcium intake will reduce the risk for each of the diseases.[1-5] Daily dietary allowance for calcium recommended by expert groups from four countries and the World Health Organization (WHO) are shown in Table I.1.[6] The large discrepancy in the recommendations underscores the difficulties and controversies associated with establishing values for human calcium requirement. Dietary allowances for calcium are based primarily on balance data. Balance measurements are relatively simple to be made but are susceptible to rather large errors since balance is calculated as the difference between two relatively large values, neither of which can be accurately obtained. Calcium balance is defined by the following equation:

$$\text{Balance} = Ca_i - (Ca_f + Ca_u + Ca_d)$$

Where Ca_i is the calcium intake, Ca_f is fecal calcium, Ca_u is urinary calcium, and Ca_d is dermal losses.

It is well established that calcium absorption involves at least two mechanisms: passive diffusion and active transport.[7] The passive

diffusion theoretically occurs down an electrochemical gradient, with calcium flux being directly proportional to luminal calcium concentration. Moreover, the passive diffusion should be nonsaturable.[8] On the other hand, the active transport can go against an electrochemical gradient, and the process should be saturable. Intestinal calcium absorption occurs by both mechanisms. The active process requires vitamin D and occurs transcellularity, i.e., calcium

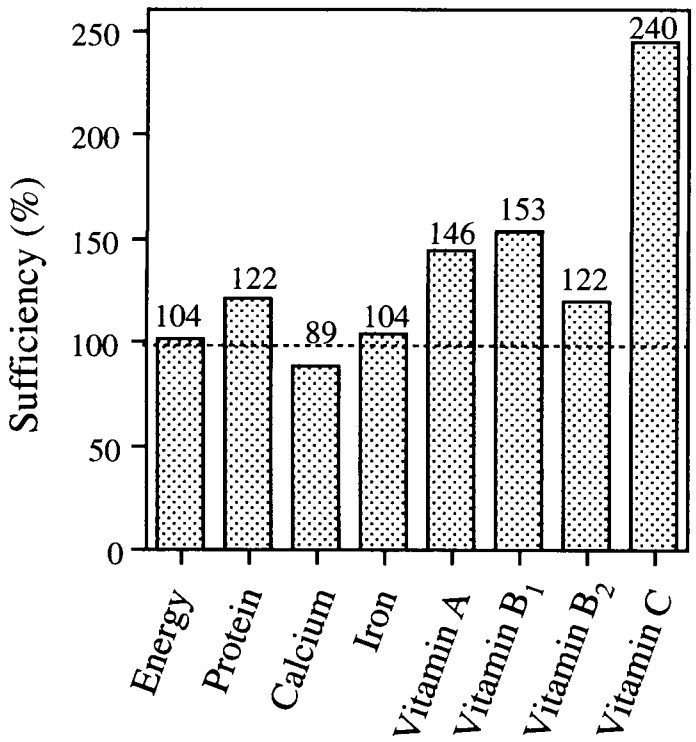

Figure I.1 The Sufficiency of Several Nutrition in Japanese in 1993 (The Report from the Ministry of Health and Welfare)

Table I.1 Recommended Dietary Allowances for Calcium from Countries and the WHO

(mg/day)

Age	United States		Canada		United Kingdom		Japan		WHO	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
0-0.5	360	360	350	350					500-600	500-600
0.5-1	540	540	400	400					500-600	500-600
1										
2			500	500			400	400		
3										
4					600	600			400-500	400-500
5	800	800	600	600						
6										
7								500		
8			700	700			500	500		
9								600		
10							600			
11			900	1000	700	700	700			
12							800	700		
13							900		600-700	600-700
14	1200	1200	1100	800			900			
15							800			
16					600	600	800			
17			900	700			700		500-600	500-600
18							700	600		
19-24	800	800	800	700	500	500				
25-49	800	800	800	700			600		400-500	400-500
>50	800	800	800	800						
Pregnant		1200		1200		1200				1000-1200
Lactating		1200		1200		1200				1000-1200

enters the mucosal cell through the brush border membrane and exists via the basal-lateral membranes.[9] The passive process is paracellular, i.e., calcium is transported from the mucosal to the serosal side by diffusing through the tight junctions between enterocytes.[7,10] Actually, Nellans and Kimberg[11] provided experimental evidence in the ileum. They indicated that the nonsaturable pathway predominates because calcium-binding protein in this segment is very low level[12] and 90% of the calcium is moved by paracellular calcium transport. At that time, it might involve a modulation of the structure of paracellular path.[13,14]

Bioavailability is independent of the physiological status of the organism, and it means potential absorbability in a form which is metabolically active. It is widely recognized a phosphate salt tends to form an insoluble complex with calcium under the alkaline pH conditions of the intestinal environment,[15,16] and the formation of calcium phosphate precipitate reduces calcium absorption into the intestine.[17-19] Since a variety of current processed foods on market, for example, ham, sausage, and many kinds of instant food, contain a significant amount of phosphate, it is important to prevent the formation of calcium phosphate and to help maintain a high concentration of soluble calcium in the intestinal lumen because the passive process is the main mechanism in calcium absorption. Considerable attention has been focused in recent years on the bioavailability of dietary calcium.[19] It is reasonable to expect that water soluble substances having acidic groups such as carboxylates or phosphorylates inhibit the formation of calcium phosphate by forming soluble complexes with calcium ions. For example, Casein phosphopeptide (CPP),[15,16,20] phosvitin, poly-L-glutamate,[16,21-23]

citrate, ATP,[24,25] alginate and chondroitin sulfate[17,21] have been reported as such inhibitors. However, there are few materials actually used in food apart from CPP. CPP is supposed to be the best substance for rendering the calcium ion soluble in several kinds of food now. However, its use is restricted because of its bitterness. In these days, more excellent inhibitors were starved to develop for food. Moreover, it is probable that the interaction between the acidic groups and calcium ion is a key factor for the appearance of the inhibitory activity. However, the mechanisms involved in the action of the inhibitors are almost unknown, and so further work is necessary to understand this precise mechanism.

In addition, increased intake of calcium decreased magnesium solubility in the ileal lumen and consequently lowered magnesium absorption.[26] The inhibitory effect of dietary calcium on magnesium absorption depends on the phosphate:magnesium ratio of the diet, which determines whether an insoluble calcium-magnesium-phosphate complex is formed in the intestine. Magnesium absorption likely depends on the concentration of soluble magnesium in the intestine.[27] It is also important to inhibit the formation of calcium phosphate precipitate for bioavailability of magnesium.

In the food industry, the formation of calcium phosphate precipitate is also a problem on an efficiency in production plant. The calcium phosphate precipitate is a major component of fouling substances both on heat exchangers in drink sterilization[28] and on a membrane in ultrafiltration.[29]

Based on the background described above, inhibition of calcium phosphate formation is a very important in the food industry and nutrition.

2) Aim of this study

Potato starch is known to contain esterified phosphoryl group in its components. Potato amylopectin contains 100-1000 ppm of the ester phosphorus.[30] The wide distribution of the ester phosphorus in various starches was observed as shown in Table I.2.[31,32]

Table I.2 Phosphorus Contents of Starches

Source	P_0[a] (ppm)	P_6[b] (ppm)	P_6/P_0 (%)
Roots			
Potato	370-970	290-800	58-86
Sweet potato	117-132	82-99	66-70
Yam	129	77	60
Kuzu	92	50	54
Braken	72	42	58
Lily	60	33	55
Seeds			
Chestnut	53	35	66
Water chestnut	30	18	60
Cereals			
Waxy maize	10	6	60
maize	61	4	7
Waxy rice	16	15	94
Rice	119	11	9
Wheat	47	1	2

[a] Phosphorus existed in the covalently bound phosphoryl group

[b] Phosphorus existed in the phosphoryl groups located at C-6 of glucosyl residue

The author's attention was focused on the utilization of esterified phosphoryl group in potato starch components because very little attention is paid their characterization and utilization in starch processing. The actions of the amylolytic enzymes were hindered by the phosphoryl groups linked to the glucosyl residues, and phosphoryl oligosaccharides (POs) were obtained as indigestible components by the enzymes. The author investigated the inhibitory effect of the POs on calcium phosphate precipitation and analyzed structures of POs. The relationship between the calcium solubilizing ability and the number of esterified phosphoryl group per molecule, and an application of POs were also described.

In chapter 1, the author proposed the method of the preparation of POs and their inhibitory effect on the formation of calcium phosphate *in vitro* and *in vivo*.

In chapter 2, the author described the structure of POs.

In chapter 3 and 4, the author described a way of enhancing the inhibitory effect of POs on the formation of calcium phosphate precipitate by using the coupling reaction of CGTase. The production and characterization of the OVA-POs conjugate are also reported.

In chapter 5, the author proposed the possibility of POs on solubilizing iron under neutral conditions.

In chapter 6, the author explored the action pattern of several α-amylases on POs, and proposed the possibility of POs as a new substrate for classifying α-amylases.

Chapter 1: Preparation of POs and its Inhibitory Effect on the Formation of Calcium Phosphate Precipitate

1.1 Introduction and aim

Potato starch is known to contain a small amount of covalently bound phosphoryl groups, 1 of 200 to 500 glucosyl residues on the average being phosphorylated. Takeda and Hizukuri have reported that the phosphoryl groups were located mostly in amylopectin, whereas the phosphorylation of amylose was very little.[33] In this chapter, potato starch was hydrolyzed, using bacterial liquefying α-amylase (BLA) [EC 3.2.1.1], glucoamylase (GA) [EC 3.2.1.3], and pullulanase [EC 3.2.1.41]. Saccharides having phosphoryl groups could be expected to provide interesting information on the inhibition of calcium phosphate formation.

1.2 Materials and methods

1.2.1 Enzymes

Alkaline phosphatase from *Escherichia coli* and β-amylase from sweet potato were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). BLA from *Bacillus amyloliquefacience* 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 from Hayashibara Biochemical Lab. (Okayama, Japan).

1.2.2 Other materials

Chitopearl BCW-2501 was purchased from Fuji Spinning Co.(Osaka, Japan), and Sephadex G-10 was from Pharmacia Fine Chemicals Co. (Uppsala, Sweden). Authentic maltooligosaccharides with DP = 1-6, 7, and 8-10 were purchased from Sigma Chemicals, Hayashibara Biochemical Lab., and Funakoshi Pharmaceutical Co.(Tokyo, Japan), respectively. D-Glucose 1,6-diphosphate (Glc-1,6-di-P) and D-glucose 1-phosphate (Glc-1-P) were purchased from Boehringer Mannheim (Mannheim, Germany), and D-fructose 1,6-diphosphate (Fru-1,6-di-P) was purchased from Fluka Chemika (Buchs, Switzerland). D-Glucose 6-phosphate (Glc-6-P) was purchased from Nacalai Tesque (Kyoto, Japan), and D-fructose 6-phosphate (Fru-6-P) and sodium alginate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Citrus pectin was a product from Sigma Chemicals, and CPP (grades I, II and III) was from Meiji Seika Kaisha (Tokyo, Japan). CPP I was a tryptic digest of casein containing over 12% CPP, CPP II was produced by removing the bitter compounds from CPP I (containing over 12% CPP), and CPP III was purified from CPP I (containing over 85% CPP). Phosphomannan was prepared from a culture broth of bisexual diploid yeast *Hansenula holstii* NRRL Y-2448 by the method of Jeanes *et al.*[34] Modified starch (4% phosphorylated, w/w) was kindly supplied by Nippon Starch Chemical Co. (Osaka, Japan). An alginate hydrolysate was prepared by heating at 100°C in 0.5 N HCl for 15 min. A pectin hydrolysate was prepared by reacting 1 ml of 0.121 U/ml of pectinase from *Aspergillus sp.* (Sigma Chemicals) with 1 ml of 1% (w/v) pectin in a 10 mM acetate buffer (pH 4.5) at 25°C for 1 h. The reaction was terminated by heat

treatment at 100°C for 5 min. After the precipitate had been centrifuged off, the pectin hydrolysate was obtained. Acetone powder of the small intestine of rats was purchased from Sigma Chemicals. All other chemicals and materials used were of analytical or commercial grade.

1.2.3 Preparation of POs

Potato starch (1 g) was suspended in 100 ml of a solution containing 6 mM NaCl, 2 mM $CaCl_2$ and 3.0 U/ml of BLA. After the suspension had gradually been heated to 100°C to liquefy the starch, the solution was cooled and incubated at 50°C for 1h. Then, 1 ml of 600 U/ml of GA, 1 ml of 200 U/ml of pullulanase and 1 ml of 300 U/ml of BLA were added, and the reaction mixture was incubated at 40°C for 2 h. The amylolytic reaction was monitored by the iodine-starch complex method and glucose oxidase method.[35] Since the action of the amylolytic enzymes was hindered by phosphoryl groups linked to the glucosyl residues, POs resided as indigestible fragments by this treatment.[36,37] After the iodine-starch color reaction of the solution had indicated negative, the solution was heated in a boiling-water bath for 10 min, and then applied on a Chitopearl BCW-2501 column (1ϕ x 2 cm) that had been equilibrated with a 20 mM acetate buffer (pH 4.5). Neutral sugars were washed out with the buffer, and POs were eluted by the same buffer containing 0.5 M NaCl. The eluted POs were precipitated with ethanol (finally 70%, v/v). To remove the salts, the ethanol precipitate was washed several times with 70% ethanol. After the washed precipitate was dissolved in deionized water, the solution was finally freeze-dried.

1.2.4 Structural evaluation of POs

The POs were dephosphorylated by an alkaline phosphatase treatment. POs (50 μl, 1%, w/v) in a 20 mM carbonate buffer (pH 9.4) containing 3 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ were incubated at 37°C for 30 h with 10 μl of 400 U/ml of alkaline phosphatase. The reaction was terminated by ultrafiltration to remove those enzymes with a higher molecular weight than 30,000.

The product was analyzed on HPAEC with a Dionex DX-300 (Dionex Corp., Sunnyvale, CA, U.S.A.) gradient chromatography system. The chromatographic conditions were as follows: column, CarboPac PA-100 (4ϕ x 250 mm, Dionex Corp.); detector, PAD; meter scale, 1 μC; temperature, ambient. Elution was done by 100 mM NaOH containing the following gradient of 1M NaOAc: 0% (v/v) at 0 min, 10% (v/v) at 12 min, 20% (v/v) at 32 min, 20% (v/v) at 37 min and 80% (v/v) at 57 min; flow rate, 1 ml/min.

The composition of each PO was determined from the retention time of the dephosphorylated compound compared with that of the authentic oligosaccharide. The average degree of polymerization (DP) of the POs were calculated from the peak areas of components of the dephosphorylated compounds on HPAEC, using the following response factors: G2, 1.0; G3, 0.989; G4, 0.979; G5, 0.969; G6, 0.958; G7, 0.948; G8, 0.938. The number of phosphoryl groups attached to the molecules was determined by evaluating the mole values from the total amount of carbohydrates and the average DP. The total amount of carbohydrates was measured by the phenol-sulfuric acid method,[38] while the amount of the covalently bound phosphoryl group was

measured as the amount of inorganic phosphates by the method of Itaya and Ui[39] after degradation with hot perchloric acid.[40]

1.2.5 Inhibitory effect on the formation of calcium phosphate precipitate

To examine the inhibitory effect, a modification of the method of Yamamoto *et al.*[17] 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 NaN_3 and 80 mM KCl. The initial pH level of this system was precisely adjusted to pH 7.4 with NaOH and HCl, before 400 µl of a solution containing 10 mM $CaCl_2$ and 5 mM NaN_3 was added to the solution. The final concentrations of calcium and phosphate were 4 mM and 10 mM, respectively. The conditions were similar to those of human intestine. 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.

1.2.6 Effect of POs on calcium absorption in rats

1.2.6.1 Digestibility of POs

A digestion test of POs was conducted by a simplified method of Hirayama *et al.*[41] Rat intestinal acetone powder was suspended in distilled water (100 mg/ml) and disrupted by an ultrasonicator. After

centrifugation, the supernatant was obtained as an enzyme solution. Then, 50 μl each of the enzyme solution was mixed with 50 μl of test solution (0.5%, w/v, POs, sucrose, and maltose), and the mixture was allowed to react at 37°C for 0, 1, 2, 3 h. After reaction, the solution was placed in a boiling water bath for 5 min to terminate the reaction. The amount of glucose was measured with a glucose oxidase method.[35] Digestibility was calculated from the amount of released glucose.

1.2.6.2 Effect of POs on calcium absorption from intestine

Five-week-old male Sprague-Dawley rats (Clea, Tokyo) were fed a solid diets MF (Oliental Yeast Co., Tokyo, Japan) for 2 weeks. After withdrawal of diets for 24 h, five rats were anesthetized with Nembutal, and their small intestines were dissected out. After a longitudinal abdominal incision, the small intestine was ligated at two sites, i.e. below the biliary duct and 30 cm away from the first site. Immediately, the sample solution was injected to the ligated intestines. The sample solution was 1 ml of 5% (v/v) POs or 5% (v/v) sucrose in a 20 mM phosphate buffer (pH 7.4) containing 28.5 mM $CaCl_2$ solution. The ligated intestine was then put back into the abdominal cavity. After 2 h, the ligated intestine was put out and the intestinal contents were flushed out with saline solution. The amount of calcium of the solution was measured by using an atomic absorption spectrometer (Spectrophotometer AA-1, Nippon Jarrell-Ash, Kyoto, Japan). Absorption of calcium was estimated as the amount disappearing during 2 h.

1.2.6.3 Effect of POs-feeding on calcium utilization

Four-week-old male Sprague-Dawley rats (Clea, Tokyo) were housed in individual stainless-steel metabolic cages in a temperature- and humidity-controlled room (23 ± 1°C and 55 ± 7% relative humidity) and a 12-h light-dark cycle. After a 7-day adaptation period in which all rats were fed a solid diets MF as control diet, the rats were separated into two groups of six animals having similar mean body weight. The one group was fed experimental diet (Table 1.1) and deionized water ad libitum for 5 weeks. POs were prepared from a potato starch hydrolysate by the method in section 2.2.3. Isolated soy protein was gifted from Fuji oil Co.(Osaka, Japan). Body weight was recorded every week and food intake was recorded every 2nd or 3rd day. For the calcium balance study, fecal and urine samples were collected from terminal 4 days. Feces and urine were first ashed at 550°C for 15 h in a muffle furnace. Each ashed sample was dissolved in 1 N HCl. The diluted solution was colorless and did not contain suspended solids. The sample was diluted with lanthanum chloride solution and calcium contents were determined by using an atomic absorption spectrometer. The absorption ratio and retention ratio of calcium were determined by the following equations:

Absorption ratio(%) = (calcium intake - fecal calcium excretion) x 100/calcium intake

Retention ratio(%) = (calcium intake - fecal calcium excretion - urinal calcium excretion) x 100/calcium intake

All data were analyzed by one-way analysis of variance. When significant F ratios were found, individual means were compared by Tukey's test. The level of significance was considered to be $p < 0.05$.

Table 1.1 Composition of Experimental Diets

(g / 100 g diet)

	Control	Test diet
ISP	20.0	20.0
Corn starch	66.8	63.4
Corn oil	5.0	5.0
Cellulose powder	0.5	0.5
Vitamin mix[a]	0.8	0.8
Chorine chloride	0.2	0.2
Mineral mix[b]	4.0	4.0
$CaCO_3$	0.4	0.4
$CaHPO_4$	0.6	0.6
KH_2PO_4	1.7	1.6
POs	-	0.35
Ca (%)	0.35	0.35
Pi (%)	0.7	0.7

Prepared according to AIN-76 prescription.

[a] AIN-76 Vitamin mix (Oriental Yeast Co., Ltd.).

[b] AIN-76 Mineral mix (Ca, P free)(Oriental Yeast Co., Ltd.).

1.3 Results

1.3.1 Preparation of POs inhibiting the formation of calcium phosphate

The mixture of POs, which had been prepared from the potato starch hydrolysate by BLA, GA and pullulanase, was analyzed on HPAEC with the results shown in Figure 1.1b. A chromatogram of authentic maltooligosaccharides and phosphoryl monosaccharides is shown in Figure 1.1a, Glc-1,6-di-P, Glc-1-P and Glc-6-P being detected at different retention times on HPAEC according to the number and the positions of phosphoryl groups linked to each molecule. The POs were clearly distinguishable from Glc-6-P, Glc-1,6-di-P, glucose and maltooligosaccharides (Figure 1.1b) by this system. Figure 1.2 shows the effect of POs mixtures (Figure 1.1b) on the formation of calcium phosphate precipitate. In comparison with the control, calcium remained soluble for the first hour after adding the oligosaccharides. After 2 hours, the amount of soluble calcium gradually decreased. This phenomenon indicates two possibilities, one being the insufficiency of binding strength between calcium and the phosphoryl group of the POs mixtures. The other possibility is an insufficient amount of the effective fraction in the oligosaccharide mixtures. In the latter case, the effective fraction would be separated.

1.3.2 Examination of the interaction between POs and calcium

To evaluate the affinity of the POs with calcium, calcium chloride was chromatographed in a Sephadex G-10 column with or without the

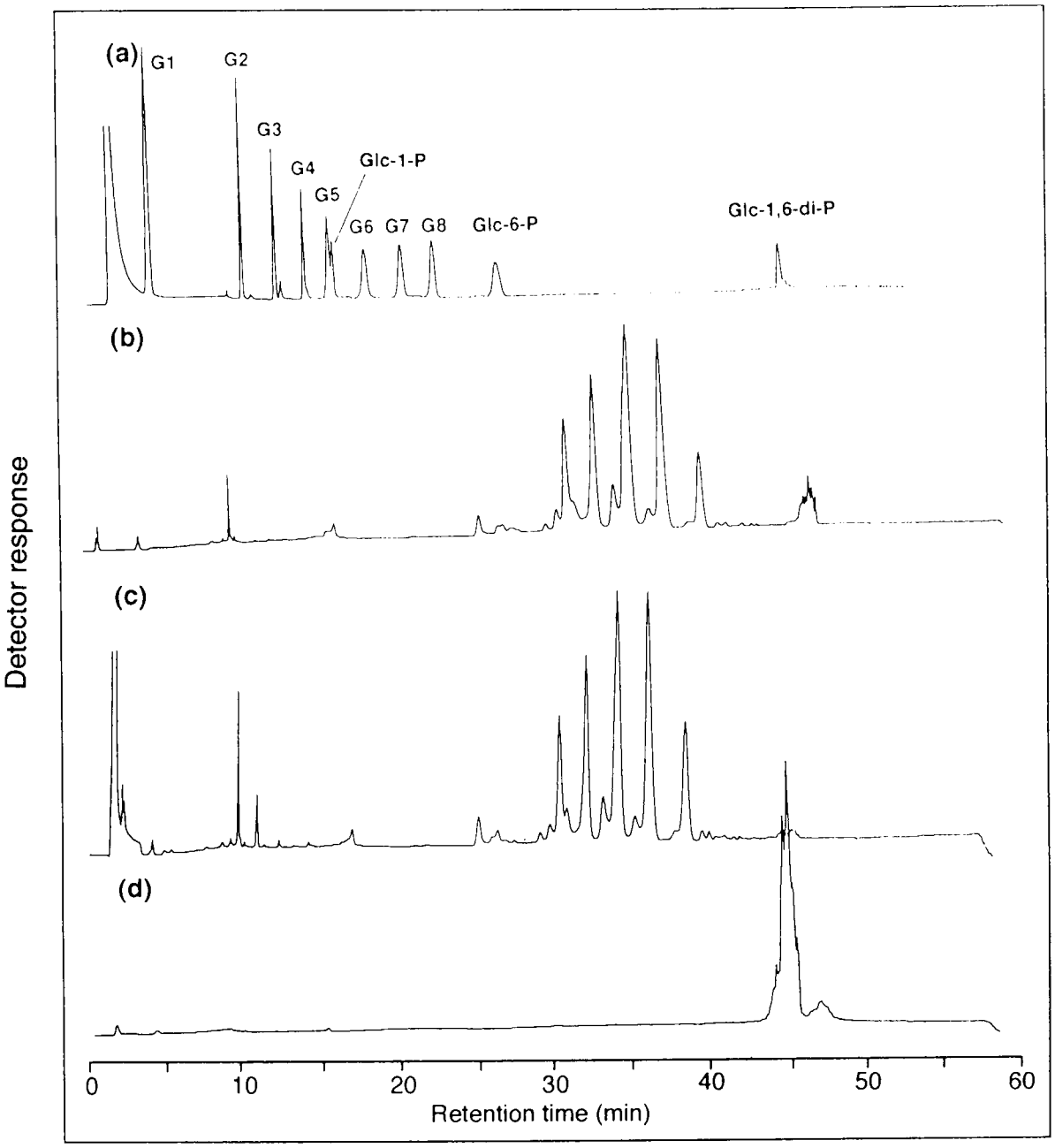

Figure 1.1 Chromatograms of the Maltooligosaccharides, Phosphoryl Monosaccharides, and POs Prepared from Potato Starch
(a) authentic maltooligosaccharides; (b) POs; (c) PO-1; and (d) PO-2. The amount of each sample injected was (a) 0.01%, 25 µl except for Glc-1,6-di-P at 0.1%, 25 µl; (b) 0.05%, 25 µl; (c) 0.05%, 25 µl; and (d) 0.2%, 25 µl. *Abbreviations*: G1-G8, glucose-maltooctaose; the others are as indicated in the text.

oligosaccharides (Figure 1.3). The elution volume of calcium, when calcium was applied with POs, was different from that when calcium was solely chromatographed. In the former case, calcium and the POs were eluted at the same position, suggesting that the POs had affinity with calcium.

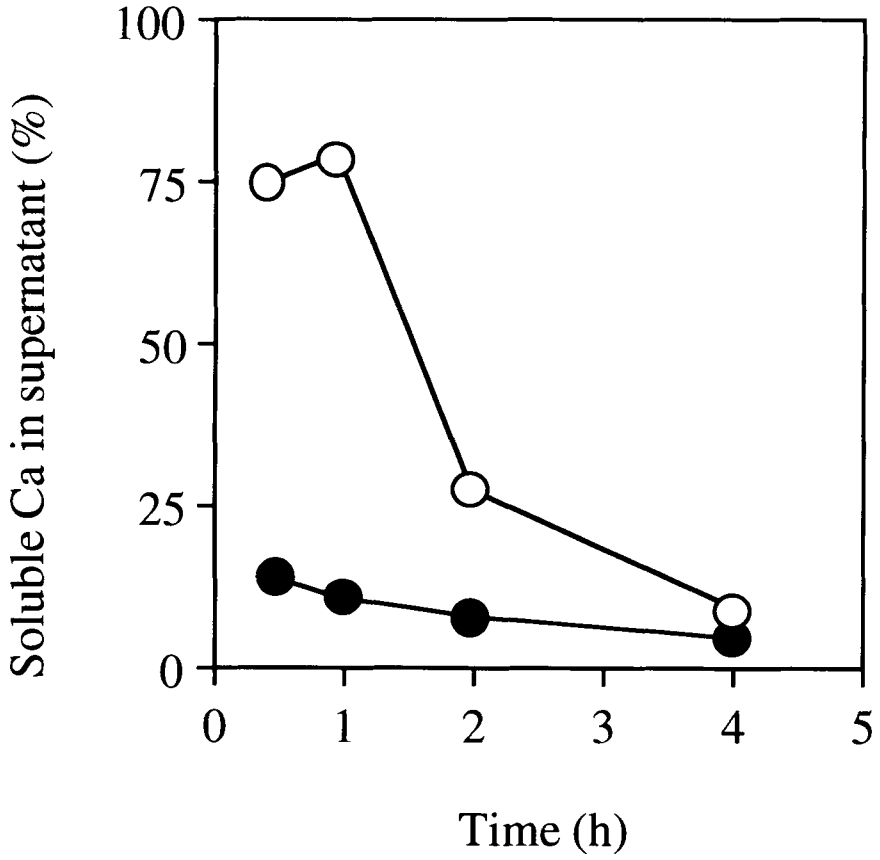

Figure 1.2 Inhibitory Effect of POs on the Formation of a Calcium Phosphate Precipitate
The concentrations of the POs (see Figure 1.1-b) in test solution were 0.5%, and those of calcium and phosphate were 4 mM and 10 mM, respectively. Symbols: ○ POs; ● deionized water as a control.

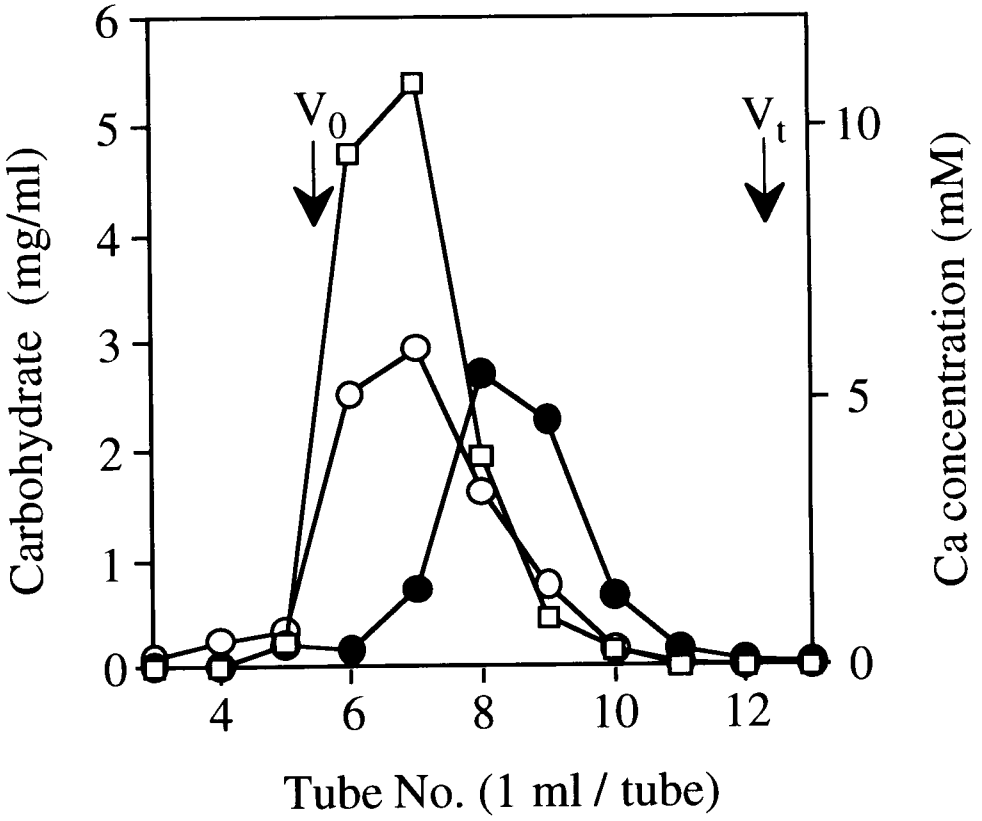

Figure 1.3 Gel Filtration Chromatogram of $CaCl_2$ with or without POs

A solution containing 100 mM calcium chloride (100 µl) was chromatographed with (○) or without (●) 10% POs in a Sephadex G-10 column (2.0 ϕ x 4 cm), and the calcium concentration in each fraction was measured. The POs concentration in each fraction was measured as the total amount of carbohydrates in the solution by the phenol-sulfuric acid method (□). The column was equilibrated and eluted with a 10 mM Tris-HCl buffer (pH 7.5) at a flow rate of 1 ml/min. V_0, void volume of column; V_t, total volume of column.

1.3.3 Fractionation of the POs

The mixture of POs (10 mg) were applied on a Chitopearl BCW-2501 column equilibrated with a 20 mM acetate buffer (pH 4.5). The adsorbed POs were eluted from the column with the same buffer containing 0.15 M (fraction PO-1) and 0.4 M NaCl (fraction PO-2), respectively. Each fraction was concentrated and then applied on a Sephadex G-10 column equilibrated with deionized water in order to remove NaCl. The eluted POs were collected and freeze-dried, the HPAEC chromatograms of fractions PO-1 (6.5 mg) and PO-2 (1.2 mg) being shown in Figure 1.1c and 1.1d, respectively.

1.3.4 Evaluation of the structures of PO-1 and PO-2

Fractions PO-1 and PO-2 were dephosphorylated with an alkaline phosphatase treatment, and the dephosphorylated oligosaccharides were then analyzed on HPAEC (Figure 1.4). No amylase activity was detected under these conditions. As shown in Figure 1.4 and Table 1.2, dephosphorylated PO-1 was composed of maltotriose, maltotetraose and maltopentaose. The average DP was calculated to be 4.02. Dephosphorylated PO-2 was composed of maltotriose, maltotetraose,

Table 1.2 Average DP and Content of Covalently Bound Phosphoryl Group in PO-1 and PO-2

	PO-1	PO-2	Glc-6-P
Average DP	4.02	5.82	1.00
Phosphoryl Group[a]	0.71	1.88	0.80

[a] Values are given as mol / mol.

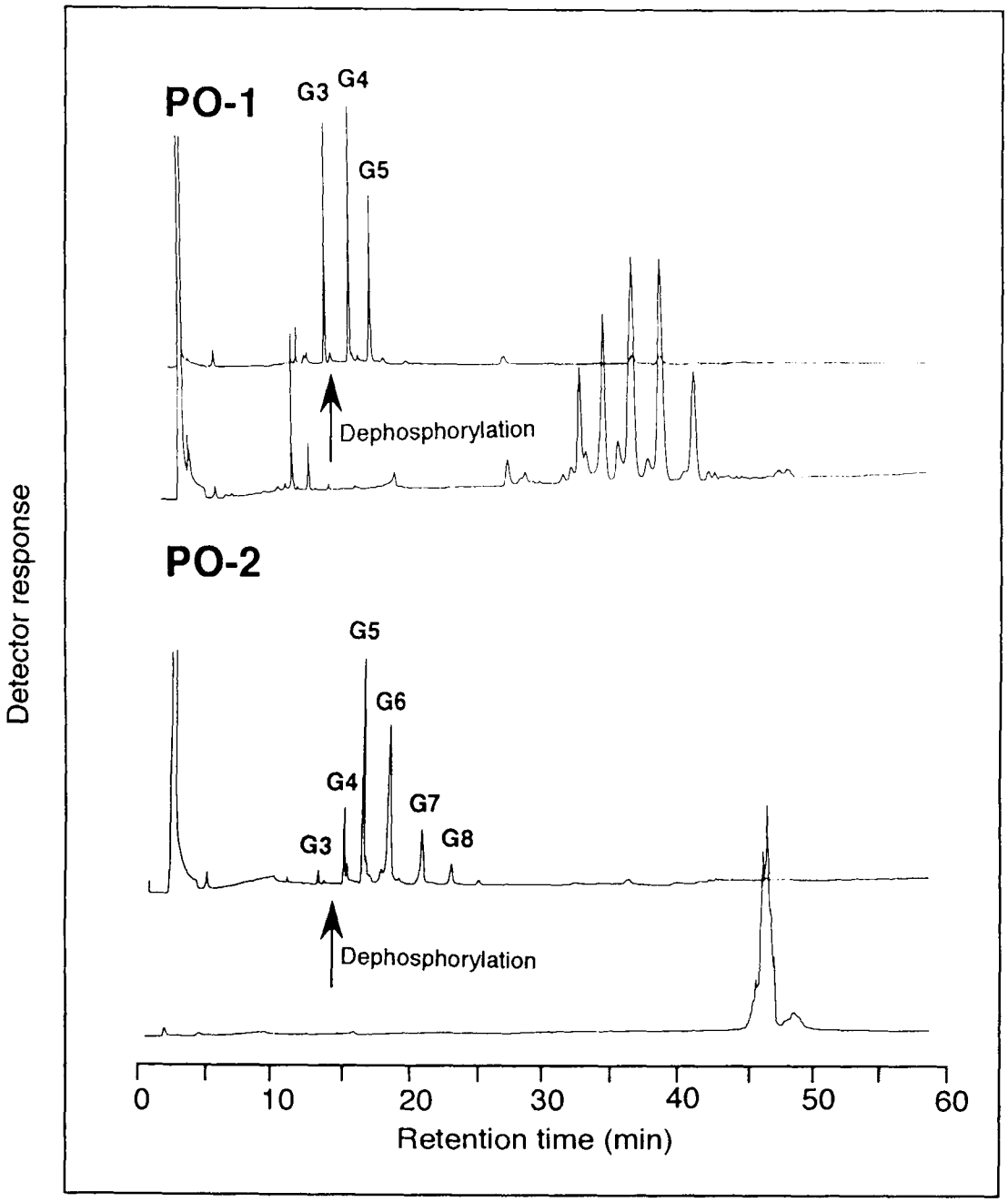

Figure 1.4 Treatment of POs with Alkaline Phosphatase
Dephosphorylated PO-1 and PO-2 were analyzed by HPAEC under the same conditions as those shown for Figure 1.1. *Abbreviations* are as listed for Figure 1.1.

maltopentaose, maltohexaose, maltoheptaose and maltooctaose. The main components were maltopentaose and maltohexaose with the average value of DP 5.82 (Table 1.2). (The average value of DP of the POs before fractionation was calculated to be 4.30.) The content of phosphoryl groups was analyzed by using Glc-6-P as standard, and calculated to be 0.8 phosphoryl group per each molecule. Fraction PO-1 had one phosphoryl group, and fraction PO-2 had at least two phosphoryl groups per each molecule. Fractions PO-1 and PO-2 were mono- and di-phosphorylated α-1,4 maltooligosaccharides, since maltose, maltotriose and a small amount of glucose were released and branched maltooligosaccharides were not obtained from the dephosphorylated PO-1 and PO-2 by the action of β-amylas [EC 3.2.1.2] (data not shown). The enzyme hydrolyzes amylose from its non-reducing end and releases β-maltose. The hydrolysis of amylopectin stops at certain places near the branched points of the α-1,6-glucosidic linkages.[42)]

1.3.5 Inhibitory effects of fractions PO-1 and PO-2 on the formation of calcium phosphate precipitate

The inhibitory effects of fractions PO-1 and PO-2, and the dependence of this inhibitory effect upon the concentration of fraction PO-2 were examined. Fraction PO-2 was found to be a more effective inhibitor than fraction PO-1 (Figure 1.5a). PO-2 was main effective fraction of the potato starch hydrolysate for inhibiting calcium phosphate formation and it stabilized soluble calcium in the supernatant during the examination period of time. However, the POs mixtures did not entirely inhibit the formation of calcium phosphate as shown in Figure 1.2. The dependence of the inhibitory effect upon the

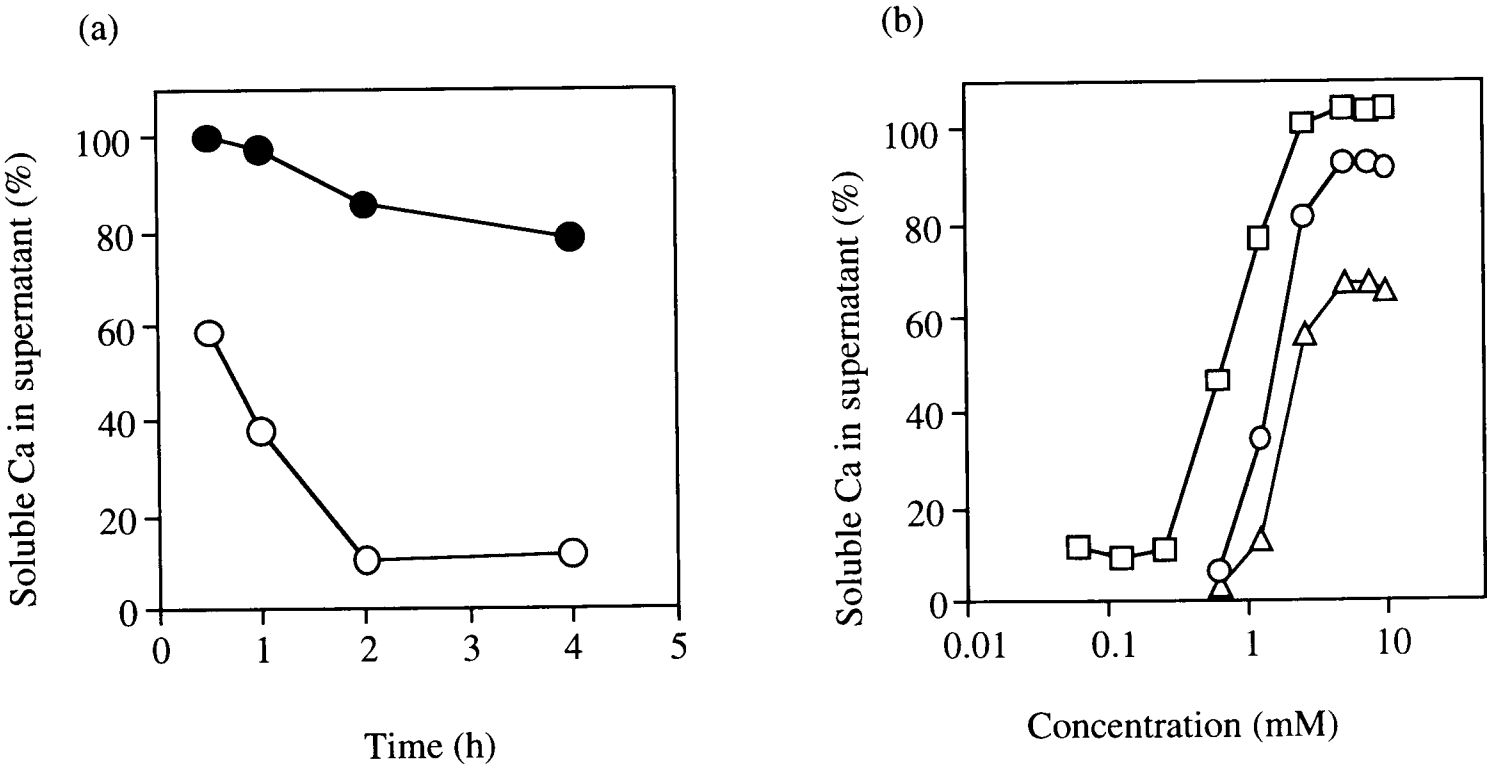

Figure 1.5 Inhibitory Effects of Fractions PO-1 and PO-2 on the Formation of Calcium Phosphate Precipitate

(a) Inhibitory effects of fractions PO-1 and PO-2. The concentration of fractions PO-1 and PO-2 was 0.5%, and those of calcium and phosphate were 4 mM and 10 mM in test solution, respectively. Symbols: ○ PO-1; ● PO-2. (b) Dependence upon concentration of the inhibitory effect of fraction PO-2 on the formation of calcium phosphate. Symbols: □ 2 h; ○ 8 h; △ 24 h.

concentration of fraction PO-2 was examined and exhibited a sigmoidal curve, as shown in Figure 1.5b. In the case of PO-1 fraction, the sigmoidal curve could not be exhibited. It was also reported that the effective substances such as alginate and CPP exhibited the sigmoidal curve and had threshold values of the concentration to exhibit the inhibitory effect.[16,17] The effect was observed at the concentration over the threshold value. The inhibitory effect of fraction PO-2 appeared at a final concentration above about 0.5 mM in the test solution, and was still found after 24 h when the final concentration of fraction PO-2 was about 10 mM (Figure 1.5b).

1.3.6 Effect of POs on calcium absorption in rats

1.3.6.1 Digestibility of POs

Sucrose and maltose could be hydrolyzed easily by rat intestinal acetone powder as shown in Figure 1.6. Digestibility of them was at least 80% after 3h. However, POs could hardly be hydrolyzed, and its digestibility was 30% at the most after 3 h.[43]

1.3.6.2 Effect of POs on calcium absorption from intestine

There was difference in two groups, and 5% POs group enhanced the absorption of calcium from intestine in this condition ($p < 0.06$) as shown in Figure 1.7.[43]

1.3.6.3 Effect of POs-feeding on calcium utilization

The final body weight and food intake of rats fed the diets were not significantly different in two groups. Figure 1.8 shows the apparent

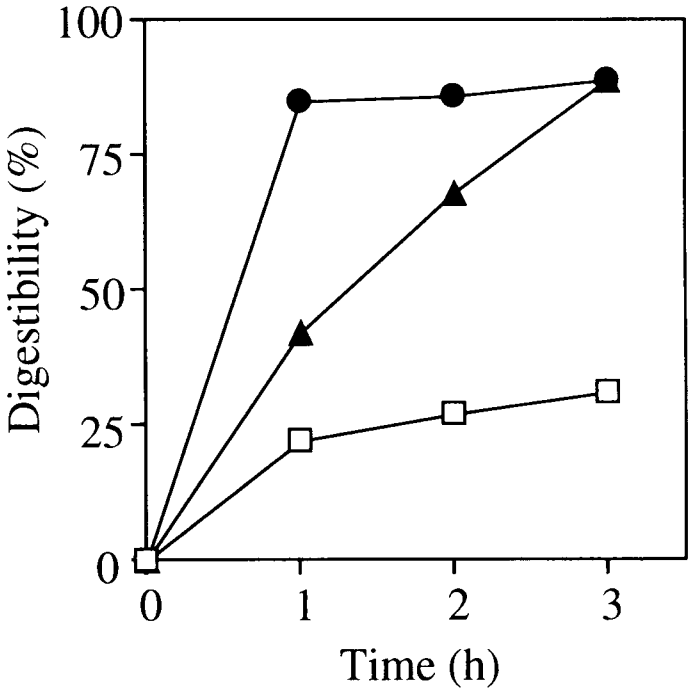

Figure 1.6 Maltose, Sucrose, and POs Digested by Rat Intestinal Acetone Powder
Symbols: □, POs; ▲ sucrose; ●, maltose.

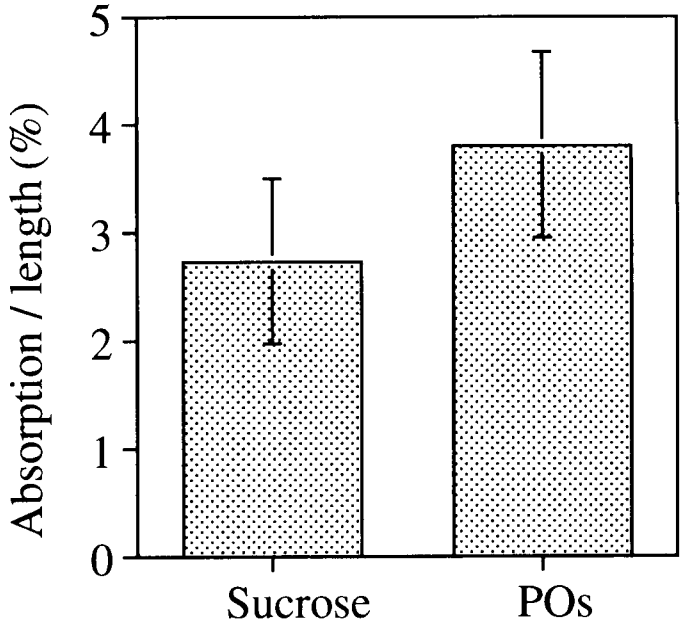

Figure 1.7 Calcium Absorption in the Rat Small Intestine with Ligated Loop
Values refer to means±standard deviations of five rats. Ten % POs or sucrose containing 57 mM Calcium chloride was injected to the small intestine.

calcium absorption ratio and retention ratio during the balance study (32-35 days).[43] Both the apparent absorption and the apparent retention ratios of calcium were increased in rats fed the POs diet, but no statistically significant differences were observed.

1.3.7 Effect of various saccharides on the inhibition of calcium phosphate formation

The inhibitory effects of various saccharides were investigated. As shown in Table 1.3, phosphomannan and modified starch (chemically phosphoryl starch) showed a strong inhibitory effect like that of pectin on the formation of calcium phosphate precipitate. The amylase-indigestible fragment of modified starch was also obtained by a similar method to prepare POs from potato starch, the amount of glucose released from the modified starch attaining 17% of total glucosyl residues at the most. The inhibitory effect of the indigestible fragments on the formation of calcium phosphate was also examined. The results showed that the fragments were more effective inhibitors than native modified starch (data not shown). Several phosphoryl monosaccharides, Glc-6-P, Glc-1,6-di-P, Fru-6-P and Fru-1,6-di-P, were also examined. Glc-1,6-di-P and Fru-1,6-di-P were found to inhibit the formation of calcium phosphate. The dependence of the inhibitory effect upon the concentration of Glc-1,6-di-P and Fru-1,6-di-P was then examined (data not shown). It is of interest to note that diphosphoryl monosaccharides such as Glc-1,6-di-P and Fru-1,6-di-P were as effective as fraction PO-2 shown in Figure 1.5b. On the other hand, the monophosphoryl monosaccharides had a very weak effect on the inhibition of calcium phosphate formation, as was found for PO-1. Alginate and pectin carrying carboxyl groups were also found to have

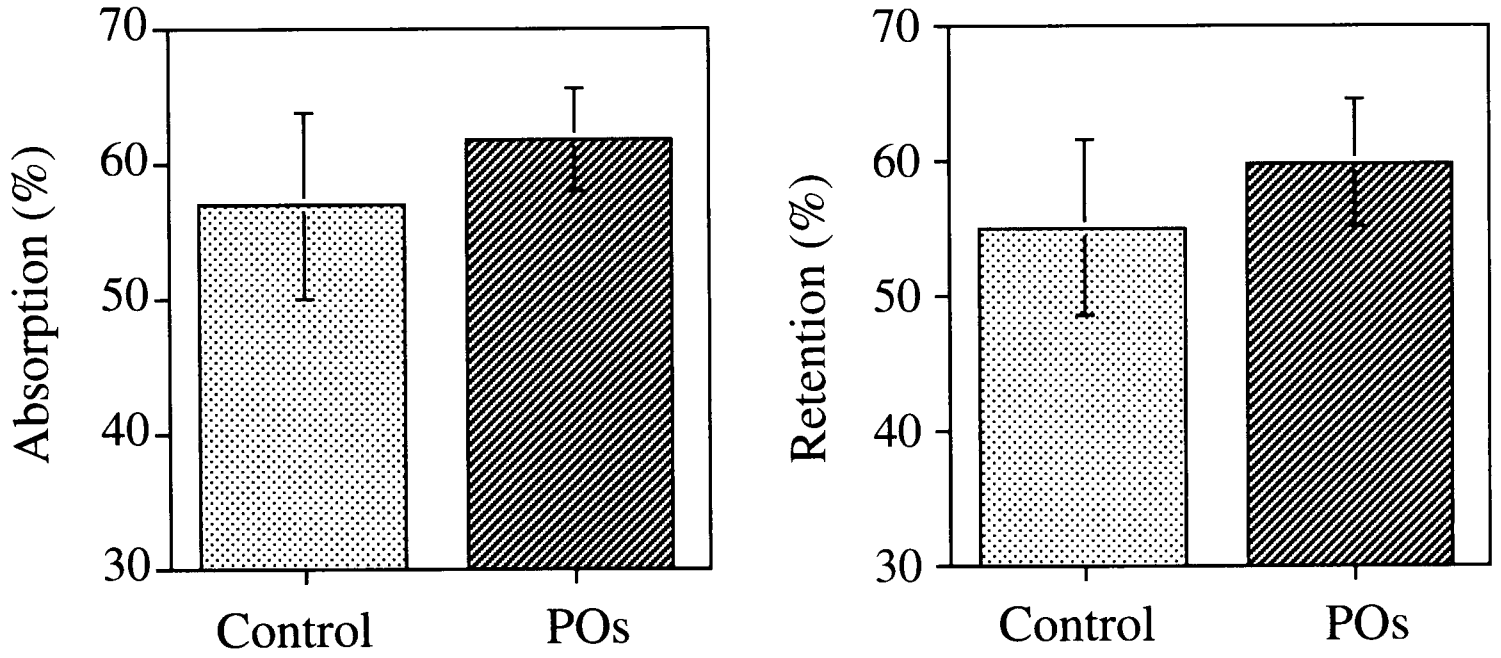

Figure 1.8 Effect of Feeding POs and Control Diets on the Apparent Calcium Absorption and Retention Ratio in Rats

Values are means ± standard deviations represented by vertical bars (6 rats/group).

Table 1.3 Inhibitory Effect of Various Saccharides and CPP on the Formation of Calcium Phosphate Precipitate

Alginate	100
Alginate hydrolysate	21
Pectin	97
Pectin hydrolysate	15
Polygalacturonate	0
Phosphomannan	100
POs	75
PO-1	38
PO-2	100
Modified starch [a]	100
Modified starch hydrolysate	95
Hyaluronate	0
Dextran sulfate	0
Chondroitin sulfate	0
Glucuronate	0
Galacturonate	0
N-Acetylneuraminate	0
Glu-1,6-di-P	95
Fru-1,6-di-P	98
Glu-6-P	13
Glu-1-P	12
Fru-6-P	12
CPP [b]	100

The concentrations of the polysaccharides and their hydrolysates in test solution were 0.5%, of the monosaccharides were 0.5 mM, and of calcium and phosphate were 4 mM and 10 mM, respectively. The percentage of soluble calcium in the supernatant after 1 h shaking to the calcium initially added was calculated.

[a] Chemically phosphoryl starch

[b] CPP III

an inhibitory effect, while the hydrolysate of these acidic polysaccharides had no effect. The other acidic polysaccharides, namely dextran sulfate, chondroitin sulfate, galacturonate and glucuronate had no effect.

1.4 Discussion

It has been reported that oligosaccharides improved intestinal bacterial flora, prevented carcinogenesis, and decreased the calorie effect of foods.[44-48] The author reports a new function of oligosaccharides in this chapter, inhibition of the formation of calcium phosphate.

The author prepared POs from potato starch, using BLA, GA and pullulanase, and they were found to have the ability to inhibit the formation of calcium phosphate precipitate. Although fractions PO-1 and PO-2 from the POs had the ability to form a complex with calcium, the fraction PO-2 had the stronger inhibitory effect and made a more stable and soluble complex with calcium ion. This ability was equal to that of CPP under the conditions shown in Table 1.3. Therefore, the fraction PO-2 was the principal part of the POs that inhibited calcium phosphate formation. On the other hand, diphosphoryl monosaccharides such as Glc-1,6-di-P and Fru-1,6-di-P were found to be more effective than monophosphoryl monosaccharides such as Glc-6-P and Fru-6-P. Additionally, Glc-1,6-di-P and Fru-1,6-di-P exhibited the sigmoidal curve and had the threshold value of the concentration on the inhibitory effect as shown in Figure 1.5b, though Glc-6-P and Fru-6-P did not. Their threshold values were the same as the PO-2 fraction. These results lead to the

assumption that at least two phosphoryl groups would be needed in a material inhibiting the formation of calcium phosphate precipitate, although the problem for the degree of the distance between the phosphoryl groups was remained.

In addition, the enhancement effect of POs on calcium absorption in rats was investigated. POs were low digestibility (Figure 1.6) and enhancement of calcium absorption in the intestine (Figure 1.7) was observed. Although the apparent absorption and the apparent retention ratios of calcium were increased in rats fed the POs diet, no statistically significant differences were observed (Figure 1.8). Further investigation was needed under several conditions.

We also found that a modified starch (chemically phosphoryl starch) showed a similar inhibitory effect. The hydrolysate of the modified starch had a stronger inhibitory effect than the native modified starch, the hydrolysate having many more phosphoryl groups in the molecule than exist in the native modified starch. From these results, it is presumed that these effects would also depend upon the density of phosphoryl groups attached to each sugar molecule, similar to that of phosphoryl groups attached to CPP which inhibits the formation of calcium phosphate.[49] Although, as shown in Table 1.3, the sugars having phosphoryl groups effectively inhibited calcium phosphate formation regardless of their molecular weight, the compounds having carboxyl groups showed a different way of effect. It has previously been reported that poly-L-glutamate and alginate[17] were effective inhibitors, and that they needed relatively high molecular weight to show their activities. A high molecular weight is needed in materials having carboxyl groups to exhibit an inhibitory

effect on the formation of calcium phosphate, while a high molecular weight is not in materials having phosphoryl groups.

Takeda *et al.*[36] have obtained 3- and 6-phosphoryl maltodextrins from potato starch, namely 6^3-phosphoryl maltotriose and 3^2-phosphoryl maltotetraose by the action of porcine pancreatic α-amylase (PPA), and 6^2-phosphoryl maltotriose and 3^2-phosphoryl maltotetraose by the action of BLA. In this study, the fraction PO-1 was completely separated into 5 POs on HPAEC as shown in Figure 1.1c, the fraction PO-1 being evaluated to be composed of maltotriose, maltotetraose and maltopentaose to which one phosphoryl group was attached. Although a more precise chemical characterization of these oligosaccharides should be done, the components of the fraction PO-1 may have a similar structure to that reported by Takeda *et al.*, and the PO-2 fraction, which contain at least two phosphoryl groups attached to the molecule, might have been newly found in potato starch. To confirm that PO-2 was not an artifact formed by transglycosylation of the enzymes used, potato starch was hydrolyzed under various conditions with less enzyme activity and less concentration of POs than that described in materials and methods section. The ratio of PO-2 to PO-1 was always constant, indicating that fraction PO-2 was not produced from PO-1 by the transglycosylation reaction of amylase.

Although there are some inhibitors of the formation of calcium phosphate, as the author have already mentioned in the introduction, there are few materials actually used in food apart from CPP. CPP is good for rendering the calcium ion soluble in several kinds of food, but its use is restricted because of bitterness. It is worth mentioning that POs have little or no bitter taste by a sensory test. The threshold value

for the bitterness of CPP II was 5 x 10^{-2}%, while no bitterness of POs was detectable in a 1% solution. This fact would enable POs to be used in many kinds of food without any problem of bitterness.

1.5 Summary

The inhibitory effect of POs, which were prepared from potato starch, on the formation of calcium phosphate were investigated. POs were fractionated by ion-exchange chromatography into two fractions, PO-1 and PO-2. Fraction PO-1 was composed of maltotriose, maltotetraose, and maltopentaose to which one phosphoryl group was attached. Fraction PO-2 was predominantly composed of maltopentaose and maltohexaose to which at least two phosphoryl groups were attached. The average DP of dephosphorylated PO-1 and PO-2 was evaluated to be 4.02 and 5.82, respectively. Fraction PO-2 was the main part having an inhibitory effect on calcium phosphate formation. The possibility of the effect of POs *in vivo* was also observed in rat. In addition, among the POs, Glc-1,6-di-P and Fru-1,6-di-P were more effective inhibitors of the formation of calcium phosphate than Glc-6-P and Fru-6-P. These results suggest that the strength of the inhibitory effect might depend on the number of phosphoryl groups attached to each sugar molecule.

Chapter 2: The Structures of POs

2.1 Introduction and aim

In chapter 1, the author has described a preliminary investigation of the structures of POs. Fraction PO-1 was a mixture of phosphoryl maltotriose, maltotetraose, and maltopentaose to which one phosphoryl group was combined. Fraction PO-2 was predominantly composed of phosphoryl maltopentaose and maltohexaose to which at least two phosphoryl groups were combined, and these were newly discovered in a potato starch hydrolysate. The average DP of dephosphorylated PO-1 and PO-2 were 4.02 and 5.82, respectively, and these were evaluated to be phosphoryl maltooligosaccharides. In this chapter, the structure of PO-1 components which was the main part of POs is described. The fraction PO-1 included many components, so that investigating the structure was essentially impossible without using the enzymatic method. Therefore, the fraction PO-1 was treated with BSA and GA, and two components which could be easily purified and analyzed were obtained. A ^{13}C-NMR analysis, in addition to the chemical analyses, was helpful in defining the structure of oligosaccharide attached phosphoryl group at C-3 of glucosyl residue. In addition to this research, the precise structure of PO-2 components was estimated. Although the structure of PO-2 components would be a more complicated structure than that in PO-1 fraction, the analytical procedure described here was helpful for examining the structure of PO-2 components.

2.2 Materials and methods

2.2.1 Enzymes

BSA from *Bacillus subtilis* was from Nagase Biochemicals Co. (Fukuchiyama, Japan), and Glc-6-P dehydrogenase was 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 enzymes were used as described in section 1.2.1.

2.2.2 Other materials

Daisopak SP-120-5-ODS-BP was from Daiso Co. (Osaka, Japan). CarboPac PA-100 (4ϕ x 250 mm) and its preparative column (22ϕ x 250 mm) were purchased from Dionex Corp. (Sunnyvale, CA, U.S.A.). All other chemicals used were described in section 1.2.2.

2.2.3 Treatment of the PO-1 with GA and BSA, and fractionation of the products

The preparation of POs from potato starch hydrolysate and their fractionation into PO-1 and PO-2 have been described in section 1.2.3.

The PO-1 fraction (125 μg) was dissolved in 5 ml of a 50 mM acetate buffer (pH 5.5), and 2 units of GA were added to the solution. After the reaction mixture had been incubated at 50°C for 15 h, 2 units of BSA were added to the solution, and the reaction mixture was incubated again under the same conditions. The solution was heated in a boiling-water bath for 10 min. After the coagulated protein had been centrifuged off, a 1 M acetate buffer (pH 4.5) was added to the

supernatant to adjust the final concentration to 10 mM. The final volume was 50 ml. The solution was then loaded on a Chitopearl BCW-2501 column connected to an FPLC system (Pharmacia Fine Chemicals Co.). The neutral saccharides were washed out with a 10 mM acetate buffer (pH 4.5), and the phosphoryl saccharides were eluted with a linear gradient of NaCl (0-120 mM). To remove NaCl, the eluate was concentrated and then applied on a Sephadex G-10 column that had been equilibrated with deionized water. After the eluate containing phosphoryl saccharides had been collected and concentrated, the solution was further subjected to HPLC in a Daisopak SP-120-5-ODS-BP column (20ϕ x 250 mm) that had been equilibrated with a 10 mM K_2HPO_4-H_3PO_4 buffer (pH 3.5). The eluted phosphoryl saccharides (S1 and S3) were collected, and after the salts had been removed by gel filtration through a Sephadex G-10 column (20ϕ x 500 mm), they were freeze-dried. The purified phosphoryl saccharides (S1 and S3) were analyzed on HPAEC with a Dionex DX-300 gradient chromatography system. The chromatographic conditions were described in section 1.2.3. The purification of each peak in the PO-1 fraction after the GA treatment and after the BSA treatment, respectively, was also carried out by using a preparative column with this system. The chromatographic conditions were the same as those shown in section 1.2.4, except for the flow rate of 8 ml/min. In addition, one-third of the eluate was directed to a PAD for detection, while the remainder was served for collecting the peaks without being affected by PAD detection.

2.2.4 Analytical methods

The total amount of carbohydrates could be measured by the phenol-sulfuric acid method.[38] The concentration of carbohydrate in POs was determined after dephosphorylation with alkaline phosphatase, because detection by the phenol-sulfuric acid method was affected by the number and the position of the phosphoryl groups linked to glucosyl residues. Each PO (50 μl, 1%, w/v) in a 20 mM carbonate buffer (pH 9.4) containing 3 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ was incubated at 37°C for 30 h with 4 units of alkaline phosphatase. The reaction was terminated by passing the reaction mixture through a ultrafiltration membrane with a nominal molecular weight limit of 30,000. The average DP values for the dephosphorylated saccharides were determined by comparing their retention times with those of the authentic oligosaccharides by an HPAEC analysis, and the concentration of the dephosphorylated saccharides was determined from the peak area by using the response factors described in section 1.2.4. The structure was then analyzed.

2.2.4.1 Analysis of dephosphorylated oligosaccharides by TLC

For analyzing the dephosphorylated saccharides, TLC was carried out by the ascending method (three-times development) with a silica gel plate (Merck, Darmstadt, Germany) and a solvent system of acetonitrile-water (8:2, v/v). Spots were visualized by spraying H_2SO_4-methanol (1:1, v/v) and then by heating at 130°C.

2.2.4.2 Determination of the reducing-terminal residue (borohydride method)

The sample (2 mM, 60 µl) was reduced by adding 5 µl of 3% (w/v) $NaBH_4$ 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. The reducing-terminal residue was measured as sorbitol by the method of Manners *et al.*,[50] and glucose was measured by the method of Miwa *et al.*[51] DP values were calculated as [glucose (mol) + sorbitol (mol)] / [sorbitol (mol)].

2.2.4.3 Determination of the non-reducing-terminal residue (rapid Smith degradation)

The non-reducing-terminal residue was determined as glycerol after a rapid Smith degradation,[52] using a sample with and without a dephosphorylation treatment.

2.2.4.4 Determination of covalently bound phosphoryl group and the phosphoryl group linked at C-6 of the glucosyl residue

The covalently bound phosphoryl group of each PO was measured as inorganic phosphate[39] after dephosphorylating with the alkaline phosphatase treatment already mentioned. The phosphoryl group at C-6 of the glucosyl residue was measured by Glc-6-P dehydrogenase as Glc-6-P after an acid hydrolysis.[53]

2.2.4.5 Spectrometric analyses

^{13}C-NMR spectra were recorded with a JEOL JNM-GX 270 system (JEOL) in D_2O, using 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt as an internal standard.

2.3 Results

2.3.1 Treatment of the PO-1 with GA and BSA, and fractionation of the products

The PO-1 fraction, which had been prepared from the potato starch hydrolysate by treating with BLA, GA and pullulanase, was analyzed on HPAEC (Figure 2.1b). The chromatogram of authentic maltooligosaccharides and phosphoryl monosaccharides is shown in Figure 2.1a. It was found that this system could be useful for the analysis of maltooligosaccharides as well as phosphoryl saccharides. For example, Glc-1-P and Glc-6-P were eluted at different retention times on HPAEC because of the different positions of the phosphoryl group linked to glucose. The PO-1 fraction was subjected to a further treatment with GA. The products were fractionated by HPAEC as shown in Figure 2.1c, and each peak was collected in a preparative column as described in section 2.2.3. BSA was added to the solution, and the products were fractionated as shown in Figure 2.1d, with each peak also being collected. The peaks collected are named as J-N shown in Figure 2.1b, J'-M' in Figure 2.1c, and S1-3 in Figure 2.1d, respectively. The solution in Figure 2.1d, which had been treated with GA and BSA, was applied on ion-exchange chromatography with Chitopearl BCW-2501 that had been equilibrated with a 10 mM acetate

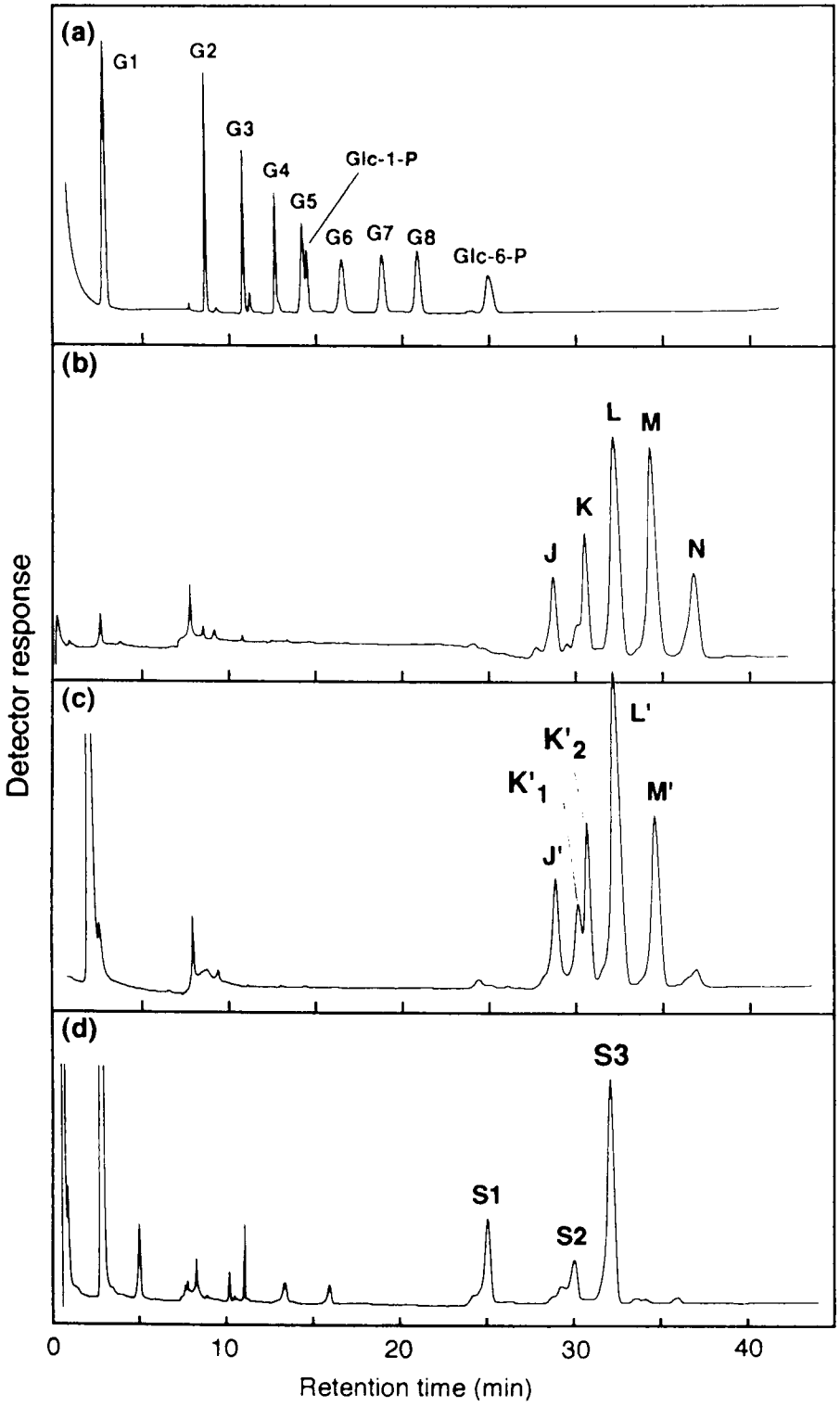

Figure 2.1 Chromatograms of the Maltooligosaccharides, Phosphoryl Monosaccharides, and POs

(a) Authentic maltooligosaccharides; (b) the PO-1 fraction prepared from potato starch; (c) the PO-1 fraction treated with GA; and (d) the PO-1 fraction treated with BSA after GA treatment. The amount of each sample injected was (a) 0.01%, 25 µl; (b-d) 0.1%, 25 µl. *Abbreviations*: G1-G8, glucose-maltooctaose; the others are as indicated in the text.

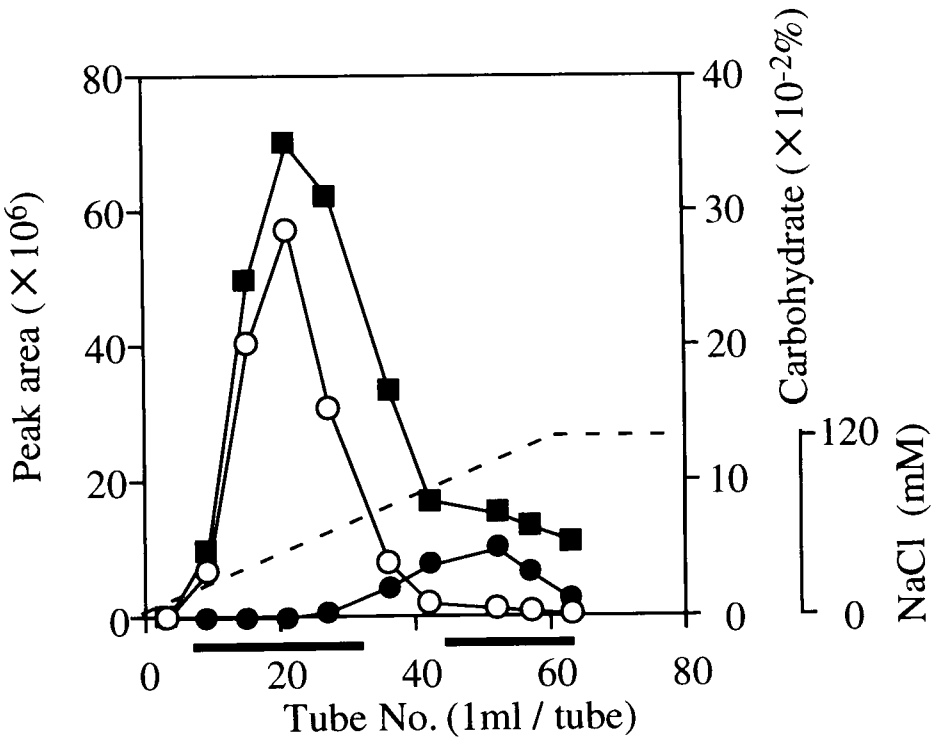

Figure 2.2 Ion-exchange Chromatogram of the PO-1 Fraction Treated by BSA after GA Treatment

The PO-1 fraction treated by BSA after the GA treatment (shown as Figure 2.1d) was chromatographed in a Chitopearl BCW-2501 column (1.5 ϕ x 4 cm) that had been equilibrated with a 10 mM acetate buffer (pH 4.5) at a flow rate of 1 ml/min. The eluted solution was collected in samples of 1ml per tube. After the neutral saccharides had been sufficiently washed out, POs were eluted with a linear gradient of NaCl (0-120 mM) shown as the dotted line. The concentration in each tube was measured as the total amount of carbohydrates in the solution by the phenol-sulfuric acid method (■), and the peak area was obtained from the PAD response by HPAEC (S1, ●; S3, ○).

buffer (pH 4.5). At this stage, S1 and S3 eluted with a linear gradient of NaCl were determined from the peak area monitored by PAD. The peak areas of some fractions are shown in Figure 2.2. These peaks (S1, tube Nos. 3-33; S3, tube Nos. 43-64) were collected and treated in a Daisopak SP-120-5-ODS-BP column (20ϕ x 250 mm) that had been equilibrated with a 10 mM K_2HPO_4-H_3PO_4 buffer (pH 3.5) for further purification. Each sample of S1 and S3 was purified to homogeneity by HPAEC, before being desalted and freeze-dried. S1 (61 μg) and S3 (26 μg) were each obtained as a white powder, although the trace peak of S2 could not be purified by this procedure.

2.3.2 Structural analyses

The results of chemical and spectrometric analyses of S1 and S3 are given as follows.

2.3.2.1 Analysis of dephosphorylated oligosaccharides by TLC

S1 and S3 were dephosphorylated with alkaline phosphatase, and the dephosphorylated oligosaccharides were then analyzed by TLC. As shown in Figure 2.3, both dephosphorylated S1 and S3 were maltotriose. HPAEC showed the same results. However, the retention times of S1 and S3 on HPAEC were obviously different (Figure 2.1d), indicating that the phosphoryl group linked position was different in S1 and S3.

Figure 2.3 Thin-layer Chromatogram of Dephosphorylated S1 and S3
Ten µl of a 0.1% sample was spotted on a TLC plate. M, authentic maltooligosaccharides G1-G6; 1, dephosphorylated S1; 2, dephosphorylated S3.

Table 2.1 Structural Analyses of S1 and S3

	S1	deP-S1[a]	S3	deP-S3[a]
Non-reducing-terminal residue	0.90	1.11	0.20	0.98
Composition of hydrolyzate				
Sorbitol	1.05	1.14	1.17	1.14
Glucose	1.72	2.00	0.93	2.05
Glc-6-P	0.03	-	0.94	-
Organic phosphate	0.91	-	0.97	-

[a] The deP-S1 or deP-S3 sample was dephosphorylated by the alkaline phosphatase treatment. Each value is expressed as mol/ mol.

2.3.2.2 Determination of the reducing-terminal residue (borohydride method)

S1 and S3 reduced with borohydride were hydrolyzed by an acid, and the resulting products were analyzed. The reducing-terminal residue was determined as sorbitol, and the concentrations of S1 and S3 were determined after dephosphorylating with alkaline phosphatase by the phenol-sulfuric acid method. One mole of sorbitol was detected in one mole of S1, dephosphorylated S1, S3, and dephosphorylated S3, indicating that both S1 and S3 had a reducing end and no phosphate linked at the reducing-terminal residue (Table 2.1). In addition, two moles of glucose were detected in one mole of S1 and dephosphorylated S1. One mole of glucose was found in one mole of S3, and two moles of glucose was in one mole of dephosphorylated S3. These findings indicate that the covalent bond between the phosphoryl groups and glucose in the S1 was not as strong as that in S3; in other words, this bond was unstable under acidic conditions and tended to release inorganic phosphate. The amounts of glucose and sorbitol in the hydrolysate of the reduced and dephosphorylated samples of S1 and S3 also indicated that their DP values were three, giving the same result as that of TLC (Figure 2.3).

2.3.2.3 Determination of the non-reducing-terminal residue (rapid Smith degradation)

To detect the non-reducing-terminal residue as glycerol, S1 and S3 were subjected to a rapid Smith degradation. Both S1 and dephosphorylated S1 produced one mole of glycerol per mole. On the other hand, glycerol could not be detected in S3 although

dephosphorylated S3 produced one mole of glycerol per mole. This indicates that S3 had a phosphoryl group linked at the non-reducing-terminal residue.

2.3.2.4 Determination of covalently bound phosphoryl group and the phosphoryl group linked at C-6 of the glucosyl residue

The amount of covalently bound phosphoryl groups per molecule was measured as inorganic phosphate after completely dephosphorylating with alkaline phosphatase. The result showed that S1 and S3 each had one mole of a covalently bound phosphoryl group per mole (Table 2.1).

The Glc-6-P content after acid hydrolysis indicated that S1 had no phosphoryl group linked at C-6 of the glucosyl residue and that S3 had one phosphoryl group linked at C-6 of the glucosyl residue.

Hizukuri *et al.* have reported that approximately 60% to 70% of the phosphoryl groups were linked to C-6 of the glucosyl residue, almost all of the remainder being linked to C-3, and a very small portion being possibly linked to C-2 of the glucosyl residue.[53] Therefore, S1 might have one phosphoryl group linked to C-2 or C-3 of the glucosyl residue, since C-1 and C-4 must be responsible for the α-1,4-glucosidic linkage. As already described, S1 was maltotriose containing one mole of non-phosphorylated reducing- and non-reducing-terminal ends, and one mole of the phosphoryl group which was not linked at C-6 of the glucosyl residue. Therefore, S1 appeared to be 3^2-phosphoryl maltotriose or 2^2-phosphoryl maltotriose. On the other hand, S3 was maltotriose containing the 6-p-glucosyl residue in

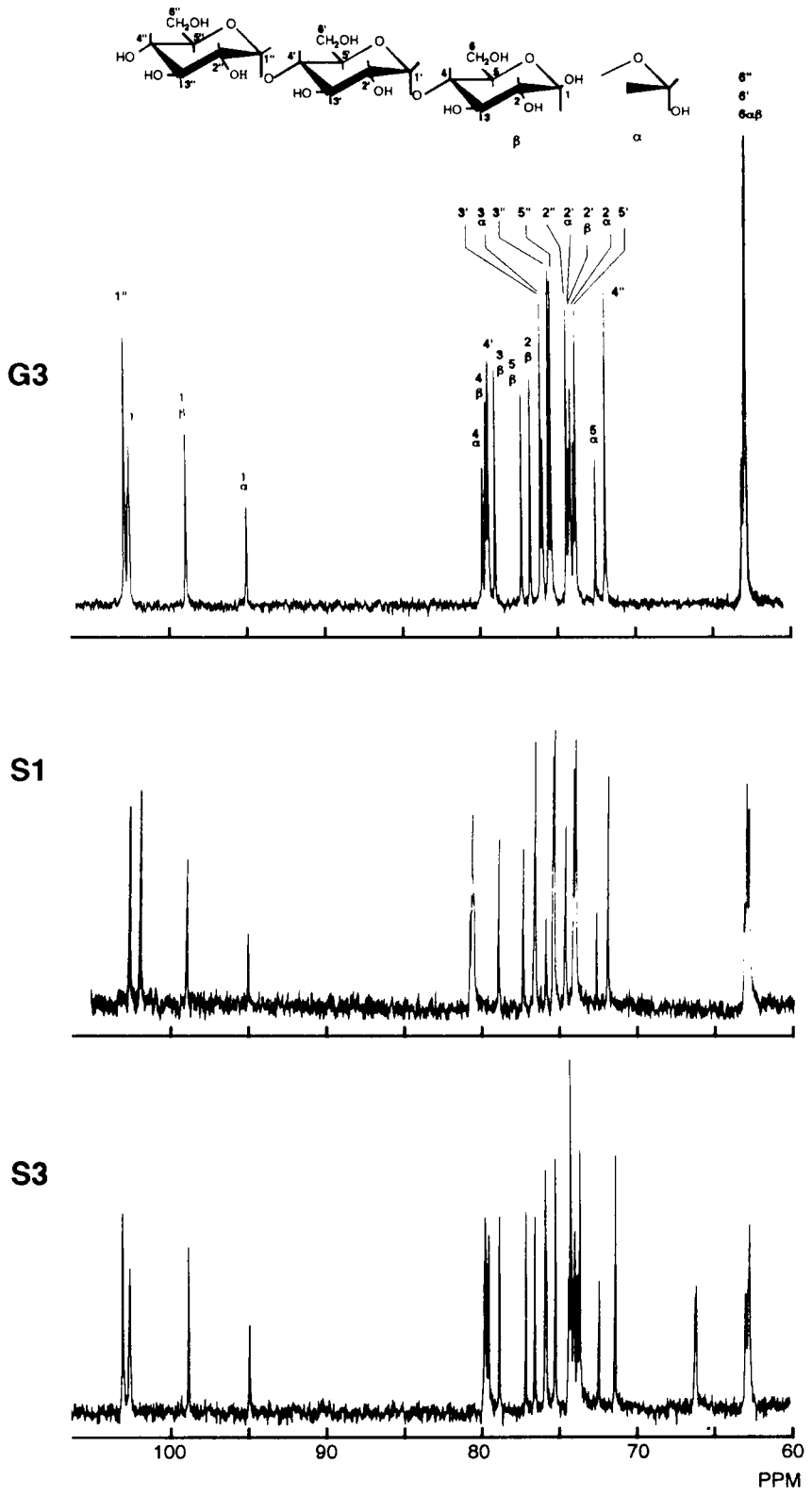

Figure 2.4 ^{13}C-NMR Spectra of Maltotriose, S1, and S3

The structure of Maltotriose (G3) is illustrated, and each carbon atom is numbered in the figure.

the non-reducing-terminal residue. Therefore, S3 was 6^3-phosphoryl maltotriose.

2.3.2.5 Spectrometric analyses

In order to ascertain the structures of S1 and S3, a spectrometric analysis by ^{13}C-NMR was carried out as shown in Figure 2.4. The chemical shift of authentic maltotriose almost agreed with that from the study by Morris *et al.*[54]

If the phosphoryl group in S1 was linked to C-3 of the glucosyl residue, the carbon signals of C-2, C-3 and C-4 of the glucosyl residue would have exhibited a split into peaks and/or a shift. If the phosphoryl group was linked to C-2 of the glucosyl residue, the carbon signals of C-1, C-2 and C-3 of the glucosyl residue would also have exhibited a split into peaks and/or a shift. The carbon signal of C-1 of all the glucosyl residues exhibited the same signal as that of authentic maltotriose (Figure 2.4). On the other hand, the carbon signal of C-4 of the middle glucosyl residue of maltotriose obviously exhibited a downfield shift (S1, δ 80.6; S3 and authentic G3, δ79.7), and that of C-4 of the non-reducing-terminal glucosyl residue in S1 and S2 exhibited the same signal as that of authentic maltotriose (δ72.1). These results and other analytical data (Table 2.1) indicate that S1 was 3^2-phosphoryl maltotriose.

One of the carbon signals of C-6 in S3 exhibited a downfield shift (δ66.6) and a split as shown in Figure 2.4. This result supports the fact that the phosphoryl group was linked to C-6 of the glucosyl residue.

Thus, the structures of S1 and S3 could be characterized as 3^2-phosphoryl maltotriose and 6^3-phosphoryl maltotriose, respectively.

2.3.3 Structural analyses of PO-1 components

Since 6^3-phosphoryl maltotriose and 3^2-phosphoryl maltotriose from the PO-1 fraction could be prepared by treating with BSA and GA, the author tried to deduce the structure of the PO-1 components on the basis of the reaction specificities of BSA and GA. First, each peak (J-N in Figure 2.1b) was obtained as a single peak by HPAEC with a preparative column. Second, it was examined which peak of the PO-1 fraction produced 6^3-phosphoryl maltotriose or 3^2-phosphoryl maltotriose.

2.3.3.1 Structures of J and K

GA could not hydrolyze J, and it remained as J' in Figure 2.1c. However, BSA could hydrolyze J to produce one mole of glucose and 3^2-phosphoryl maltotriose (S1), with the release of one mole of glucose from the reducing-terminal residue (Table 2.2). Peak K produced two mole of glucose and S1 with BSA. These results indicate that J and K were 3^3-phosphoryl maltotetraose and 3^4-phosphoryl maltopentaose, respectively.

2.3.3.2 Structures of M and N

GA could hydrolyze M to produce one mole of glucose and L'. L' could not be hydrolyzed by BSA and had the same retention time as that of S3, indicating that L' was 6^3-phosphoryl maltotriose (S3). Peak N produced S3 by releasing one mole of glucose from the non-reducing end with GA and one mole of glucose from the reducing-terminal

Table 2.2 Products[a] from the Digestion of Components in Fraction PO-1

Native component (min)	Dephosphorylated[b] (min)	Released Glc (mol/ mol)		Product (min)
		GA treatment	BSA treatment[c]	
J (28.4)	G4 (13.6)	-	1.12	S1 (25.0)
K (30.3)	G5 (14.5)	-	2.21	S1 (25.0)
L (32.5)	G3 (11.0)	0.385	-	S3 (32.5), S2 (30.0)
M (34.0)	G4 (13.6)	1.18	-	S3 (32.5)
N (36.4)	G5 (14.5)	0.852	1.14	S3 (32.5)

[a] Products are shown by their retention times with HPAEC.

[b] Native components were dephosphorylated with alkaline phosphatase as described in section 2.2.4, and maltooligosaccharides were detected by HPAEC as shown as Figure 2.1.

[c] BSA was reacted after the GA treatment as described in section 2.2.3.
Abbreviations are as given in Figure 2.1.

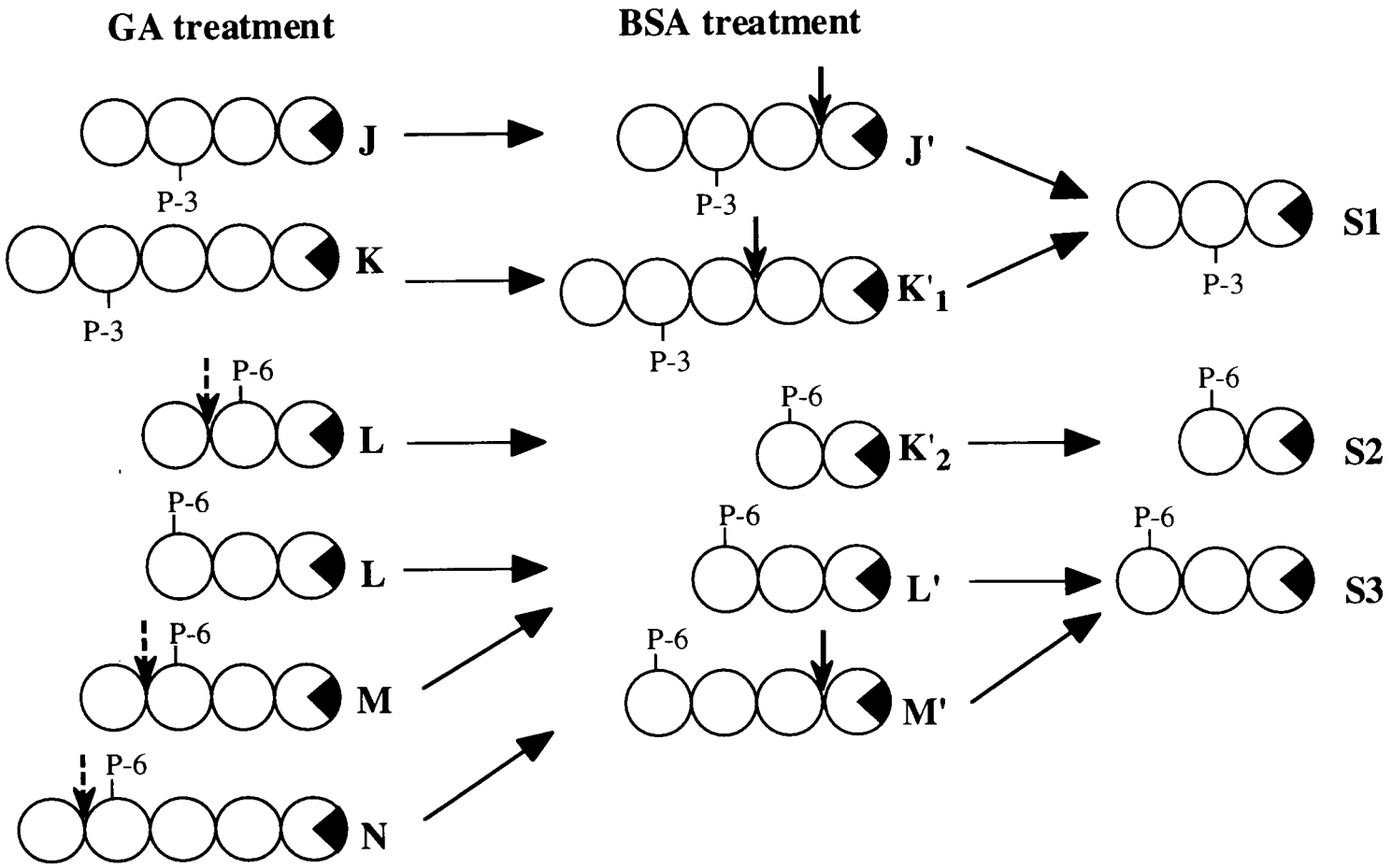

Figure 2.5 Actions of GA and BSA on the PO-1 Fraction

Symbols: P-3 and P-6, phosphoryl groups linked at C-3 and C-6 of the glucosyl residues, respectively; ○ , glucosyl residue; ◔ , reducing glucosyl residue; --➤, action of GA; ➤, action of BSA.

residue with BSA (Table 2.2). These results indicate that M and N were 6^3-phosphoryl maltotetraose and 6^4-phosphoryl maltopentaose, respectively, and that M' produced by GA was 6^4-phosphoryl maltotetraose. The peaks of M and M' had the same retention time by the HPAEC analysis, although their structures differed in the phosphorylation site.

2.3.3.3 Structure of L

Treatment with GA produced K'_2 from L, with the release of 0.385 mole of glucose from the non-reducing end (Table 2.2), but most of L remained as L' in Figure 2.1c. BSA could not hydrolyze L' and K'_2. Furthermore, maltotriose and maltose were obtained by the dephosphorylation treatment from L' and K'_2 (data not shown). These results imply that peak L contained about 60% of 6^3-phosphoryl maltotriose (S3) and 40% of 6^2-phosphoryl maltotriose, which could produce 6^2-phosphoryl maltose (K'_2) with GA, judging from the amount of glucose released. Therefore, S2 seemed to be 6^2-phosphoryl maltose.

Thus, the analyses of the products by GA and BSA hydrolysis enabled us to define the structures of the PO-1 components. These hydrolytic reactions are illustrated in Figure 2.5. It is concluded that the PO-1 fraction was made up of the oligosaccharides attached with the phosphoryl group at C-3 (3^3-phosphoryl maltotetraose (J) and 3^4-phosphoryl maltopentaose (K)) and the oligosaccharides attached with the phosphoryl group at C-6 (6^3-phosphoryl maltotriose (L), 6^2-phosphoryl maltotriose (L), 6^3-phosphoryl maltotetraose (M), and 6^4-phosphoryl maltopentaose (N)).

2.4 Discussion

The structures of S1 and S3 derived from potato starch were defined as 3^2-phosphoryl maltotriose and 6^3-phosphoryl maltotriose, respectively, by chemical and spectrometric analyses. These products and the substrate specificities of GA and BSA made the structures of all the PO-1 components clear.

The HPAEC system with PAD seems to be useful for analyzing phosphoryl substrates as the PO-1 fraction was separated into 5 peaks on HPAEC as shown in Figure 2.1b. It was shown that the HPAEC system could separate POs with different positions for the phosphoryl group linked to the glucosyl residue such as 6^3-phosphoryl maltotetraose and 3^3-phosphoryl maltotetraose, but could hardly separate POs with different positions for the phosphoryl glucosyl residue in the molecule such as 6^3-phosphoryl maltotriose and 6^2-phosphoryl maltotriose, or 6^3-phosphoryl maltotetraose and 6^4-phosphoryl maltotetraose.

The author can summarize the reaction specificities of GA and BSA for POs: GA could hydrolyze 6^2-phosphoryl maltotriose (L), 6^3-phosphoryl maltotetraose (M) and 6^4-phosphoryl maltopentaose (N), and release one mole of glucose from the non-reducing end. BSA could hydrolyze 3^3-phosphoryl maltotetraose (J), 3^4-phosphoryl maltopentaose (K) and 6^4-phosphoryl maltopentaose (N), and release one mole of glucose or maltose from the reducing end (Figure 2.5).

GA is an exo-type amylase that consecutively removes glucose from the non-reducing end of α-1,4 glucan. This study has shown that GA removed the glucose molecules from the non-reducing end up to

the 6-phosphoryl glucosyl residue and up to one glucosyl residue from the 3-phosphoryl glucosyl residue (Figure 2.5) as reported by Takeda *et al.*[36] This reaction of GA might be very slow, especially in the case of the phosphoryl group at C-6 of oligosaccharides, since insufficient hydrolysis of the PO-1 fraction by the GA treatment was observed. This phenomenon agrees with that of GA from *Aspergillus niger* as reported by Takeda *et al.*[36]

On the other hand, BSA hydrolyzes amylose to produce glucose, maltose and maltotriose,[55,56] and then the enzyme splits maltotriose to glucose and maltose after sufficient hydrolysis. Acting on the PO-1 fraction after treatment by GA, BSA removed glucose from the reducing end up to two glucosyl residues from the 6-phosphoryl glucosyl residue, and removed glucose or maltose from the reducing end up to one glucosyl residue from the 3-phosphoryl glucosyl residue (Figure 2.5). These different hydrolytic properties are of interest for studying the action mechanisms of BSA and GA.

Transglycosylation is also known as one action of BSA. A small amount of transferred products was detected at a early retention time in Figure 2.1d. These products were neutral saccharides and not POs, because they were not adsorbed on the ion exchange resin.

The enzymatic analysis described here was helpful for examining the structure of PO-2 components. The structure of PO-2 components was also estimated as follow. The components in PO-2 fraction were scarcely hydrolyzed by the action of BSA. It indicated that the glucosyl residues attached to the phosphoryl groups of PO-2 components would be neighboringly located. In addition, all of the phosphoryl linkages

attached to C-3 of glucosyl residues of POs were hydrolyzed by 0.35 N acetic acid treatment, nevertheless those of C-6 were not (Figure 2.6). A novel acid phosphatase from *Aspergillus niger* KU-8 also have been succeed to obtain. The enzyme could hydrolyze the phosphoryl linkages attached to C-6 of glucosyl residues of POs, nevertheless those of C-3 could not (Figure 2.6).[57] 6^3-Phosphoryl maltotriose (S3) or 3^2-phosphoryl maltotriose (S1) could be obviously detected by the acid treatment or the acid phosphatase treatment, respectively, followed by the hydrolysis of BSA and GA (Figure 2.7). However, these treatment-resistant PO-2 slightly remained. It clearly indicated that two of the phosphoryl groups attached to C-6 and C-3 existed in PO-2 components. However, the phosphoryl groups attached to C-2 could not be detected in this study. From these results, the possible structures of PO-2 components were as shown in Figure 2.8.

The phosphorylation ratio of C-6 to C-3 in this analysis was determined directly as 7 to 3, while Hizukuri *et al.*[53] estimated the ratio from the contents of 6-p-glucosyl residues and total covarently phosphates because the contents of 3-p-glucosyl residues had not been able to determine directly. This analytical methods seem to be also useful to define this ratio for starches from different potato species and from potatoes grown under different conditions.

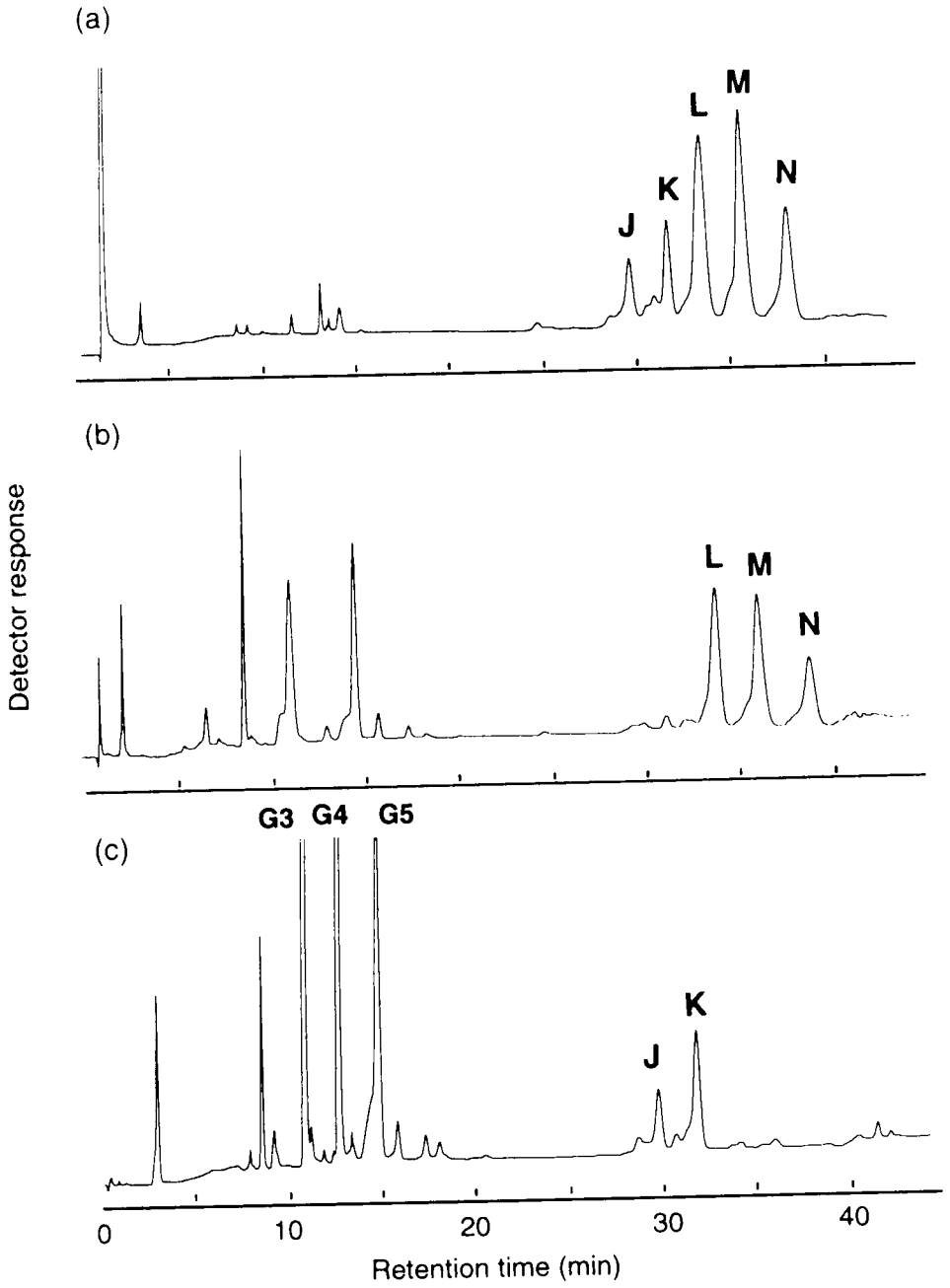

Figure 2.6 HPAEC Chromatograms of PO-1 Fraction Treated with 0.35N Acetic Acid or Acid Phosphatase

(a), PO-1 fraction prepared from potato starch; (b), treatment with 0.35N acetic acid for 8h; (c), treatment with acid phosphatase from *A. niger* KU-8. The amount of each sample injected was 0.1%, 25 µl. *Abbreviations*: J, 3^3-phosphoryl maltotetraose; K, 3^4-phosphoryl maltopentaose; L, 6^3-phosphoryl maltotriose and 6^2-phosphoryl maltotriose; M, 6^3-phosphoryl maltotetraose; N, 6^4-phosphoryl maltopentaose; G3-G5, maltotriose-maltopentaose.

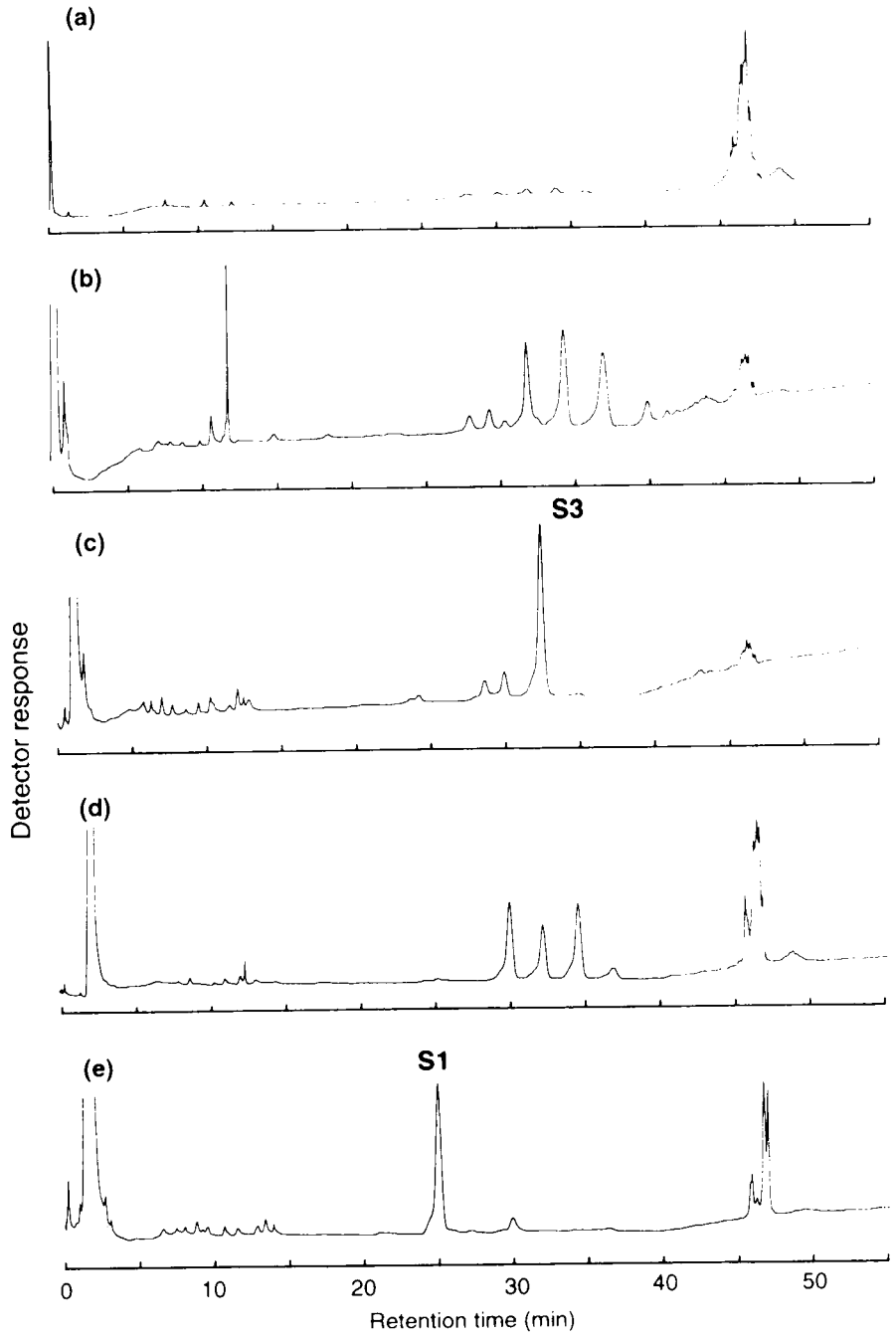

Figure 2.7 HPAEC Chromatograms of PO-2 Fraction Treated with 0.35N Acetic Acid or Acid Phosphatase

(a), treatment with BSA and GA; (b), treatment with GA after 0.35N acetic acid for 8h; (c), treatment with BSA and GA after 0.35N acetic acid for 8h; (d), treatment with GA arter the action of acid phosphatase from *A. niger* KU-8; (e), treatment of BSA and GA arter the action of acid phosphatase from *A. niger* KU-8. The amount of each sample injected was 0.1%, 25 μl. *Abbreviations*: S1, 3^2-phosphoryl maltotriose; S3, 6^3-phosphoryl maltotriose.

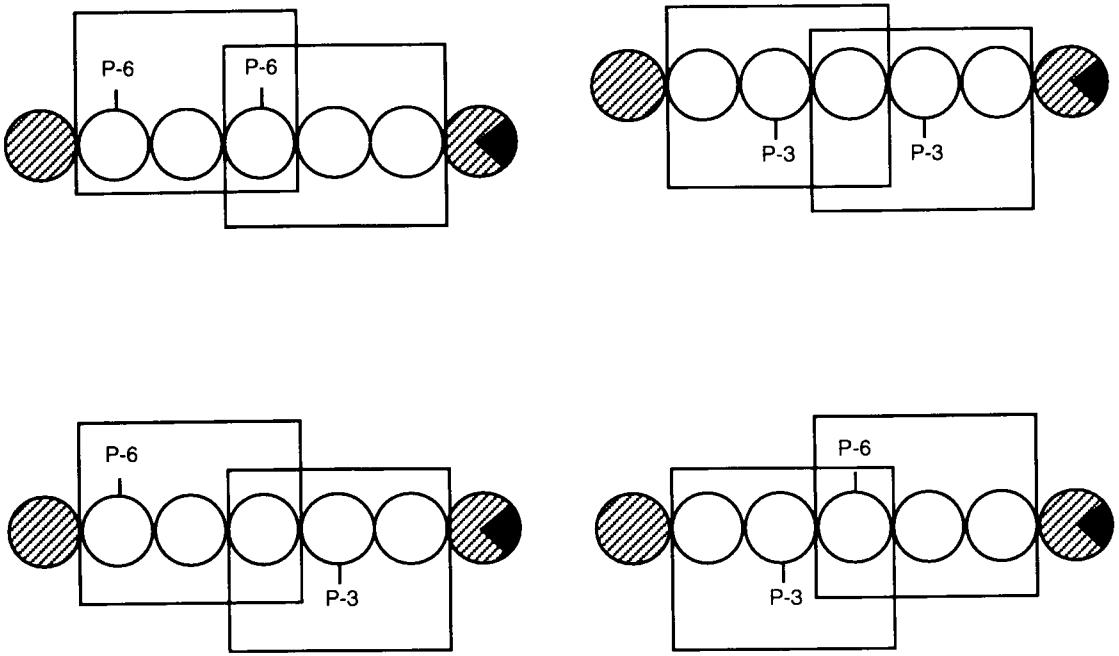

Figure 2.8 Possible Structure of PO-2 Components

Symbols: P-3 and P-6, phosphoryl groups linked at C-3 and C-6 of glucosyl residues; ○ , glucosyl residue; ◍ , glucosyl residue which possibly exists; ◑ , reducing end. The square indicates the units of indigestible fragments by the action of BSA and GA.

2.5 Summary

In this chapter, the structures of the PO-1 components, which was the main fraction of POs, were investigated. By treating with BSA after GA, 3^2-phosphoryl maltotriose was produced from the oligosaccharides attached the phosphoryl group at C-3 in PO-1 components, and 6^3-phosphoryl maltotriose was produced from the oligosaccharides attached the phosphoryl group at C-6 in PO-1 components. These products were characterized spectrometrically as well as chemically, including measurement of the amounts of the non-reducing-terminal residue, reducing-terminal residue, and covalently bound phosphoryl group. A small amount of 6^2-phosphoryl maltose was also detected after treating with GA alone, indicating that 6^2-phosphoryl maltotriose existed in the PO-1 fraction. According to the reaction specificities of GA and BSA, it was concluded that the PO-1 fraction was made up of the oligosaccharides attached with the phosphoryl group at C-3 (3^3-phosphoryl maltotetraose and 3^4-phosphoryl maltopentaose) and the oligosaccharides attached with the phosphoryl group at C-6 (6^3-phosphoryl maltotriose, 6^2-phosphoryl maltotriose, 6^3-phosphoryl maltotetraose, and 6^4-phosphoryl maltopentaose).

Chapter 3: A Way of Enhancing the Inhibitory Effect of POs on the Formation of Calcium Phosphate Precipitate (I); Utilization of the Coupling Reaction of Cyclomaltodextrin glucanotransferase

3.1 Introduction and aim

Although both PO-1 and PO-2 fractions can form complexes with calcium, fraction PO-2 made more stable and soluble complexes with the calcium ion as shown in chapter 1. This capacity of PO-2 fraction was equal to that of CPP. On the other hand, the capacity of PO-1 fraction was inferior to PO-2 fraction, while PO-1 fraction was the main part of POs. From the results of the inhibitory effect on the formation of calcium phosphate precipitate and the structural analyses, it concluded that these abilities were dependent upon the number of the covalently bound phosphoryl groups in the molecule. In this chapter, the author endeavored to increase the components that were linked at least two phosphoryl groups in the molecule of POs. The author describes a way of enhancing the inhibitory effect of POs on the formation of calcium phosphate precipitate by using the coupling reaction of CGTase [EC 2.4.1.19] and analyzed the structures of the effective fractions. The relationship between the calcium solubilizing ability and the number of covalently bound phosphoryl group per molecule was also described.

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 the intermolecular transglycosylation (coupling reaction). The enzyme also

catalyzes the disproportionation reaction between two molecules of oligosaccharides and hydrolysis reaction of α-1, 4-glucan or CD.[58] Some ways of applications of this enzyme to food industry have been reported.[59,60]

3.2 Materials and methods

3.2.1 Enzymes and materials

CGTase from *Bacillus macerans* was purchased from Amano Pharmaceutical Co. (Nagoya, Japan). Other enzymes and materials were used as described in section 1.2 and 2.2.

3.2.2 Preparation of CGT-POs

The preparation of POs from potato starch hydrolysate and their fractionation into PO-1 and PO-2 have been described in section 1.2.3. The POs (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 48h. 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 the ion-exchange chromatographies by the method mentioned in section 1.3.3. The products were analyzed on HPAEC system as shown in section 1.2.4.

3.2.3 Analytical methods

The total amount of carbohydrates was measured by the phenol-sulfuric acid method.[38] The structure of CGT-POs was then analyzed as follows:

3.2.3.1 Chemical analyses

The methods of chemical analyses were followed as shown in section 2.2.4. Determination of contents of the reducing-terminal residue was analyzed by borohydride methods. After an acid hydrolysis, the reducing-terminal residue was measured as sorbitol. Glucose was measured by the method using glucose oxidase. The phosphoryl group at C-6 of the glucosyl residue was also measured as Glc-6-P with Glc-6-P dehydrogenase after an acid hydrolysis. DP values were also calculated as [glucose (mol) + sorbitol (mol) + Glc-6-P (mol)] / [sorbitol (mol)]. The covalently bound phosphoryl group of each PO after an acid hydrolysis was measured as inorganic phosphate after dephosphorylating with the alkaline phosphatase treatment.

3.2.3.2 Enzymatic analyses

After the treatment of POs with CGTase, the fractionated products (1 mg : 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 for 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 on HPAEC system using PAD. In this procedure, 3^2-phosphoryl maltotriose (S1) and 6^3-phosphoryl maltotriose (S3) were obtained, and their contents were calculated from PAD response, using the following response factors: S1, 0.93; S3, 1.0.

3.2.4 Detection of the transglycosylation reaction of CGTase on each component of PO-1 fraction

Each component (J-N in Figure 3.1a) of PO-1 fraction was purified by HPAEC with preparative column as shown in section 2.3.3. The action of CGTase on these substrates (1 μg) was processed with 20 units of CGTase at 50°C for 48h. The reaction was stopped by heat treatment in a boiling-water bath for 5 min. The products were analyzed by TLC. One μl of a 1% (w/v) sample was spotted on a TLC plate. TLC was carried out by the ascending method with silica gel plate (Merck, Darmstadt, Germany) and a solvent system of ethanol-water-acetic acid (35:15:1, v/v). Spots were visualized by spraying H_2SO_4-methanol (1:1, v/v) and then by heating at 130°C.

3.3 Results

3.3.1 Preparation of POs treated with CGTase

The POs prepared from the potato starch hydrolysate was analyzed on HPAEC with the results shown in Figure 3.1a. After CGTase reaction on POs, the products (CGT-POs) were also analyzed on HPAEC as shown in Figure 3.1b. The peaks such as components in PO-2 fraction increased, and the peaks of K, M, and N decreased with CGTase treatment. These facts suggested that the coupling reaction of the components in PO-1 fraction obviously occurred with CGTase, and the transglycosylation products (CGT-PO2) were obtained. The amounts of CGT-PO2 produced at various POs concentrations were analyzed as shown in Figure 3.2. The content of CGT-PO2 became maximum at 10-40% of POs in reaction mixture, and final content of

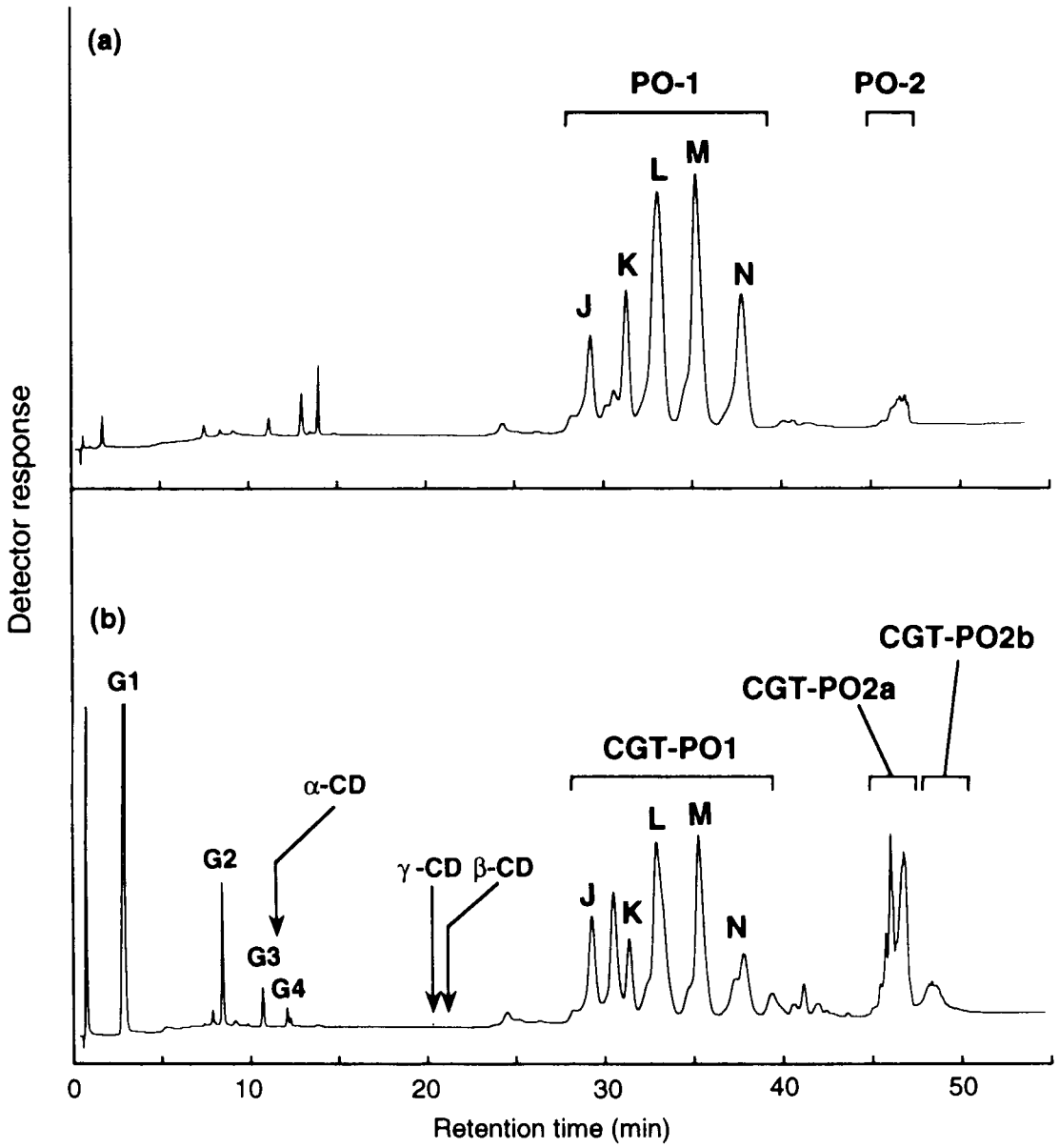

Figure 3.1 Chromatograms of the POs and the CGTase-Treated POs
(a) POs; (b) POs treated with CGTase. The amount of each sample injected was 0.15%, 25 μl. *Abbreviations*: J, 3^3-phosphoryl maltotetraose; K, 3^4-phosphoryl maltopentaose; L, 6^3-phosphoryl maltotriose and 6^2-phosphoryl maltotriose; M, 6^3-phosphoryl maltotetraose; N, 6^4-phosphoryl maltopentaose; the others are as indicated in the text.

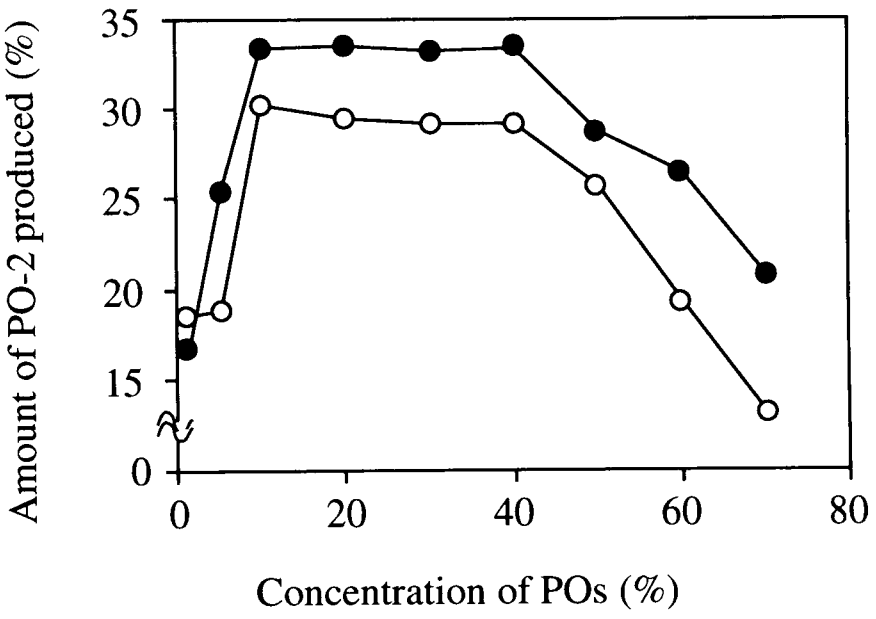

Figure 3.2 Effects of the Concentration of POs on the Transglycosylation with CGTase
The various concentrations of the POs were treated by CGTase for 24h (○) and 48h (●).

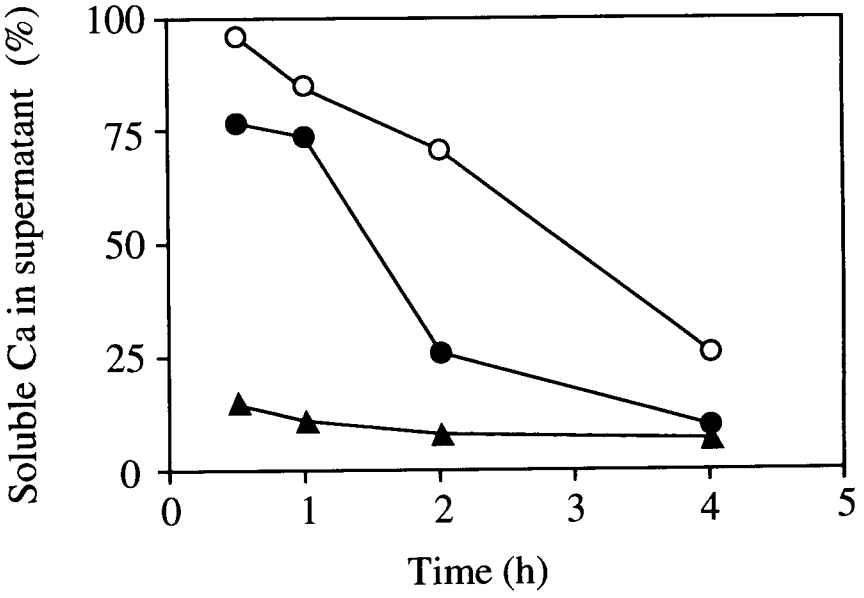

Figure 3.3 Inhibitory Effects of POs and POs Treated with CGTase on the Formation of Calcium Phosphate Precipitate
The concentrations of the POs and the POs treated with CGTase in test solution were 0.5%, and those of calcium and phosphate were 4 mM and 10 mM, respectively. Symbols: ●, POs; ○, CGTase treated POs; ▲, deionized water as control.

CGT-PO2 in CGT-POs was from 10% to about 33% on HPAEC analysis. The reaction would be hindered by the evolution of viscosity of POs when a concentration over 40% of POs 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.

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

Figure 3.3 shows the effect of CGT-POs on the formation of calcium phosphate precipitate by the method described in section 1.2.5. In comparison with the control, soluble calcium remained for the first one hour after addition of POs. After 2 hours, the amount of soluble calcium gradually decreased. On the other hand, soluble calcium remained for 2 hours after addition of CGT-POs. As a results, the inhibitory effect of POs could be improved by CGTase treatment. This phenomenon indicates that the amount of the effective fraction in the POs increased by CGTase reaction.

3.3.3 Fractionation of the CGT-POs

The POs solution (1%, w/v, 300 ml) was prepared with a 10 mM acetate buffer (pH 4.5). The solution was applied on a Chitopearl BCW-2501 column equilibrated with the same buffer. The adsorbed 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 NaCl. The eluted saccharides were collected and freeze-dried.

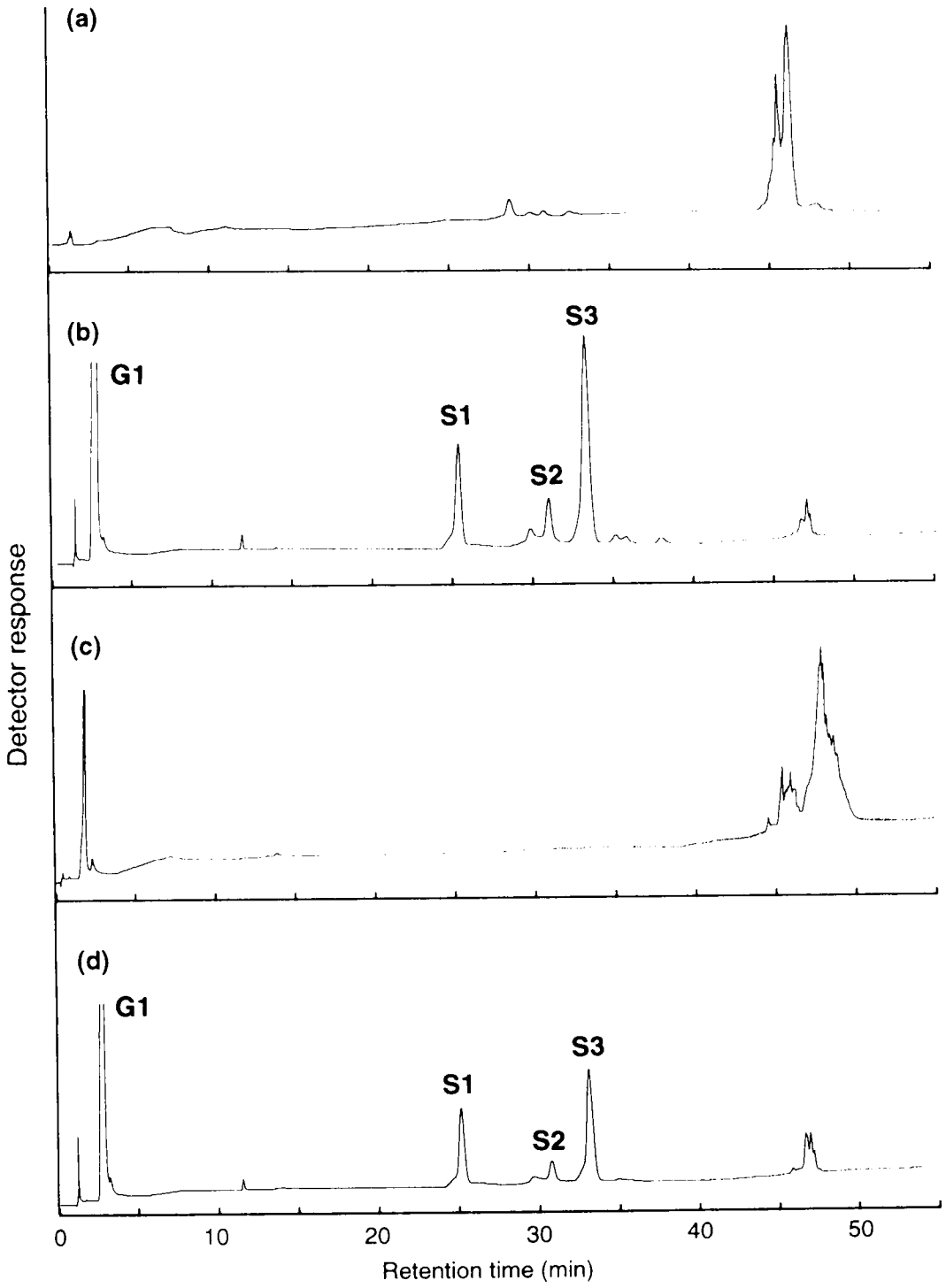

Figure 3.4 Chromatograms of the CGT-PO2a, the CGT-PO2b, and their Hydrolysates with BSA and GA

(a), CGT-PO2a fractionated by ion-exchange chromatography; (b), CGT-PO2a treated with BSA and GA; (c), CGT-PO2b fractionated by ion-exchange chromatography; (d), CGT-PO2b treated with BSA and GA. The concentration and amount of each sample injected was 0.15%, 25 μl. *Abbreviations*: S1, 3^2-phosphoryl maltotriose; S2, 6^2-phosphoryl maltose; S3, 6^3-phosphoryl maltotriose.

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 CGT-PO2a and CGT-PO2b fractions are shown in Figure 3.4a and 3.4c, respectively.

Table 3.1 Average DP and Content of Covalently Bound Phosphoryl Group in POs before and after CGTase Reaction

Sample	DP	P_o[a]
PO-1	4.02	1.18
CGT-PO1	3.12	1.07
PO-2	5.65	2.09
CGT-PO2a	7.89	2.06
CGT-PO2b	11.06	3.19

[a] The covalently bound phosphoryl group. Values are given as mol/mol.

Table 3.2 Content of Organic Phosphate Attached to C-3 and C-6 of Glucosyl Residues in POs before and after CGTase Reaction (%)

Sample	C-3[a]	C-6[a]	C-6[b]
PO-1	29.0	71.0	71.0
CGT-PO1	13.8	86.2	86.0
PO-2	-	-	44.0
CGT-PO2a	28.7	71.3	78.4
CGT-PO2b	44.4	55.6	59.8

[a] Calculated from HPAEC-PAD responce.

[b] Calculated by Glc-6-P dehydrogenase methods.

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

3.3.4.1 Chemical analyses

The results of chemical analyses of CGT-PO1, CGT-PO2a, and CGT-PO2b were shown in Table 3.1. The average DP values were calculated 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 acidic conditions, however other phosphoryl bonds were not stable and tended to release inorganic phosphate.[53] The average DP values of CGT-PO2a and CGT-PO2b were extended to 7.89 and 11.06, respectively, from 5.65 of PO-2. The average DP values of fraction CGT-PO1 decreased to 3.12 from 4.02 of fraction PO-1. It indicated that the components in PO-1 fraction was used for the coupling reaction of CGTase and produced CGT-PO2a and CGT-PO2b which had two and three phosphoryl groups in molecule, respectively.

3.3.4.2 Enzymatic analyses

Each CGT-PO fraction was individually treated with BSA and GA. The products were analyzed on HPAEC system. After action of GA, glucose was detected in each fractionated CGT-PO and PO-2 fraction. Although most of PO-2 components were indigestible and remained after BSA hydrolysis, CGT-PO2a and CGT-PO2b could be hydrolyzed (Figure 3.4b and d), and produced 3^2-phosphoryl maltotriose (S1) and 6^3-phosphoryl maltotriose (S3). It supported that CGT-PO2a and CGT-PO2b were produced from the components in PO-1 fraction by the

coupling reaction of CGTase. However, a small amount of indigestible CGT-PO2a and CGT-PO2b were remained (Figure 3.4b and d). They were most likely to be PO-2 components. The ratio of C-3 to C-6 phosphoryl substances indicated the ratio of POs transferred from PO-1 components. The contents of phosphoryl groups attached to C-6 and C-3 of glucosyl residues could be measured as the contents of 3^2-phosphoryl maltotriose (S1) and 6^3-phosphoryl maltotriose (S3) except for a small amount of indigestible components such as PO-2 fraction (Table 3.2). Furthermore, contents of 6-p-glucosyl residues were also measured by the enzymatic methods. A small difference in values was observed because the indigestible components such as the PO-2 fraction. In CGT-PO1 fraction, the content of the phosphoryl groups at C-3 of glucosyl residue decreased and that of the phosphoryl groups at C-6 increased by the action of CGTase. The ratio of C-3 to C-6 phosphoryl groups in CGT-PO2a was similar to that in PO-1 components. The ratio of C-3 phosphoryl group in CGT-PO2b was relatively higher than the other samples (Table 3.2). It indicated that both phosphoryl substances were used as a donor in CGT-PO2a and that more C-3 phosphoryl substrates of POs were used as donor for making the component of CGT-PO2b.

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

The dependence of the inhibitory effect upon the concentration of CGT-PO2a or CGT-PO2b was examined and was exhibited the sigmoidal curve as shown in Figure 3.5. In case of PO-1 fraction, the sigmoidal curve could not be showed. It was also reported that the effective substances such as alginate and CPP exhibited the sigmoidal

curve and had threshold value for the inhibitory effect depended on their concentrations.[16,17] The stable inhibitory effect on calcium phosphate formation was obtained at the 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-PO2a for the inhibitory effect was 7-fold higher than that of CGT-PO2b (Figure 3.5). The threshold value of CGT-PO2a was the same as one of PO-2 fraction (Figure 1.5). Although both of CGT-PO2a and CGT-PO2b were the effective inhibitor on the formation of calcium phosphate precipitate, the fraction CGT-PO2b was more effective inhibitor than fraction CGT-PO2a. The difference in these effects would depend upon the density of phosphoryl groups attached to each sugar molecule (Table 3.2). It was concluded that both of CGT-PO2a and CGT-PO2b enhanced the inhibitory effect of POs.

3.3.6 Detection of the transglycosylation reaction of CGTase in each component of PO-1 fraction

The transglycosylation reaction was obviously observed when 3^4-phosphoryl maltopentaose and 6^4-phosphoryl maltopentaose were used as substrates, respectively (Figure 3.6). On the other hand, the transglycosylation reaction could not be detected when the other substrates were used in the condition. These results indicated that 3^4-phosphoryl maltopentaose and 6^4-phosphoryl maltopentaose were main substrates in transglycosylation catalyzed by CGTase.

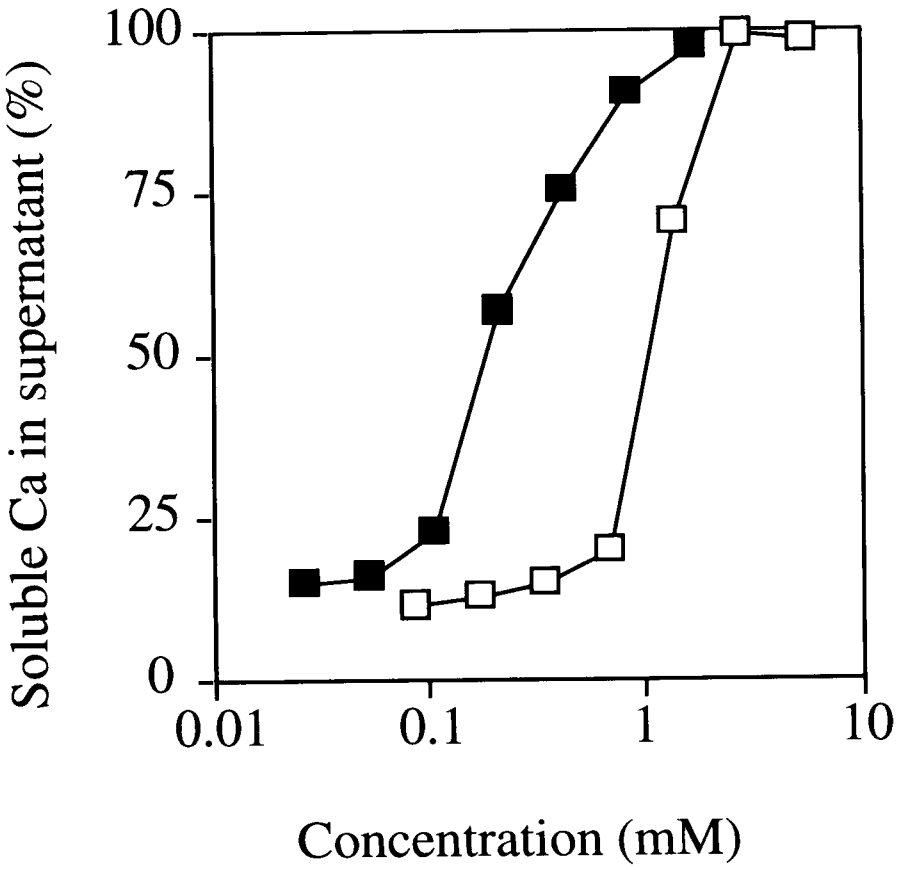

Figure 3.5 Dependence of Inhibitory Effects on the Formation of Calcium Phosphate Precipitate upon the Concentration of CGT-PO2a and CGT-PO2b

The concentrations of CGT-PO2a and CGT-PO2b in test solution were represented as the horizontal axis, and those of calcium and phosphate were 4 mM and 10 mM, respectively. Symbols: □, CGT-PO2a; ■, CGT-PO2b.

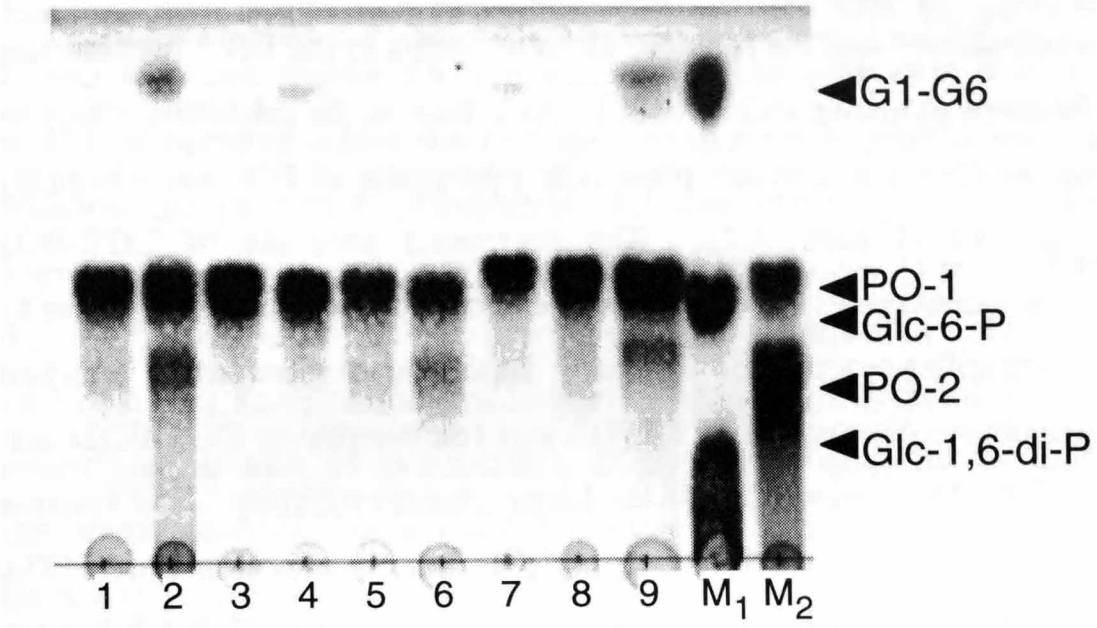

Figure 3.6 TLC of Each PO-1 Component after CGTase Treatment

1, POs; 2, CGT-POs; 3, 6^2-phosphoryl maltotriose treated with CGTase; 4, 6^3-phosphoryl maltotriose treated with CGTase; 5, 6^3-phosphoryl maltotetraose treated with CGTase; 6, 6^4-phosphoryl maltopentaose treated with CGTase; 7, 3^3-phosphoryl maltotriose treated with CGTase; 8, 3^3-phosphoryl maltotetraose treated with CGTase; 9, 3^4-phosphoryl maltopentaose treated with CGTase; M_1, glucose, authentic maltooligosaccharides (G2-G6), PO-1 fraction, and PO-2 fraction; M_2, Glc-6-P and Glc-1,6-di-P.

3.4 Discussion

The transglycosylation of components in the PO-1 fraction was accomplished and the increase of components in the PO-2 fraction was observed as shown in Figure 3.1. As a results, the inhibitory effect on the formation of calcium phosphate precipitate of POs was obviously improved (Figure 3.3). The structural analysis of CGT-PO2 components shows the extension of chain length of glucosyl residues by the coupling reaction of CGTase. Based on the number of attached phosphoryl groups, the CGT-PO2 was fractionated to CGT-PO2a and CGT-PO2b fractions by ion-exchange chromatography. The fraction CGT-PO2b was more effective inhibitor 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 (Figure 3.5). These results support the hypothesis; the inhibitory effect on the formation of calcium phosphate precipitate depends upon the number of carrying phosphoryl groups per molecule described in chapter 1.

The transglycosylation reaction could be obviously observed when 3^4-phosphoryl maltopentaose and 6^4-phosphoryl maltopentaose were used as substrates (Figure 3.6). No products of CGTase reaction were detected when the other substrates were used in the conditions. The TLC system was useful to confirm the transglycosylation of POs since the substances linked one or at least two phosphoryl groups to each molecule had different Rf values, and obviously distinguished from neutral oligosaccharides. The peaks of 3^4-phosphoryl maltopentaose (K) and 6^4-phosphoryl maltopentaose (N) were also obviously decreased as shown in Figure 3.1b. These results indicated that 3^4-

phosphoryl maltopentaose and 6^4-phosphoryl maltopentaose would be important substrates in transglycosylation catalyzed by CGTase. In this reaction, glucose was also detected on HPAEC and TLC as shown in Figure 3.1b and Figure 3.6, respectively. This hydrolysis reaction would be occurred while the transglycosylation was progressed. In addition, the peak of 6^3-phosphoryl maltotetraose (M) was slightly decreased as shown in Figure 3.1b. From this result, it is considered that 6^3-phosphoryl maltotetraose could not work as donor molecule and could work as acceptor molecule. Further studies are contemplated to investigate a way of preparation of the large quantity of each component in PO-1 fraction and determine the precise mechanism of the transglycosylation of POs.

The structure of components in the CGT-PO2 fraction was estimated as follow. The CGT-PO2 components was easily hydrolyzed by BSA (Figure 3.4), and 3^2-phosphoryl maltotriose (S1) and 6^3-phosphoryl maltotriose (S3) were produced by the action of GA and BSA. They had been also obtained from the fraction PO-1 by the action of BSA and GA as described in section 2.4. These facts indicated that the CGT-PO2 fraction was obviously produced from the fraction PO-1 by the coupling reaction of CGTase. From these results, the possible structure of CGT-PO2a fraction were as shown in Figure 3.7. Almost of the glucosyl residues in reducing site of CGT-PO2a would be lacked by the hydrolysis because the average DP value from Table 3.1 was about 8. In this context, the structure of components in CGT-PO2b fraction would be estimated as construct from the products from the coupling reaction of CGT-PO2a components and 3^2-phosphoryl maltotriose according to the results in Tables 3.1 and 3.2.

However, further study is needed to determine the precise structure of them.

In this research, the author proposed a method for the enhancement of the inhibitory effect of POs on the formation of calcium phosphate precipitate.

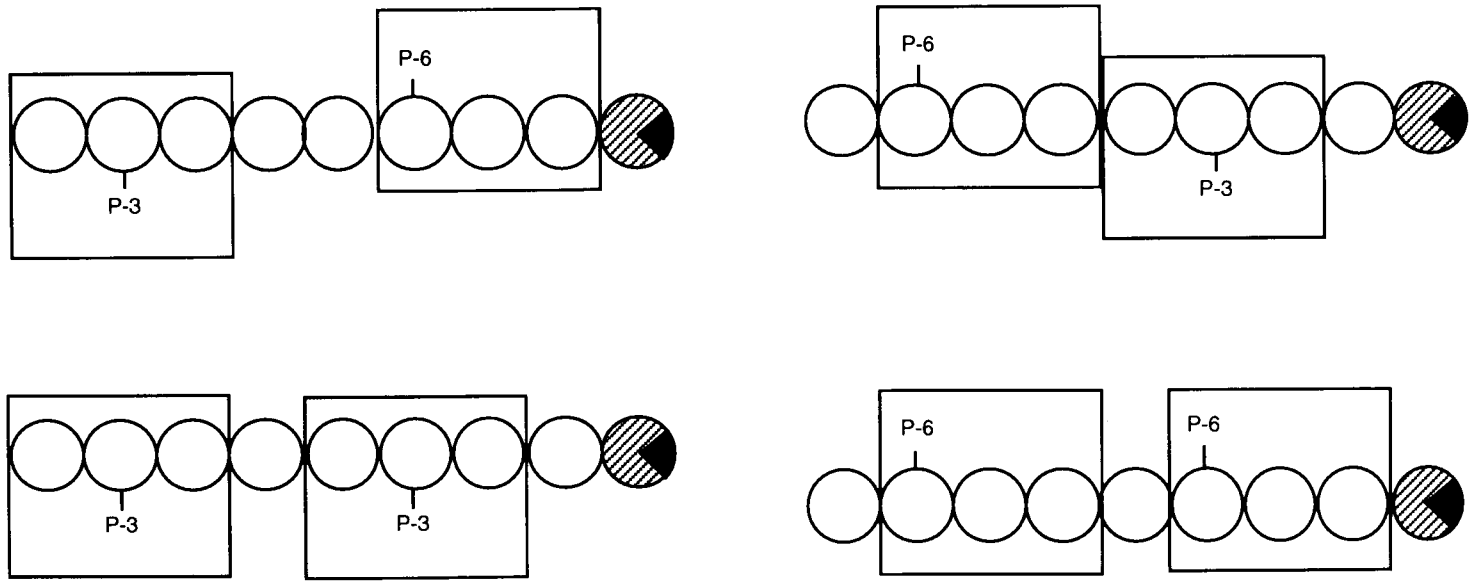

Figure 3.7 Possible Structure of CGT-PO2a Components
Symbols: P-3 and P-6, phosphoryl groups linked at C-3 and C-6 of glucosyl residues; ○, glucosyl residue; ◍ , glucosyl residue which possibly exists; ◑ , reducing end. The square indicates the units of indigestible fragments by the action of BSA and GA.

3.5 Summary

In chapter 1, the POs were prepared from potato starch hydrolysate and were investigated the inhibitory effect on the formation of calcium phosphate precipitate. In this chapter, the enhancement of the inhibitory effect by using the coupling reaction of CGTase was investigated. In the reaction, the effect of POs was obviously improved and the conversion of the structure of POs was observed. By the coupling reaction of CGTase, the fraction of the POs with one phosphoryl group decreased, and the fraction with at least two phosphoryl groups increased from 10% to 33%. Phosphoryl maltopentaose was the main substrates for the coupling reaction of the enzyme. The products (CGT-POs) obtained after CGTase reaction were fractionated by an ion-exchange chromatography into three fractions, CGT-PO1, CGT-PO2a, and CGT-PO2b. The fractions of the CGT-PO2a and the CGT-PO2b exhibited strong inhibitory effect such as PO-2 fraction in the POs from potato starch. The fraction CGT-PO2a was maltooligosaccharides with an average DP of 7.89 to which two phosphoryl groups were attached. The fraction CGT-PO2b was maltooligosaccharides with an average DP of 11.06 to which three phosphoryl groups were attached. In addition, the fraction CGT-PO2b was more effective than the fraction CGT-PO2a since the threshold value of the concentration of CGT-PO2a to exhibit the inhibitory effect was 7-fold higher than one of CGT-PO2b. These results support the hypothesis that the strength of the inhibitory effect would depend on the number of phosphoryl groups attached to each sugar molecule.

Chapter 4: A Way of Enhancing the Inhibitory Effect of POs on the Formation of Calcium Phosphate Precipitate (II); Utilization of the Ovalbumin-PO-1 fraction Conjugate

4.1 Introduction and aim

Maillard reaction occurs in food processing procedure and it is free from chemical reagents.[61] In this way, it is a safe manner of processing food and we can prepare a conjugate which is safely used as a food additive. For example, production of protein-dextran conjugates is a good means in improving functional properties of protein such as solubility in water, emulsifying activity, and foaming properties.[61-64] Recently, it was reported that whey protein-Glc-6-P, or egg white protein-Glc-6-P conjugates showed the inhibitory properties on the formation of calcium phosphate precipitate.[65] Such conjugates having phosphoryl groups would be expected as one of interesting compounds to exhibit the inhibitory effect on the formation of calcium phosphate precipitate.

The POs were prepared from potato starch hydrolysate, and they have the ability to inhibit calcium phosphate formation as described in chapter 1. They were fractionated by ion-exchange chromatography into two fractions; PO-1 and PO-2 fractions. The inhibitory effect on the formation of calcium phosphate precipitate of PO-2 fraction was equal to that of CPP. On the other hand, the capacity of PO-1 fraction, which was main part of POs, was inferior to PO-2 fraction. In previous chapter, the author described a way of enhancing the inhibitory effect of POs on the formation of calcium phosphate

precipitate by using the coupling reaction of CGTase. In this chapter, the author describes another way for enhancing the inhibitory effect of POs by making a OVA-PO-1 conjugate. Some properties of the OVA-PO-1 conjugate were discussed comparing with OVA-Glc-6-P conjugate.

4.2 Materials and methods

4.2.1 Materials

The fraction PO-1 was prepared from potato starch as described in 1.2.3. OVA from chicken egg was purchased from Sigma Chemicals. Ampholine, isoelectric point markers, and molecular weight markers were purchased from Bio Rad (California, U.S.A.). Polyacrylamide gel was purchased from Tefco Co. (Nagano, Japan). Protease Amano A and M from *Aspergillus oryzae* and P from *Aspergillus melleus* were purchased from Amano Pharmaceutical Co.(Nagoya, Japan). Chymotrypsin from Bovine Pancreas and trypsin from Porcine Pancreas were purchased from Wako Pure Chemical Industries. Other chemicals and materials used were of analytical or commercial grade.

4.2.2 Preparation of OVA-saccharides conjugates by Maillard reaction

One mg OVA was dissolved in 1 ml of distilled water (1 mg/ml). To the solution 1 mg of PO-1, Glc-6-P, or glucose was added. The protein-saccharide solutions were adjusted to pH 8.0 with 1N NaOH and lyophilized. The dried samples were stored at 50°C and 65% relative humidity maintained with saturated KI solution for 0-20 days. After 0-20 days' incubation, each sample was dissolved in 100 ml of

distilled water and filtrated with membrane (pore size 0.5 μm) to remove precipitate. Saccharides which remained in the solution were removed by ultrafiltration using a membrane with nominal molecular weight limit of 30,000. Then, the solutions of protein-saccharide conjugates were obtained, and the final volume of each solution was prepared to 1 ml.

4.2.3 Measurements of browning value in OVA-saccharides conjugates

The OVA-saccharide mixture solutions (1ml) were offered for measurements of browning value and content of free amino groups as the indicators of Maillard reaction. Browning value was measured by spectrophotometry at 420 nm by a Shimazdu spectrophotometer, Model UV-240.

4.2.4 Measurement of the content of amino groups in OVA-saccharides conjugates.

The content of free amino groups in soluble conjugates were measured by the method of Fields *et al.*[66] using trinitrobenzene sulfonate which is specific reagent for amino groups. The content of soluble protein were measured by the method of Lowry *et al.*[67]

4.2.5 Estimation of molar ratios of OVA to saccharides in conjugate for inhibitory effect on the formation of calcium phosphate

OVA (1.0 mg) was dissolved in distilled water (1 ml) containing the PO-1 fraction (0.08 - 2.0 mg), Glc-6-P (0.06 - 1.0 mg) in each mole ratio, and then OVA-saccharide conjugates by Maillard reaction

were prepared by the method mentioned above. After incubation for 7 days (PO-1 fraction) and 4 days (Glc-6-P), protein conjugates were obtained, and then the inhibitory effect on the formation of calcium phosphate was investigated at appropriate molar ratio by the method described in section 1.2.5.

4.2.6 Determination of some properties of OVA-saccharides conjugates

4.2.6.1 Determination of the molecular weight

The molecular weights of protein-saccharide conjugates were estimated with the results from SDS-PAGE. The conjugates (1.5 μg) were treated with sodium dodecyl sulfate and 2-mercaptoethanol, and analyzed by 12% (w/v) SDS-PAGE using the system of Laemmli.[68] Protein was stained with Coomassie Brilliant Blue R-250 after electrophoresis. In addition, gel filtration was also carried out to measure the molecular weight of the conjugates by HPLC using a TSK-gel G-3000SW column (7.5ϕ x 300 mm, Tosoh Ltd.) eluted with 50 mM phosphate buffer (pH 7.0) containing 0.3 M NaCl at a flow rate of 0.7 ml/min at room temperature with detection by the absorbance at 220 nm. In this analysis, Thyoglobin (M_r:670,000), Gamma globurin (M_r:158,000), OVA (M_r:45,000), Myoglobin (M_r:17,000), Vitamin B-12 (M_r:1,350) were used as molecular markers.

4.2.6.2 Determination of the isoelectric point

Each conjugate (1.5 μg) was solubilized with sample solution (pH 3-10 ampholine 0.2 ml and glycerol 3.0 ml in 10 ml distilled water), and analyzed by IEF-PAGE with 4% (w/v) polyacrylamide gel using

the system of Vesterberg.[69] Protein was stained with Coomassie Brilliant Blue R-250 after electrophoresis.

4.2.6.3 Measurement of emulsifying properties

The emulsifying properties were measured by the method of Shimbo *et al.*[70] with a slight modification. The solutions of the conjugates or native OVA (0.2%, w/v) were prepared in 25 mM Tris-HCl buffer (pH 7.0) or 25 mM acetate buffer (pH 3.0) to investigate the effects of pH on the emulsifying properties. These buffers containing 0.1 M $CaCl_2$ were also used to investigate the effects of salt on the emulsifying stability. Kerosene (0.5 g) was added to each sample solution (0.5 g) in a tube. The mixtures were then homogenized in a Polytron mixer for 30 sec at room temperature. The emulsion prepared was transferred to a graduated tube and kept at 30°C, and the separation of the water and oil phases was measured as a function of time. The stability of emulsion was defined as [height of emulsion phase] x 100 / [(height of emulsion phase) + (height of separated phase)]. When the emulsion was prepared, droplet sizes of the emulsion were immediately measured with particle size analyzer (LA-700; Horiba Co., Ltd., Kyoto, Japan) at 20°C.

4.2.6.4 Digestibility of the conjugates

The conjugates were hydrolyzed by some proteases: protease Amano A, M, P, chymotrypsin, and trypsin. Each protease solution (3%, w/v, 1μl) was added to the conjugate solution (1%, w/v, 100μl) in 50 mM phosphate buffer containing 6 mM NaN_3 and incubated at 37°C for 17h, and analyzed by HPLC using a Poly LC column (4.6ϕ x 200 mm, Sypress Ltd.) eluted with a 10 mM formic acid at a flow rate of

0.5 ml/min at room temperature with detection by the absorbance at 215 nm.

4.2.6.5 Determination of denaturation temperature

Two mg of the conjugates in 50μl of the 10 mM phosphate buffer was placed in a silver pan, and a DSC thermogram was recorded from 25°C to 120°C with a differential scanning calorimeter attached to a thermal analysis station (DSC100, Seiko Instruments). The rate of heating was 1°C/min.

4.3 Results

4.3.1 Inhibitory effects of OVA-saccharides on the formation of calcium phosphate precipitate

After removal of precipitate and remaining saccharides, each conjugate sample for 0-20 days' incubation was obtained. The inhibitory effects of the conjugates on the formation of calcium phosphate precipitate were examined. Although OVA itself and OVA-glucose conjugate had no effect, OVA-Glc-6-P, and OVA-PO-1 conjugate showed the effects as shown in Figure 4.1. The inhibitory effect appeared whithin 2 days of the formation of OVA-PO-1 conjugate and OVA-Glc-6-P conjugate. The OVA-PO-1 conjugate could express the highest inhibitory effect after 7 days' treatment and maintain the high inhibitory effect during examination period. The highest inhibitory effect of OVA-Glc-6-P conjugate could be detected after 2 days' treatment, and the effect disappeared during 20 days' treatment because OVA-Glc-6-P conjugate became insoluble and precipitated during the period. The content of the soluble protein

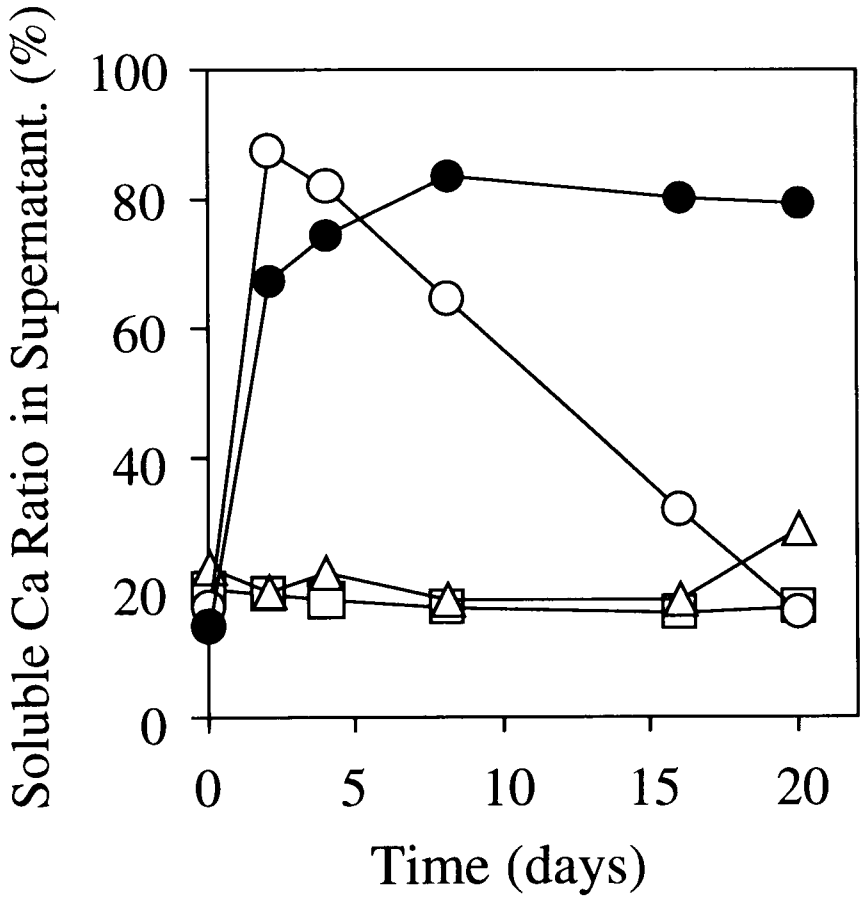

Figure 4.1 Inhibitory Effects of OVA-Saccharides Conjugates on Calcium Phosphate Formation after 0-20 days' Maillard Reaction

The reaction mixture consisting of 0.1% conjugates, 4 mM calcium, and 10 mM phosphate, were shaken at 30°C for 4h, and soluble calcium in the supernatant was measured. Symbols; the OVA-PO-1 conjugate (●), the OVA-Glc-6-P conjugate (○), and the OVA-glucose conjugate (△). OVA alone (□) as a control.

decreased with the same decline as the decline of the inhibitory effect (Figure 4.1). After 20 days' incubation, most of OVA-Glc-6-P conjugate became insoluble, while most of OVA-PO-1 conjugate did not. In addition, the content of free amino groups in the conjugate was determined using trinitrobenzene sulfonate. As the treatment period for the formation of the conjugates was prolonged, the free amino groups of OVA-saccharides conjugates obviously decreased (data not shown), since the free amino groups reacted with reducing terminal residues of saccharides by the process of the Maillard reaction. The content of free amino groups of OVA-PO-1 conjugate reached a minimum level after 7 days' treatment, and OVA-Glc-6-P conjugate reached a minimum level after 4 days' treatment. These results affirmed the occurrence of Maillard reaction and indicated the optimum treatment periods of both conjugates, while the slightly difference was observed with the expression of the inhibitory effect (Figure 4.1). The conjugate solutions were also offered for

Figure 4.2 Developments of the Color in the Conjugate Solutions after Maillard Reaction
The concentration of the conjugates in the solutions were prepared at 5%. The optimum incubation period in Maillard reaction was determined from the inhibitory effect as shown in Figure 4.1. Symbols; the OVA-PO-1 conjugate obtained after 7 days' incubation (1), the OVA-Glc-6-P conjugate obtained after 4 days' incubation (2), OVA alone (C) as a control.

measurement of browning value, which is one of the indicators of the Maillard reaction. The color of OVA-Glc-6-P conjugate became brown within 2 days and reached a maximum value after 4 days' treatment at this conditions (Figure 4.2), and then the color gradually disappeared because the conjugate turned insoluble and precipitated. However, the color of OVA-PO-1 conjugate was slightly brown after 7 days' reaction (Figure 4.2), and the color did not change for next 10 days. The conjugates with monosaccharide quickly developed brown color, though those with polysaccharides hardly did.[71] As shown in Figure 4.2, the phosphomonosaccharide like Glc-6-P quickly developed brown color, but the phosphooligosaccharide like PO-1 hardly did (Figure 4.2).

Although fractions PO-1 and PO-2 had the ability to make complex with calcium ion, PO-2 fraction had a stronger effect and was better for inhibiting the formation of calcium phosphate than PO-1 fraction. It was surmised that this difference in the effect depended upon the number of phosphoryl groups carrying per molecule as described in chapter 1 and 3. The results obtained in this work are consistent with the hypothesis. The OVA-PO-1 conjugate had stronger inhibitory effect on formation of calcium phosphate than intact PO-1 itself, and the effect was almost equal to that of PO-2. In results, a way of practical use as the conjugate was a effective way in improving the inhibitory effect of PO-1.

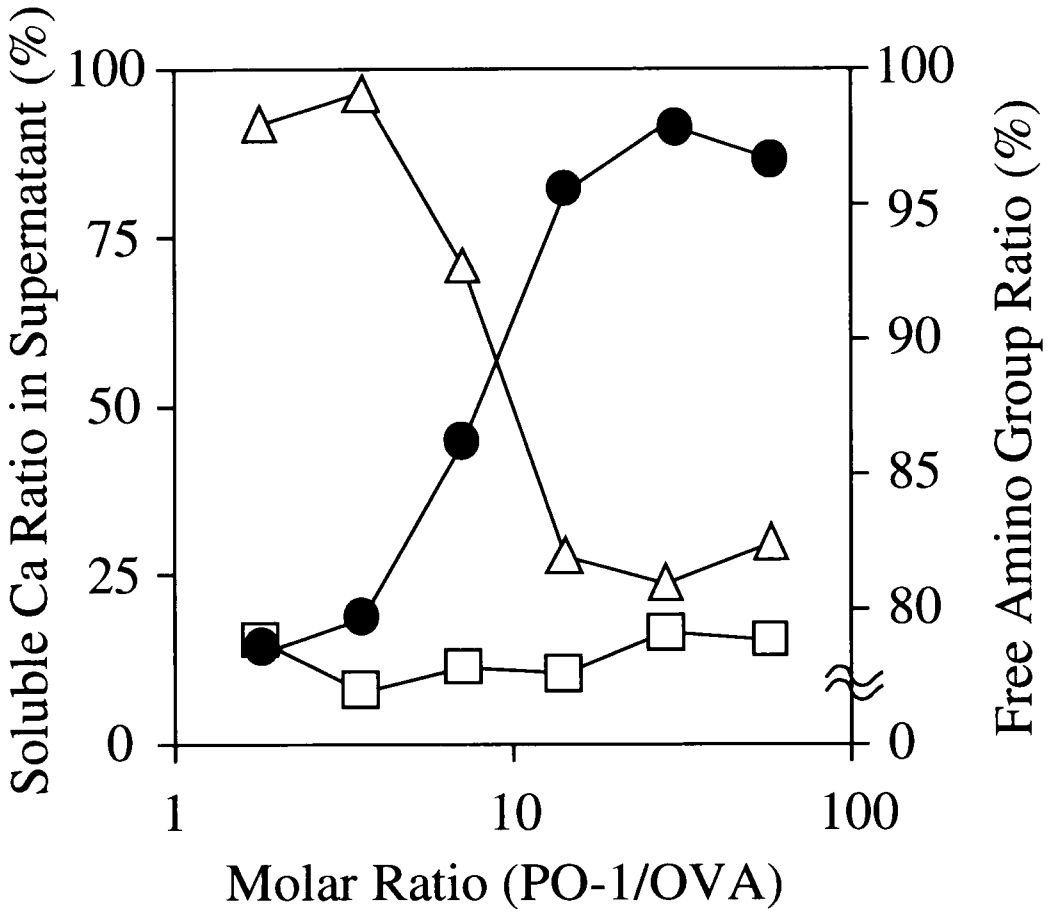

Figure 4.3 Relation Between Inhibition of Calcium Phosphate Formation and Molar Ratio of PO-1 to OVA

PO-1 fraction and OVA (molar ratio of PO-1/OVA; 1.8, 3.6, 7.2, 14.4, 28.8, 57.6) were treated for 7 days at 50°C, 65 % relative humidity. The reaction mixture; consisting of 0.1% conjugates, 4 mM calcium, and 10 mM phosphate.

Symbols; ●, soluble calcium ratio in the supernatant; △, free amino group ratio in the conjugate solution; □, soluble calcium ratio in the supernatant of the mixture of PO-1 and OVA before Maillard reaction as a control.

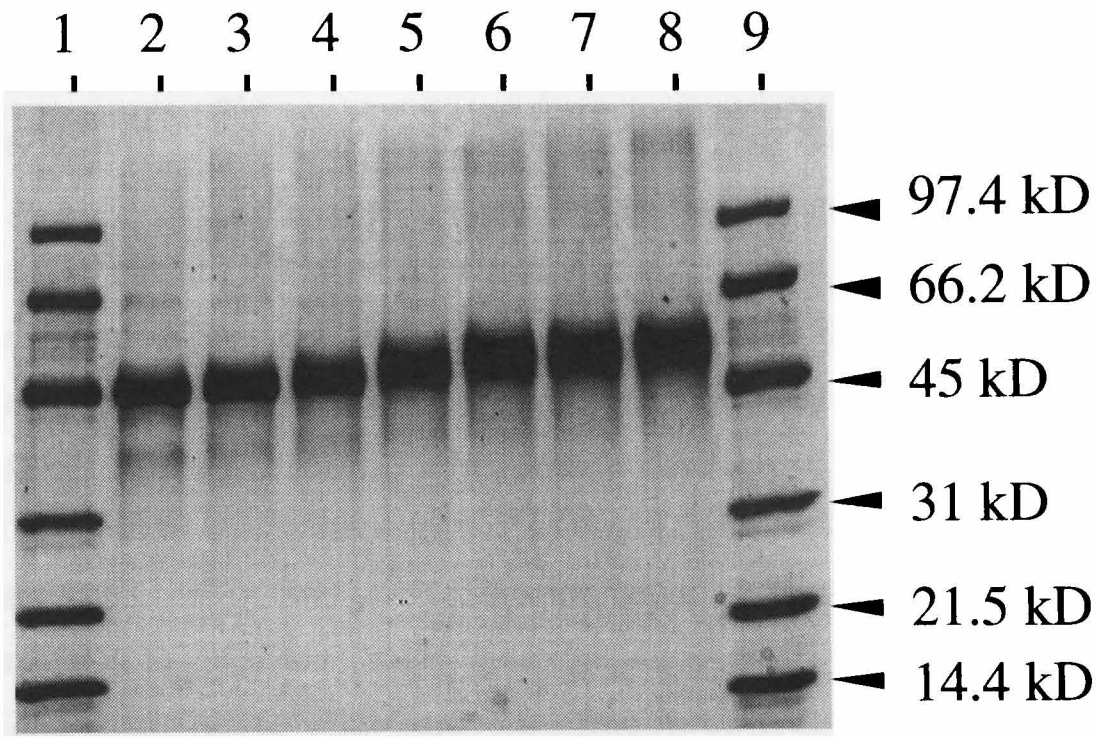

Figure 4.4 SDS-PAGE of the OVA-PO-1 Conjugates Produced with Various Ratio

Lane 1and 9, molecular weight makers; Lane 2, OVA; Lane 3-8, conjugate: the molar ratio of PO-1 to OVA are 1.8 (Lane 3), 3.6 (Lane 4), 7.2 (Lane 5), 14.4 (Lane 6), 28.8 (Lane 7), 57.6 (Lane 8).

4.3.2 Relation between inhibition of calcium phosphate formation and molar ratio of PO-1 to OVA in conjugate

Various molar ratio of OVA-PO-1 conjugate was produced with Maillard reaction during the optimum incubation period (7 days) to determine the minimum molar ratio of PO-1 to OVA in expressing inhibitory effect on calcium phosphate formation. The effect significantly appeared when the molar ratio of PO-1 to OVA was higher than 3.6, and sufficiently effect was obtained when that of PO-1 to OVA was 10 (Figure 4.3). In this conditions, free amino group ratio decreased to 80% at the most. An OVA molecule has 35 free amino groups (20 residues of Lys and 15 residues of Arg).[72] The molecular weight of the OVA-PO-1 conjugate increased from 45kD to 53kD at the most, as the molar ratio of PO-1 to OVA increased (Figure 4.4, Table 4.1). These results indicated that 10 molecules of PO-1 was necessary for OVA to express the inhibitory effect on calcium phosphate formation. In this context, isoelectric point of the conjugates changed from 4.5 to 4.0 (Table 4.1). It indicated that phosphoryl groups of PO-1 turned the isoelectric point of OVA. In case of the OVA-Glc-6-P conjugate, the molecular weight increased at most from 45kD to 60kD, and isoelectric point changed from 4.5 to 3.5 after 4 days' incubation (Table 4.1). The optimum incubation period was also determined from the result of former experiment (Figure 4.1). It indicated almost free amino groups of OVA were reacted with Glc-6-P. The reaction of OVA and Glc-6-P was more rapidly than that of OVA and PO-1.

Table 4.1 Molecular Weight and pI of OVA and its Conjugates

	M.W.		saccharides (mol/mol)	pI
	SDS-PAGE	gel filtration		
native OVA	45 kD	45 kD	-	4.5
OVA-PO-1	53 kD	55 kD	10～12	4.0
OVA-Glc-6-P	60 kD	61 kD	40～43	3.5

Table 4.2 Emulsifying Properties of the Conjugates

		native OVA	OVA-PO-1	OVA-Glc-6-P
pH 7	stability (%)	70.6	84.1	77.1
	MDD[a] (μm)	45.0	48.3	41.4
$+Ca^{2+}$	stability (%)	74.4	80.2	92.6
	MDD[a] (μm)	82.3	68.6	64.7
pH 3	stability (%)	69.6	80.6	93.8
	MDD[a] (μm)	27.1	34.6	27.1
$+Ca^{2+}$	stability (%)	82.9	89.7	96.3
	MDD[a] (μm)	60.9	52.8	47.8

[a] MDD; median droplet diameter

4.3.3 Some properties of the conjugates.

Some properties of the OVA-saccharide conjugates were examined and compared with native OVA. First, the emulsifying stability and the droplet size of emulsion were investigated (Table 4.2). Emulsifying capacity is an important functional property of a protein in various emulsion-based food systems.[73] The droplet size of emulsion is a key parameter of emulsifying stability and large droplets coalesce much faster than small ones.[74] The emulsion of the OVA-PO-1 conjugate was slightly stable comparing with one of native OVA. Especially, the OVA-Glc-6-P conjugate was stable in acidic condition. Furthermore, the effect of calcium ion on stability of emalsion was expressed in the OVA-Glc-6-P conjugate, though the effect of calcium ion was slightly observed in OVA-PO-1 conjugate. It would be due to the amount of phosphoryl groups incorporated to protein because it would be able to catch the calcium ion, though native OVA had at most 2 phosphoryl groups in molecule.[75] The emulsifying property of OVA was still remained through the Maillard reaction, although dynamic improvement of the property of OVA observed hardly.

Second, digestibility of the conjugates were investigated. Although chymotrypsin and trypsin could hardly hydrolyze, protease Amano A, M, and P could almost hydrolyze OVA-PO-1 and OVA-Glc-6-P conjugates as same as native OVA. The patterns of the chromatograms from HPLC analyses were almost the same in this condition (data not shown). In addition, the results from DSC indicated the denaturation temperatures of the OVA-PO-1 and OVA-Glc-6-P conjugates were almost same as native OVA at about 83°C. Difference could not be

observed as was observed between OVA and its derivative from disulfide-reduced and carboxymethylated treatment.[76] The denaturation temperatures of the derivatives are clearly higher than that of native OVA and the structures of the derivatives were clearly different from native OVA.[76] Therefore, it is most likely that the structure of the OVA-PO-1 conjugate hardly changed from native OVA since some properties of them were almost the same. However, further studies are contemplated to decide the precise structure of the OVA-PO-1 conjugate.

4.4 Discussion

The structure of PO-1 components has been determined as shown in Chapter 2. Approximately 70% of the phosphoryl groups were linked to C-6 of the glucosyl residues, and their linkages still remained after treatment with 0.7 N HCl in 100 °C for 4 h as described there. This result indicated that the PO-1 fraction was almost stable during Maillard reaction and food processing. The variety of molar ratio of PO-1 to OVA means that any phosphate groups would be able to add protein molecule through the Maillard reaction. Furthermore, phosphoryl group of PO-1 is located at non-reducing residue or one glucosyl residue apart from non-reducing residue, but not at reducing end (Figure 2.5). In case of Glc-6-P, phosphoryl group and reducing glucosyl residue are located at the same glucosyl residue. When saccharides react with protein by Maillard reaction, reducing residue of saccharides react with amino group of protein. The ionic functional group might promote unfold and insolubilize protein.[77] Therefore, it may be important which glucosyl residue in the molecule carries phosphoryl group. It was concluded that location of phosphoryl groups

in PO-1 would be advantageous in producing conjugate. A few glucosyl residues between glucosyl residue attached to the phosphoryl group and reducing glucosyl residue reacted to protein molecule in molecule of PO-1 components would be able to work as the spacer.

In this chapter, the author described a improvement of PO-1 fraction and addition of a new function to protein: inhibitory effect of calcium phosphate formation by usage of saccharide carrying with phosphoryl groups. Phosphoryl protein which has a similar function of phosphopeptide like CPP can be easily produced, although oligosaccharides existed between protein and phosphoryl group. Although CPP is good for rendering the calcium ion soluble in several kinds of food, its use is restricted because of its bitterness. In addition, the reaction of monosaccharides and protein was so fast that the color developed brown quickly in comparing the reaction of oligosaccharides and protein. The usage of the OVA-Glc-6-P conjugate is also restricted because of its brown color (Figure 4.2). However, the OVA-PO-1 conjugate had neither bitter taste nor color. The number of phosphoryl groups incorporated into the protein molecule could be altered by varying ratio of POs used (Figure 4.3). The protein-POs conjugate is easily produced and safe as a food additive.[61] The OVA-PO-1 conjugate could be feasible in an industrial use.

4.5 Summary

The PO-1 was conjugated with OVA through the Maillard reaction. The conjugate was prepared by treating at 50°C, 65% relative humidity. The inhibitory effect of the conjugate on the formation of calcium phosphate precipitate was examined. The optimum treatment period (7 days) for expression of the inhibitory effect of PO-1 on the formation of calcium phosphate was determined. In this period, PO-1 molecule combined about 10 molecules with one molecule of OVA. The average molecular weight of OVA-PO-1 conjugate increased from 45kD to 53kD. The isoelectric point of the conjugate also changed from 4.5 to 4.0. The structure of OVA hardly changed during the Maillard reaction since the some properties of native OVA and OVA-PO-1 conjugate were not so different. On the other hand, OVA-Glc-6-P conjugate was rapidly produced in this condition than OVA-PO-1 conjugate. However, the OVA-Glc-6-P conjugate quickly developed brown color, while OVA-PO-1 conjugate hardly did. These results indicated that making a conjugate with OVA was a noteworthy way to enhance the inhibitory effect of PO-1 on the formation of a calcium phosphate and that the OVA-PO-1 conjugate could be a better food additive than the OVA-Glc-6-P conjugate.

Chapter 5: Effect of POs on Iron Solubility

5.1 Introduction and aim

Iron is an essential metallic element in human physiology and nutrition. The absorption of iron depends on its form, the conditions within the gastrointestinal tract, the amount and chemical form of the iron ingested, and the quantities and properties of other compounds in the diet.[78] Food iron is classified into heme and non-heme forms. Heme iron, which is mainly contained in meat and blood, is absorbed more efficiently than non-heme iron via the heme receptor on the brush border membrane,[79,80] but then joins the same iron pool as non-heme iron in the mucosal cells. Non-heme iron is in three forms: inorganic ferrous (Fe^{2+}) and ferric iron (Fe^{3+}) and several protein-bound irons such as transferrin- and lactoferrin-bound iron in the intestinal lumen. The ferric iron is hardly dissolved in the intestinal canal at the physiological pH level so that its absorption is extremely low. On the other hand, ferrous iron is relatively easily absorbed.[81]

Unlike heme iron, inorganic iron needs to be rendered soluble in the gastrointestinal tract to be effectively absorbed. For example, CPP and lactoferrin enhances intestinal iron absorption by increasing the solubility of inorganic iron.[82,83] Lactoferrin is one of the functional proteins in milk components containing iron,[84] but has no phosphoryl groups. In addition, it would be significantly absorbed in the intestinal mucosa by an alternative mechanism to which is used in the transportation of soluble iron salts.[85] One mole of lactoferrin can solubilize over 70 moles of iron. The author have proposed a new function of oligosaccharides, namely the inhibitory effect on the

formation of calcium phosphate precipitate by POs produced from a potato starch hydrolysate. In this chapter, the author investigated the effect of POs on iron solubility, and the relationship between the solubilizing effect and number of phosphoryl groups attached to each sugar molecule in the presence of inhibitory ligands such as bicarbonate and phosphate salts; these salts form insoluble complexes with iron. It is also known that the pancreatic juice contains the bicarbonate ion, and that a variety of processed foods generally contain phosphate salts.

5.2 Materials and Methods

5.2.1 Materials

Lactofferin from bovine milk was purchased from Wako Pure Chemical Industries. Other enzymes and materials were used as described in section 1.2 and 2.2.

5.2.2 Measurement of the ability of POs to solubilize iron

The method for preparing POs from potato starch and the fractionation into PO-1 and PO-2 have been described in section 1.2.3. These two fractions were subjected to an investigation of their iron solubilizing effects by the method of Kawakami *et al.*[83] Sample solutions containing an appropriate concentration of each PO were mixed with 25 µl of 100 mM ferric chloride. After adding deionized water to adjust the total volume to 850 µl, each solution was mixed with 150 µl of a 0.2 M sodium bicarbonate solution (pH 8.0) or a 0.2 M sodium phosphate buffer (pH 7.5) to adjust the pH to 6.5-7.4. After incubating at 37°C for 48 h, each solution was centrifuged at 10,000 x *g* for 10 min to remove the precipitate. The iron content of the

supernatant after reduction with hydroxylamine was measured as the ferrous form of iron by the spectrophotometric method using *o*-phenanthroline.[86] The molecular ratio of iron to POs was calculated by assuming the average molecular weights as 830 (PO-1) and 1260 (PO-2).

5.2.3 Measurement of iron oxidization

Sample solutions (100 μl), including 2 mM covalently bound phosphoryl group, were prepared. The effect on iron solubility and iron oxidization were investigated with ferrous chloride (the final concentration was 1 mM) as the iron. Each sample was incubated at 37°C for 10 min, 1, 3 or 24 h. The precipitate was removed after this incubation. The ferrous and total soluble iron content were measured. The ferric iron was calculated as [total soluble iron (%) - ferrous iron (%)]. The percentage of soluble iron per total iron initially added was calculated.

5.3 Results

5.3.1 Ability of POs to solubilize iron

As shown in Figure 5.1, PO-1 solubilized equivalent moles of iron, whereas PO-2 solubilized 10-fold equivalent moles of iron. The ability of the PO solution containing phosphate salt was identical to that of the bicarbonate-containing solution (data not shown). The ability of the PO-2 fraction was equal to that of CPP.[83] This implies that the iron-solubilizing ability depended upon the number of covalently bound phosphoryl group per molecule.

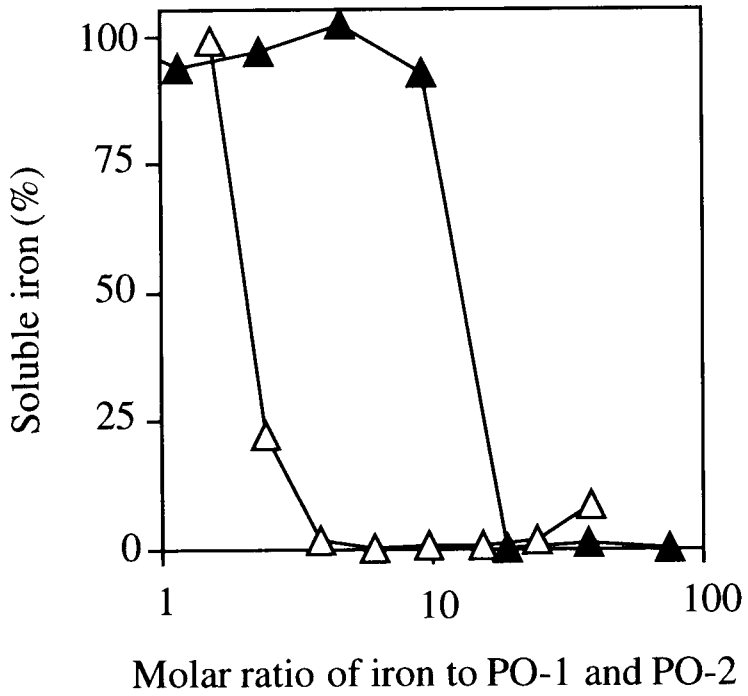

Figure 5.1 Effect of PO-1 and PO-2 on Iron Solubility in the Presence of the Bicarbonate Ion
The pH of the solutions ranged from 7.0 to 7.4. The iron and bicarbonate ion concentrations were kept constant at 2.5 mM and 30 mM, respectively. Symbols: △ PO-1; ▲ PO-2.

5.3.2 Measurement of iron oxidization

Although POs could solubilize iron after 24 h, almost all the iron was immediately oxidized to ferric iron from the ferrous form (Table 5.1). This phenomenon was also observed with other phosphoryl substances such as Glc-1-P, Glc-6-P, Glc-1,6-di-P, and CPP. Lactoferrin also gave the same results (data not shown). However, maltopentaose as the control did not solubilize or oxidize ferrous iron, and almost all iron was precipitated after 24 h. Each substrate possessing phosphoryl group was an effective solubilizer of iron and formed a stable and soluble complex with ferric iron.

Table 5.1 Effects of Various Phosphoryl Substances on Iron Solubility

Sample	Soluble iron (%)					
	10 min.			24 h		
	Fe^{2+}	Fe^{3+}	Total	Fe^{2+}	Fe^{3+}	Total
PO-1	18.9	67.2	86.1	2.3	74.9	77.2
PO-2	14.8	78.2	93.0	3.8	82.0	85.8
Glc-1-P	29.0	60.9	89.9	0.5	81.5	82.0
Glc-6-P	23.2	64.2	87.4	1.8	79.2	81.0
Glc-1,6-di-P	5.60	87.4	93.0	0.0	80.7	80.7
Maltopentaose	64.4	26.0	90.4	0.0	10.7	10.7
CPP	12.0	85.8	97.8	0.25	95.3	95.6

5.4 Discussion

The PO-1 molecule solubilized equivalent moles of iron, and the PO-2 molecule solubilized more than 10-fold equivalent moles of iron. It indicated that the iron solubilizing ability depends upon the number of covalently bound phosphoryl group per molecule. It supported the same hypothesis mentioned in the chapter 1 in case of inhibitory effect of a calcium phosphate precipitate. Although at least two moles of phosphoryl groups in the molecule could solubilize the mole over equivalent moles of iron, the precise mechanism was unknown. In the same context, lactoferrin has two specific binding sites for iron in molecule,[85] and one mole of lactoferrin can solubilize over 70 moles of iron. This is much higher than the specific ability, and the mechanism also remains unproved.[83]

For the effective absorption of inorganic iron, the ferrous form (Fe^{2+}) is considered to be better than the ferric form (Fe^{3+}); however, Ebihara *et al.*[87] have reported that there was no significant difference in the bioavailability of ferrous and ferric iron. Although the oxidation of iron is caused by CPP,[88] the high availability of iron was also observed.[89,90] The reduction of ferric iron would be accomplished by the gastric juices, and by other digestive secretions.

In conclusion, the results suggest that POs, if orally administered, would be able to prevent from precipitation of iron as well as calcium in the intestines. Although there are some other solubilizers of non-heme iron, there are few materials actually used in food apart from the CPP, ascorbic acid. They are effective for rendering the iron ion soluble in several kinds of food, although their usage is restricted

because of bitterness or acidity. It is worth mentioning that POs have not been essentially found not to have taste by a sensory test as shown in section 1.4.

5.5 Summary

The solubility of iron ions with POs from a potato starch hydrolysate was investigated in the presence of bicarbonate and phosphate salts. PO-1, having one phosphoryl group, solubilized equivalent moles of iron, and PO-2, having two phosphoryl group, solubilized more than 10-fold equivalent moles of iron. The number of phosphoryl groups attached to the molecule influenced the solubility of iron. The ability of PO-2 was equal to that of CPP which is currently used as an iron solubilizer for food.

Chapter 6: Action pattern of Neopullulanase on POs

6.1 Introduction and aim

Neopullulanase [EC 3.2.1.135] catalyzed the hydrolysis of both α-1, 4- and α-1, 6-glucosidic linkages, and the formation of both α-1, 4- and α-1, 6-glucosidic linkages by transglycosylation.[91,92] These four reactions are typically catalyzed by α-amylase, pullulanase, CGTase, and 1,4-α-D-glucan branching enzyme, respectively. As neopullulanase catalyzes these four reactions by one active center,[91-93] it has been very difficult to detect its activity independently of other α-amylase family enzymes by the simple method using maltooligosaccharides.[92,94] Therefore, a simple system for the analysis of the action pattern of neopullulanase has been required.

BSA and GA were useful for the structural analysis for the components in PO-1 fraction, and their reaction specificities on PO-1 were determined in chapter 2. In this chapter, the action pattern of neopullulanase on PO-1 components was investigated and compared with those of various α-amylases. The possibility of using POs for characterization and the comparison of the action patterns of neopullulanase and other several α-amylases are also discussed.

6.2 Materials and Methods

6.2.1 Enzymes and materials

PPA, human saliva α-amylase (HSA), and Taka-amylase A (TAA) [EC 3.2.1.1] were purchased from Sigma Chemicals. Neopullulanase from *Bacillus stearothermophilus* was purified by the methods of

Kuriki *et al.*[91] Other enzymes and materials were used as described in section 1.2 and 2.2.

6.2.2 Preparation of the products from the PO-1 fraction after neopullulanase treatment

The preparation of POs have been described in section 1.2.3. The PO-1 fraction (2.5 g) was dissolved in 20 ml of a 10 mM phosphate buffer (pH 7.0) and incubated with 20 units of neopullulanase at 50°C for 24h. 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 purified by the ion-exchange and the ODS chromatographies as described in section 2.2.3.

6.2.3 Analytical methods

The total amount of carbohydrates was measured by the phenol-sulfuric acid method. The precise concentration of POs was determined by dephosphorylated oligosaccharides, following the treatment of alkaline phosphatase. Each component of POs (50 μl, 1%, w/v) in a 20 mM carbonate buffer (pH 9.4) containing 3 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ was incubated at 37°C for 30 h with 4 units of alkaline phosphatase. The reaction was terminated by removing the enzyme by an ultrafiltration membrane with nominal molecular weight limit of 30,000. The retention time of dephosphorylated compounds were compared with the authentic oligosaccharides on HPAEC analysis, and the concentration of the dephosphorylated compounds was determined by the peak area by using the response factors as shown in section 1.2.4. The structure was then analyzed as follows:

6.2.3.1 Enzymatic analyses

After the treatment with neopullulanase, each component (1 mg) after purification of the products from the reaction was 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 for 15 h. The solution was heated in a boiling-water bath for 10 min to stop the reaction. The products after the amylase treatment and these dephosphorylated compounds obtained from the reaction were analyzed on HPAEC system using PAD and TLC, respectively. TLC was also carried out by the method described in 2.2.4.1.

6.2.3.2 Chemical analyses

The methods of chemical analyses were followed as described in section 2.2.4. Determination of content of the reducing-terminal residue was analyzed by borohydride methods. The reducing-terminal residue was measured as sorbitol after an acid hydrolysis. Glucose was measured by using glucose oxidase. DP values were calculated as [glucose (mol) + sorbitol (mol)] / [sorbitol (mol)].

The non-reducing-terminal residue was determined as glycerol after a rapid Smith degradation using the sample with and without a dephosphorylation.

The content of the phosphoryl group of each PO was measured as inorganic phosphate after dephosphorylating with the alkaline phosphatase treatment described above. The phosphoryl group at C-6 of the glucosyl residue was measured as Glc-6-P after an acid hydrolysis as shown in section 2.2.4.4.

6.2.3.3 Determination of action patterns of neopullulanase and various α-amylases on PO-1 fraction

The PO-1 fraction (75 μg) after the sufficient reaction with GA was dissolved in 50 μl of deionized water and incubated with 0.25 units of neopullulanase or various α-amylases at 40°C for 10 min, 3h, 6h, and 24h. The reaction was stopped by heat treatment in a boiling-water bath for 5 min. After centrifugation, the supernatants of the reaction mixtures were analyzed on HPAEC. The chromatographic conditions were shown in section 1.2.4. The action patterns of neopullulanase, BSA, TAA, HSA, PPA, and BLA were determined. For further analyses, the products were purified by the ion-exchange and the ODS chromatographies by the method described in section 2.2.3.

6.3 Results

6.3.1 Preparation of the products from the PO-1 fraction after neopullulanase treatment

The PO-1 fraction was a mixture of 6^3-phosphoryl maltotriose, 6^2-phosphoryl maltotriose (peak L), 6^3-phosphoryl maltotetraose (peak M), and 6^4-phosphoryl maltopentaose (peak N) from the oligosaccharides attached with the phosphoryl groups at C-6, and 3^3-phosphoryl maltotetraose (peak J), and 3^4-phosphoryl maltopentaose (peak K) from the oligosaccharides attached with the phosphoryl groups at C-3 as described in chapter 2. The chromatogram after the digestion of PO-1 fraction by neopullulanase was shown in Figure 6.1b, and the products were fractionated in P1, P2, and P3 by anion-exchange column chromatography and the Daisopak SP-120-5-ODS-BP column. In results, P1, P2, and P3 were purified to homogeneity as

shown in Figure 6.2a, c, and d, respectively. They were desalted and freeze-dried. P1 (8 mg), P2 (7 mg), and P3 (25 mg) were obtained as white powder.

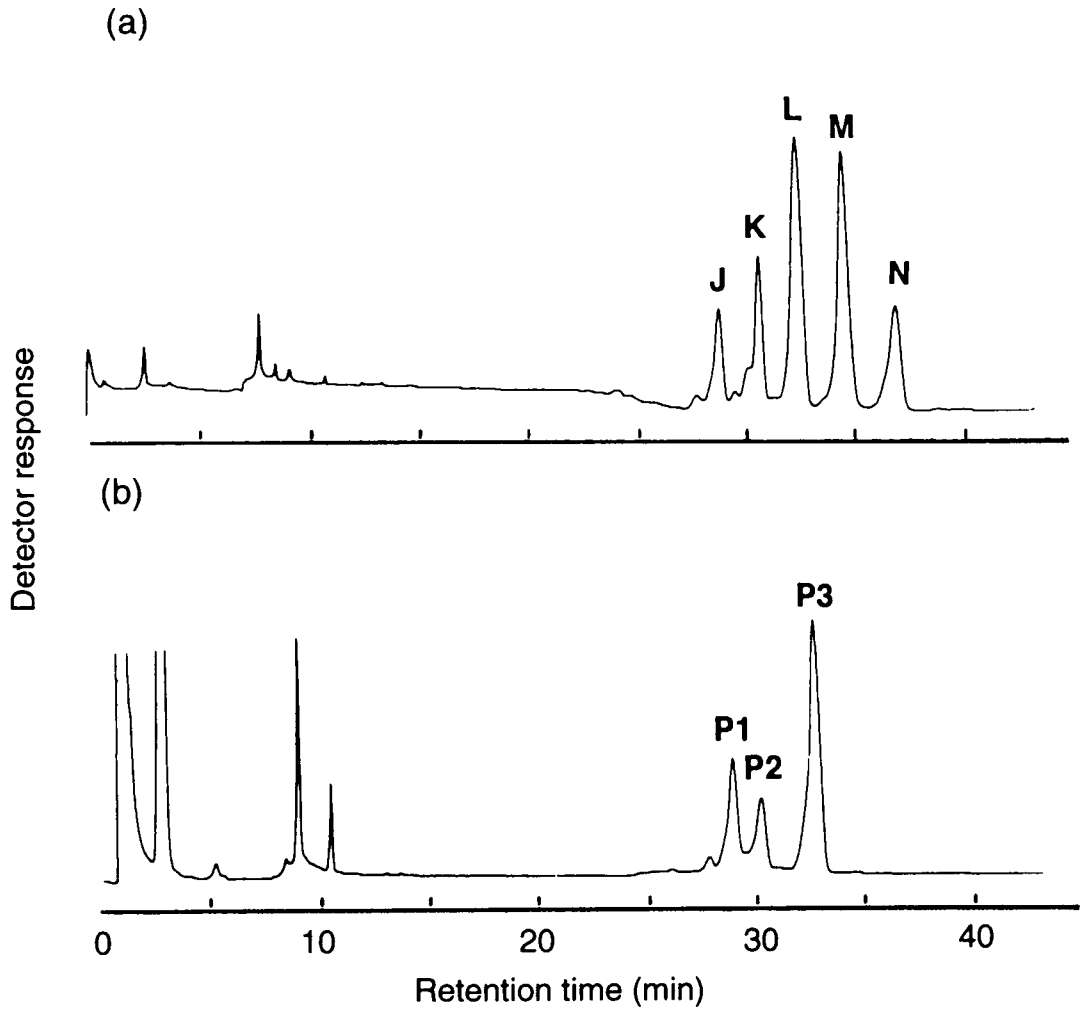

Figure 6.1 HPAEC Chromatograms of PO-1 Fraction before and after Neopullulanase Treatments
(a) PO-1 fraction prepared from potato starch; (b) PO-1 fraction treated with neopullulanase. The amount of each sample injected was 0.1%, 25 μl.

6.3.2 Structure analyses

The structures of P1, P2, and P3 were analyzed as follows.

6.3.2.1 Enzymatic analyses

The purified P1, P2, and P3 components were individually treated with BSA or GA. After dephosphorylation treatment, the products were detected as neutral oligosaccharides by TLC system as shown in Figure 6.3. The products from P2 were shown in lanes 1-3. The dephosphorylated P2 was detected as maltose (lane 1). The results of lane 2 and 3 shows that BSA and GA could not hydrolyze P2. The products from P3 were shown in lanes 4-6. The dephosphorylated P3 was detected as maltotriose (lane 4). The results of lane 5 and 6 shows that BSA could not hydrolyze P3, however GA hydrolyzed it because of the detection of glucose and maltose, respectively. On HPAEC analysis of P3 treated by GA, glucose and P3' were also detected (Figure 6.2e). The conclusion was that P3 and P3' were phosphoryl maltotriose and phosphoryl maltose, respectively. The products from P1 were shown in lanes 7-9. The dephosphorylated P1 was detected as maltotetraose (lane 7). The results of lane 8 and 9 shows that BSA could hydrolyze P1 because of the detection of glucose and maltotriose, and that GA could not hydrolyze P1, respectively. On HPAEC analysis of P1 treated by BSA, glucose and P1' were also detected (Figure 6.2b). The conclusion was that P1 and P1' were phosphoryl maltotetraose and phosphoryl maltotriose, respectively.

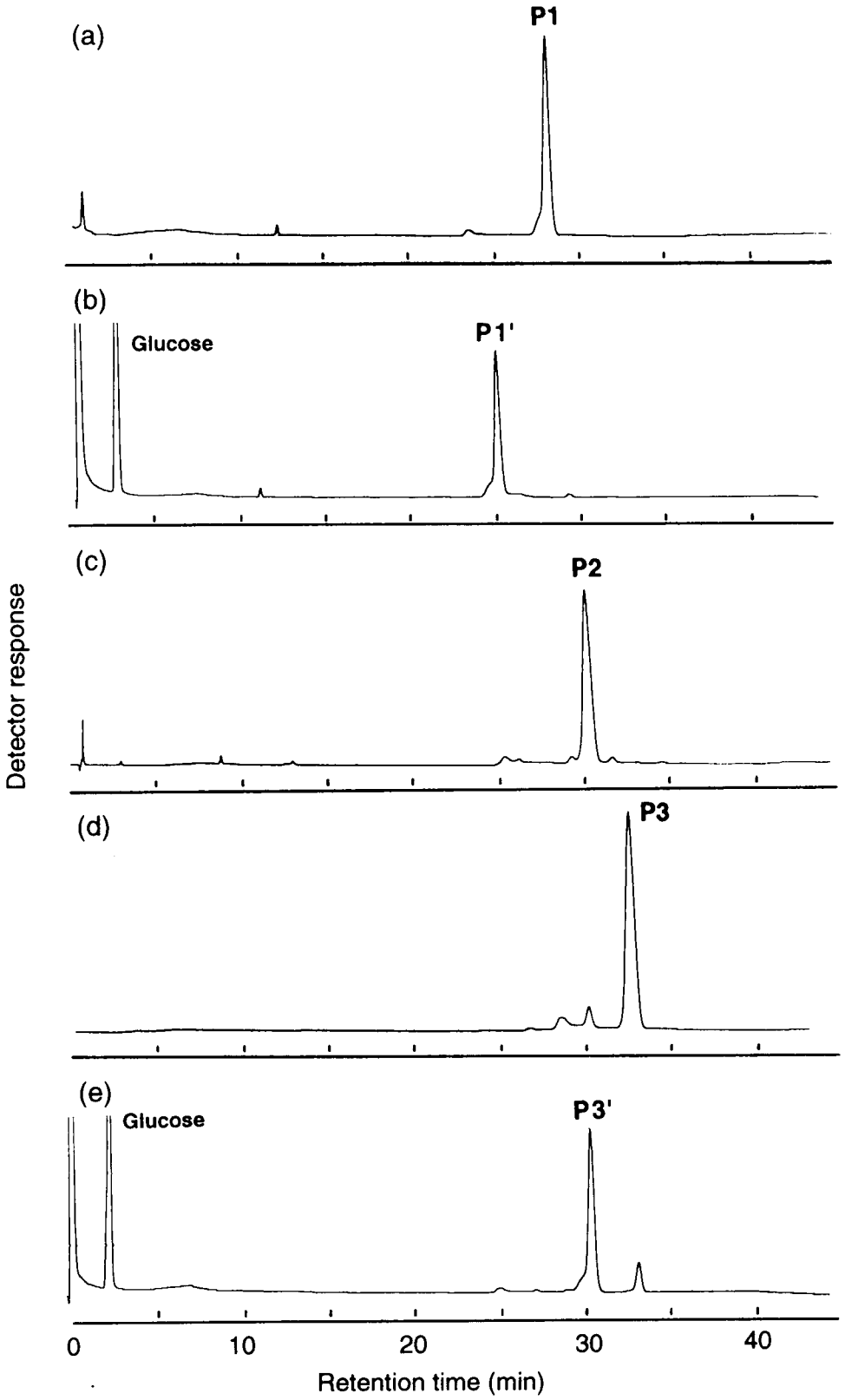

Figure 6.2 HPAEC Chromatograms of Purified Products of PO-1 Fraction after Neopullulanase Treatments

P1 (a), P2 (c), and P3 (d) fractions isolated by column chromatographies, and the products from P1 after the treatment of BSA (b), the products from P3 after the treatment of GA (e). The amount of each sample injected was 0.2%, 25 µl.

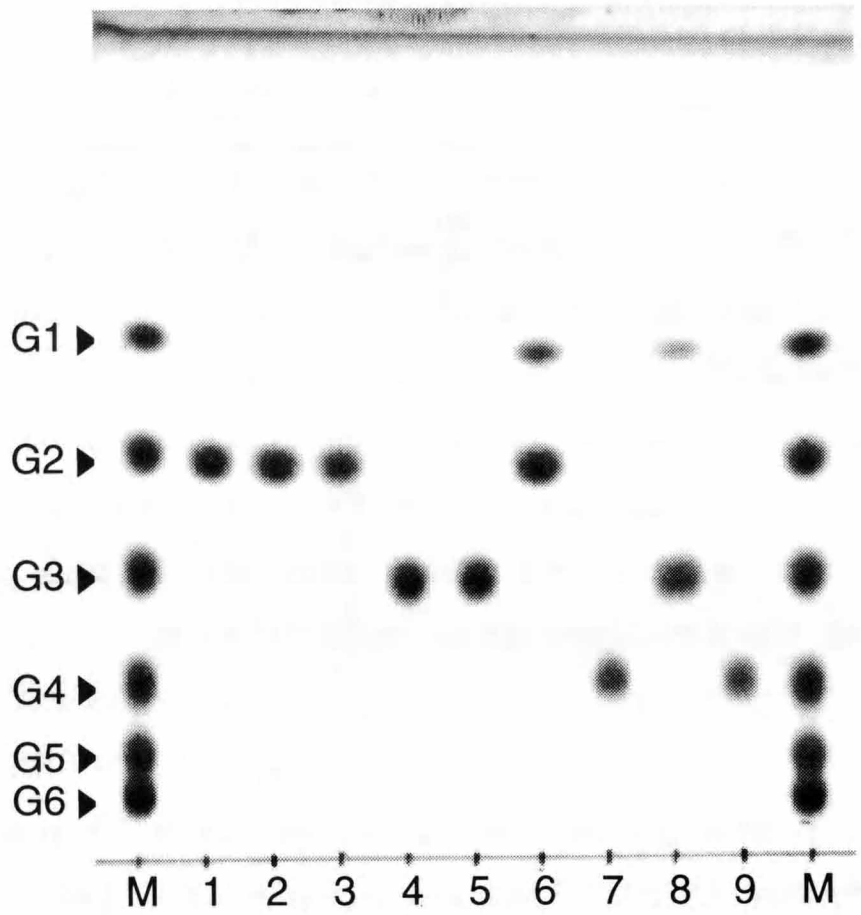

Figure 6.3 TLC of the Dephosphorylated P1, P2, and P3, before and after Action of GA and BSA

Two microliter of 0.3% samples were spotted on TLC plate. M, authentic maltooligosaccharides; 1, dephosphorylated P2; 2, dephosphorylated P2 after the treatment with BSA; 3, dephosphorylated P2 after the treatment with GA; 4, dephosphorylated P3; 5, dephosphorylated P3 after the treatment with BSA; 6, dephosphorylated P3 after the treatment with GA; 7, dephosphorylated P1; 8, dephosphorylated P1 after the treatment with BSA; 9, dephosphorylated P1 after the treatment with GA.

6.3.2.2 Chemical analyses

The reducing-terminal residue of P1, P2, and P3 was determined by the sorbitol production by the borohydride methods (Table 6.1). One mole of sorbitol was produced from one mole of intact P1, P2, and P3, and those after dephosphorylation. These results indicated that P1, P2, and P3 have the reducing-terminal residue in their molecules, and that there was no phosphoryl group linked at the reducing-terminal residue (Table 6.1). In addition, after the reduction of reducing-terminal residue with subsequent acid hydrolysis, three mole of glucose were detected from one mole of P1 and dephosphorylated P1 molecules. No glucose was detected in P2 but one mole of glucose was detected in one mole of dephosphorylated P2. One mole of glucose was detected in one mole of P3 molecule and two mole of glucose were detected in one mole of dephosphorylated P3 (Table 6.1). These facts indicated that covalent bond between phosphoryl group and glucose in P1 was weaker than that of P2 and P3. In other words, the covalent bond between phosphoryl group and glucose is unstable under acidic condition and tend to release inorganic phosphate. The amounts of glucose and sorbitol in the hydrolysates of reduced samples of dephosphorylated P1, P2, and P3 also indicated that their DP values were four, two, and three, respectively, as shown in Figure 6.3.

To detect the non-reducing-terminal residue as glycerol, P1, P2, and P3 were subjected to the rapid Smith degradation (Table 6.1). One mole of P1, P3, and those after dephosphorylation produced one mole of glycerol, respectively. On the other hand, one mole of glycerol could not be detected from one mole of P2, although one mole of

Table 6.1 Structural Analyses of P1, P2, and P3

	P1		P2		P3	
	Intact	dephospho-rylated	Intact	dephospho-rylated	Intact	dephospho-rylated
Non-reducing-terminal residue	1.07	1.18	0.26	1.01	1.23	1.19
Composition of hydrolysate[a]						
Sorbitol	1.12	0.99	0.93	0.98	0.94	1.09
Glucose	2.83	3.06	0.05	1.26	1.14	2.05
Glc-6-P	-	-	1.04	-	1.18	-
Organic phosphate	1.15	-	1.03	-	1.01	-

[a] The sample (2 mM, 60 µl) was reduced by addition of 5 µl of 3% $NaBH_4$ in 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. The values are expressed as mol/ mol.

dephosphorylated P2 produced one mole of glycerol. This suggests that P2 has one phosphoryl group linked at the non-reducing-terminal residue.

The amounts of covalently bound phosphoryl groups of P1, P2, or P3 were measured as inorganic phosphates after complete dephosphorylation with alkaline phosphatase. The result showed that P1, P2, and P3 had one mole of covalently bound phosphoryl group per each molecule (Table 6.1)

The content of Glc-6-P after acid hydrolysis of P1, P2, or P3 was investigated. The results indicated that P1 had no phosphoryl group linked at C-6 of the glucosyl residue, and that P2 and P3 had one phosphoryl group linked at C-6 of the glucosyl residue.

As the results from enzymatic and chemical analyses, P2 should be maltose containing one mole of the reducing-terminal residue and phosphoryl group linked at C-6 of the glucosyl residue, and GA and BSA could not react on it; 6^2-phosphoryl maltose. P3 should be maltotriose containing one mole of the non-reducing-terminal residue, one mole of reducing terminal residue, and the covalently bound phosphoryl group linked at C-6 of glucosyl residue, and GA could react on it but BSA could not; 6^2-phosphoryl maltotriose. These results were completely consistent with the results shown in Figure 6.2. On the other hand, the PO-1 fraction was composed of oligosaccharides with covalently bound phosphoryl group at either C-6 or C-3 of glucosyl residue as described in chapter 2. Three moles of glucose were detected in one mole of P1 molecule after the reduction, and the covalently bound phosphoryl groups at C-3 of oligosaccharides were

unstable under acidic condition and tended to release inorganic phosphate.[53] Therefore, P1 should be maltotetraose containing one mole of the non-reducing-terminal residue, the reducing-terminal residue, and phosphoryl group which was linked at C-3 of glucosyl residue. Furthermore, BSA could react on it but GA could not. P1 was also identified as 3^3-phosphoryl maltotetraose.

It was indicated that 3^3-phosphoryl maltotetraose (P1), and 6^2-phosphoryl maltose (P2) and 6^2-phosphoryl maltotriose (P3) were produced after the action of neopullulanase on PO-1 fraction. In the context, P1' and glucose were produced by the action of BSA on P1, and P3' and glucose were produced by the action of GA on P3. Therefore, P1' and P3' were 3^2-phosphoryl maltotriose and 6^2-phosphoryl maltose, respectively. The action properties of neopullulanase on PO-1 components could be represented as shown in Figure 6.4.

6.3.2.3 Comparison of action patterns of neopullulanase and various α-amylases on PO-1 fraction

The author had described the action patterns of BSA on PO-1 fraction as described in chapter 2. The action patterns of PPA, HSA, TAA, and BLA on PO-1 fraction were also investigated. The limiting cleavage points of neopullulanase and these α-amylases to PO-1 fraction were indicated in Table 6.2. The hydrolysis of PO-1 fraction by the action of TAA and BLA could not be observed under the conditions described in section 6.2.3.3. However, PPA and HSA could hydrolyze PO-1 components, and these limiting cleavage points were the same as

Table 6.2 Limiting Cleavage Points of Neopullulanase and Various α-Amylases on POs

Amylase	G-G-G (3-P)	G-G-G-G (3-P)	G-G-G-G-G (3-P)	6-P G-G	6-P G-G-G	6-P G-G-G-G
NP	none	none	↑	none	↑	↑
BLA	none	none	none	none	none	none
BSA	none	↑	↑	none	none	↑
TAA	none	none	none	none	none	none
HSA	none	↑	↑	none	none	↑
PPA	none	↑	↑	none	none	↑

The arrow indicates action sites of enzyme reactions. *Abbreviations*: NP, neopullulanase; others are as given in the text.

Table 6.3 Action Patterns of Neopullulanase and Various α-Amylases on Maltooligosaccharides

Amylase	G-G-G	G-G-G-G	G-G-G-G-G
NP	↑	↑	↑ ↑
BLA	none	none	none
BSA	↑	↑ ↑	↑ ↑
TAA	↑	↑ ↑	↑ ↑
HSA	↑ ↑	↑	↑
PPA	↑	↑	↑

Long and short arrows indicate relatively fast and slow enzyme reactions, respectively. *Abbreviations*: NP, neopullulanase; others are as given in the text.

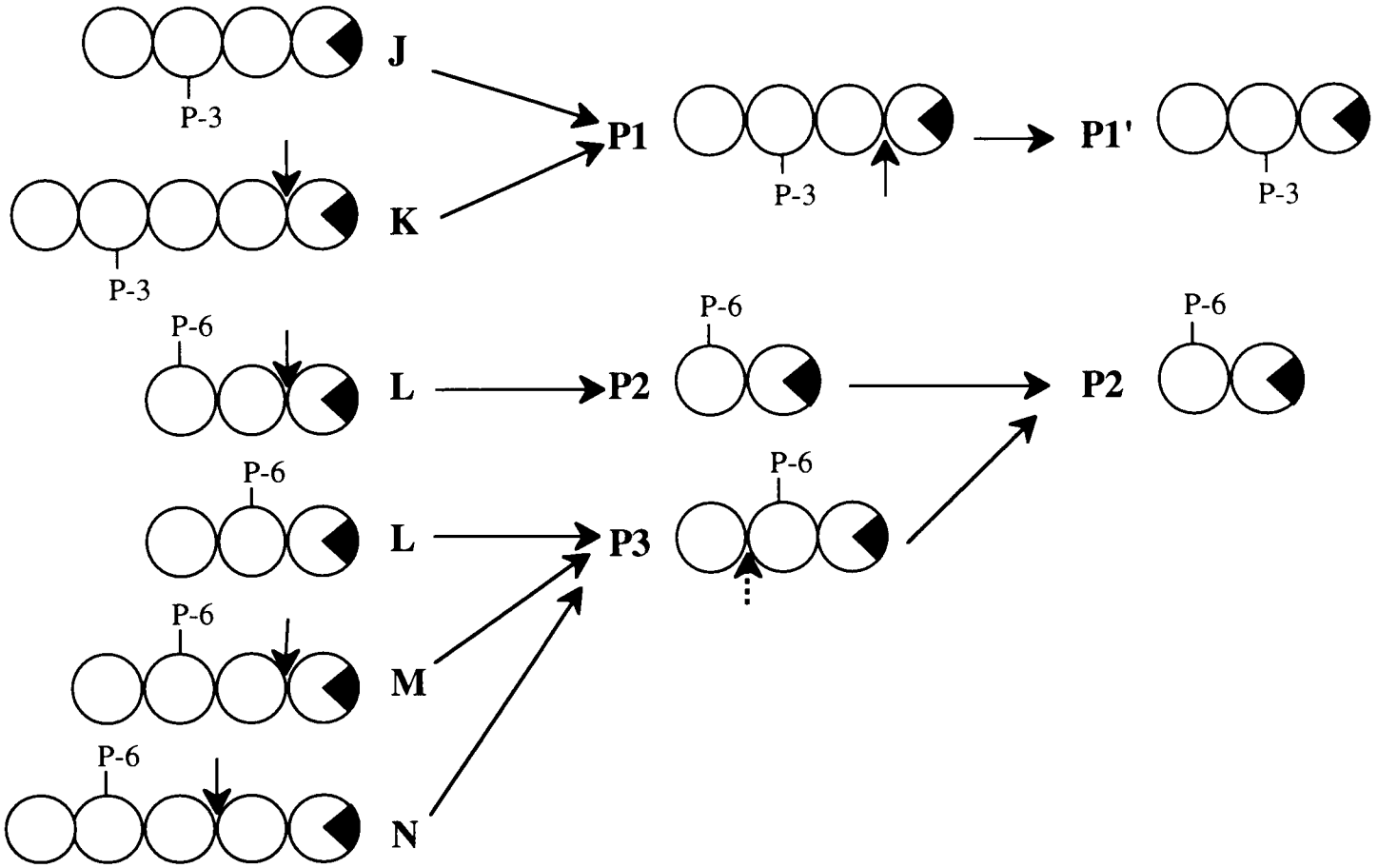

Figure 6.4 Actions of Neopullulanase, BSA and GA on PO-1 Fraction
Symbols: P-3 and P-6, phosphoryl groups linked at C-3 and C-6 of glucosyl residues, ○ ; glucosyl residue, ◔ ; reducing glucosyl residue, ↓; action of neopullulanase, ↑; action of BSA, ↟ ; action of GA.

that of BSA. The specificity of neopullulanase was quite different from α-amylases, PPA, HSA, TAA and BLA on PO-1 components.

6.4 Discussion

According to the structural analyses of P1, P2, and P3 which were obtained from PO-1 fraction by the neopullulanase treatment, the limiting cleavage point of neopullulanase on PO-1 fraction could be determined as follows: neopullulanase could hydrolyze the glucosyl linkage at the reducing site up to one glucosyl residue from the 6-p-glucosyl residue and up to two glucosyl residues from the 3-p-glucosyl residue, respectively (Figure 6.4 and Table 6.2). It was of interest that position of phosphoryl group which was linked to the carbon influenced the hydrolyzing point of α-1,4-glucosidic linkage. In this context, the Michaelis constants (Km) of neopullulanase were varied depending on the substrate with anomalous linkages at C-6 of glucosyl residues. The enzyme hydrolyzed 6^3-*O*-α-glucosyl maltotriose to produce panose and glucose.[91,95] The Km (mM) value of the enzyme for 6^3-*O*-α-glucosyl maltotriose (105 mM) was the almost same as that for 6^3-phosphoryl maltotriose (94 mM). Furthermore, they were two times of that for maltotriose (52 mM). It indicated that Km value of the enzyme increased with the phosphoryl group at C-6 of the non-reducing terminal residue of maltotriose as well as the glucosyl residue at the same position. From these results, it is considered that the suitable binding of neopullulanase for hydrolyzing was inhibited by the phosphoryl group at C-6 of glucosyl residue and the glucosyl residue at C-6 of glucosyl residue in the same way.

The reaction specificities of BSA led us to determine the structure of PO-1 components in chapter 2 and the limiting cleavage points of BSA were indicated as shown in Table 6.2. The limiting cleavage points of HSA and PPA on PO-1 components were the same site as that of BSA (Table 6.2) in this experiment. The results agreed with those reported by Takeda *et al.*[36] They obtained 6^3-phosphoryl maltotriose and 3^2-phosphoryl maltotriose from phosphoryl α-1,4 glucan by action of PPA.[36] The hydrolysis of PO-1 components could not be observed by the action of BLA and TAA (Table 6.2). It is reasonable to infer from the facts that POs were produced from potato starch by the sufficiently action of BLA (section 1.2.3) and TAA could hardly hydrolyze branched substrate.[96] It indicated that the covalently bound phosphoryl groups would be recognized as hindrance like branched substrate. Therefore, POs may be an excellent substrate in detecting the activities of neopullulanase or BSA type α-amylase without the influence of BLA type α-amylase, which would be present widespread in nature.

The action patterns of several α-amylases[55,97,98] and neopullulanase[92,95] on maltooligosaccharides were summarized in Table 6.3 from numerous studies. However, it was difficult to distinguish and characterize neopullulanase among several α-amylases from the action patterns on maltooligosaccharides. In addition, neopullulanase catalyzes not only the hydrolysis, but also the transglycosylation.[92,95] From these results, it was concluded that the POs were useful substrate for characterization and distinguished neopullulanase from other several α-amylases. Further studies are

needed to obtained kinetic parameters of these α-amylases on each purified substrate of PO-1 components.

Primary amino acid sequences of various starch hydrolases and related enzymes from various origins are now available and have been compared with each other.[99] The existence of four highly conserved regions in these α-amylases has been reported.[100] The regions are most likely to constitute the active center of each enzyme. The individual properties of some substrates leads us to notice the importance of a little difference in the subsite structure in the active center of each enzyme. Indeed, the substrate preference of neopullulanase was altered toward α-1,6-branched oligosaccharides by manipulating the volume of side chain of Ile-358, which was located in the active center. Replacing Ile-358 with Trp, which has a bulky side chain, reduced the acceptability of α-1,6-branched oligo- and polysaccharides as substrates. In contrast, replacing Ile-358 with Val, which has a smaller side chain, increased the preference for α-1,6-branched oligosaccharides and pullulan as substrates.[93] Reaction specificity of neopullulanase and various α-amylases to POs would be also of interest in relation to be these altered enzymes by protein engineering. The possibility of POs as a new substrate in classifying α-amylase was suggested, and this research might be a help in studying the specificity and the subsite structures of neopullulanase and α-amylase.

6.5 Summary

The action pattern of neopullulanase on the PO-1 fraction was investigated. Neopullulanase hydrolyzed PO-1 and produced 3^3-phosphoryl maltotetraose from oligosaccharides attached with the phosphoryl groups at C-3, and 6^2-phosphoryl maltotriose and 6^2-phosphoryl maltose from oligosaccharides attached with the phosphoryl groups at C-6. Neopullulanase hydrolyzed the glucosyl linkage at the reducing site up to one glucosyl residue far from the 6-p-glucosyl residue and up to two glucosyl residues far from the 3-p-glucosyl residue, respectively. The reaction specificity of neopullulanase was compared with that of BLA, TAA, BSA, HSA, and PPA by using the PO-1 components as the substrate. The specificity of neopullulanase on PO-1 components was quite different from that of α-amylases; PPA, HSA, TAA, and BLA.

General Discussion

1) A new function of oligosaccharides; the inhibitory effect on the formation of calcium phosphate precipitate

It has been reported that oligosaccharides improved intestinal bacterial flora, prevented carcinogenesis, and decreased calorie effects of foods.[44-48] In this thesis, the author reported a new function of oligosaccharides, inhibition on the formation of calcium phosphate precipitate. An attention was aimed at high covalently bound phosphoryl group content in amylopectin of potato starch.[53] This investigation was concentrated on efforts to prepare POs from potato starch by using several amylases. The POs were founded to have the ability to inhibit calcium phosphate formation by making a complex with calcium ion. Both fractions PO-1 and PO-2 from the POs which have the ability to form a soluble complex with calcium. However, the ability of PO-1 fraction was inferior to PO-2 fraction (Figure 1.5).

In chapter 2, POs were treated with GA or BSA and the products were determined by the chemical and spectrometric analyses. From these results of the analyses, it was concluded that the PO-1 fraction was made up of oligosaccharides attached with phosphoryl group at C-3 (3^3-phosphoryl maltotetraose and 3^4-phosphoryl maltopentaose) and oligosaccharides attached with phosphoryl group at C-6 (6^3-phosphoryl maltotriose, 6^2-phosphoryl maltotriose, 6^3-phosphoryl maltotetraose, and 6^4-phosphoryl maltopentaose). In addition, PO-2 fraction was newly found in potato starch. Although PO-2 components had more complicated structure than those of PO-1 components, the estimation of

the structure of PO-2 components was accomplished by help of the enzymatic procedure (Figure 2.8).

2) Role of the covalently bound phosphoryl group for the inhibitory effect on the formation of calcium phosphate precipitate

The inhibitory effects of various saccharides on the formation of calcium phosphate precipitate were investigated. It is interest to note that diphosphoryl monosaccharide such as Glc-1,6-di-P or Fru-1,6-di-P was as effective as fraction PO-2 (Table 1.3). On the other hand, the monophosphoryl monosaccharide had very weak effect on the inhibition of calcium phosphate formation, as it was found for PO-1. These results lead us to the assumption that at least two phosphoryl groups are needed in exhibiting the inhibitory effect. The author also found that phosphomannan[34] and modified starch (chemically phosphoryl starch) had strong inhibitory effects on the formation of calcium phosphate precipitate (Table 1.3). The hydrolysate of the modified starch, containing more phosphoryl groups in the molecule than the native modified starch, had a stronger inhibitory effect than the native modified starch. By the coupling reaction with CGTase, a fraction of the POs with one phosphoryl group decreased, and a fraction of the POs with at least two phosphoryl groups increased (chapter 3). The inhibitory effect of POs treated by CGTase was stronger than that of the POs (Figure 3.3). Additionally, several phosphoryl groups could easily incorporate into protein molecule with the POs (chapter 4). The OVA-PO-1 conjugate had more phosphoryl groups in the molecule compared with native OVA (Table 4.1). The inhibitory effect appeared in the conjugate (Figure 4.3). These results

also support the hypothesis that the strength of the inhibitory effect would depend on the number of phosphoryl groups attached to molecule. Furthermore, it was found that the number of phosphoryl groups attached to the molecule had influence on the solubility of iron (Chapter 5).

Although saccharides having phosphoryl group effectively inhibited calcium phosphate formation regardless of short DP, and some compounds having carboxyl groups as functional groups showed a different effect. It has been reported that poly-L-glutamate and alginate[17] were indeed effective inhibitors, but relatively high molecular weights are needed for exhibiting the inhibitory effect. A high molecular weight is needed to exhibit an inhibitory effect on the formation of calcium phosphate precipitate for substances having carboxyl groups but not phosphoryl groups (Table 1.3). Other acidic polysaccharides, dextran sulfate, chondroitin sulfate, galacturonate and glucuronate had no effects under the conditions. The conclusion of chapter 1 is that the substances having covalently bound phosphoryl group were effective inhibitors on the formation of calcium phosphate precipitate.

3) Action properties of several amylases on POs

In chapter 2, the author studied the reaction specificities of GA and BSA on POs. GA could hydrolyze 6-phosohoryl oligosaccharides up to just the 6-p-glucosyl residue from non-reducing site. On the other hand, using 3-phosphoryl oligosaccharides as substrates, GA could not hydrolyze up to the 3-p-glucosyl residue from non-reducing site and one glucosyl residue remained at non-reducing site. After the action of

BSA, glucose was liberated from the reducing site of POs up to two glucosyl residues from the 6-p-glucosyl residue and one glucosyl residue from the 3-p-glucosyl residue (Figure 2.5). In chapter 6, it was found that neopullulanase hydrolyzed the glucosyl linkage at the reducing site up to one glucosyl residue from the 6-p-glucosyl residue and up to two glucosyl residues from the 3-p-glucosyl residue. The limiting cleavage points of HSA and PPA on PO-1 components were the same as that of BSA (Table 6.2). BLA and TAA could not hydrolyze PO-1 components. These differences of hydrolytic properties are interesting in studying the reaction mechanisms on amylases. It was considered that the POs were useful substrate for characterization of several α-amylases.

4) Further work

4)-1 Characterization of the ratio of C-6 to C-3 of glucosyl residue linked phosphoryl group in starch

By BSA and GA treatment, 3^2-phosphoryl maltotriose was produced from oligosaccharides attached with the phosphoryl group at C-3, and 6^3-phosphoryl maltotriose produced from oligosaccharides attached with the phosphoryl group at C-6 (Table 6.2). A small amount of 6^2-phosphoryl maltose would be also produced. From the ratio of the content of these oligosaccharides, the phosphorylation ratio of C-6 to C-3 was determined as 7 to 3. This analytical method can be a useful way in defining the ratio for starches from different potato species and from potatoes grown under different conditions. Further work is needed for investigation of the ratio of C-6 to C-3 in several starches from different species. Additionally, it may be more

interesting if starch from different stage of tuber development is analyzed toward exploration of mechanism of the phosphorylation in starch synthesis.

4)-2 The possibility as a new substrate for amylase and phosphatase

When the PO-1 fraction was treated by amylases like GA, BSA, and neopullulanase, difference of hydrolytic properties are observed between oligosaccharides linked the phosphoryl groups at C-6 and oligosaccharides linked the phosphoryl groups at C-3 (Table 6.2). Furthermore, the specificity of neopullulanase on PO-1 components was quite different from those of α-amylases; PPA, HSA, TAA, and BLA. These different hydrolytic properties are of interest in studying the action mechanism of amylase. The possibility of POs as a new substrate in classifying α-amylase was suggested, and this research should be an aid in studying the specificity and the subsite structures of neopullulanase and α-amylase. The conserved regions are most likely to constitute the active center of each enzyme. The individual properties of some substrates leads us to notice the importance of a little difference in the subsite structure in the active center of each enzyme. Further studies are needed to obtained kinetic parameters of these α-amylases on each purified component of PO-1 fraction.

Additionally, a novel specificity of acid phosphatase are investigated by using PO-1 fraction as a substrate as described in section 2.4. It is indicated that the POs can be a useful substrate for phosphatase as well as amylase.

4)-3 Calcium absorption *in vivo*

It is very important to examine whether the enhancement of the calcium absorption occurs *in vivo*. Further careful studies on the calcium absorption *in vivo* should be done under various conditions and the existence of the POs and its derivatives, i.e., the conjugates and CGT-POs. It is very interesting to investigate whether the number of phosphoryl groups attached to each molecule influenced the calcium absorption. Additionally, the effect on other essential metallic element absorption in human (e.g., iron, magnesium, and so on) should be also verified.

4)-4 Application of POs to food

Although there are some inhibitors on the formation of calcium phosphate precipitate that were already mentioned in the introduction, there are few materials actually used in food apart from CPP.[16,82] CPP is good for rendering the calcium ion soluble in several kinds of food, however, its use is restricted because of bitterness. POs have no bitter taste by a sensory test (section 1.4). Furthermore, POs are safe as a food additive because it is just a hydrolysate of potato starch. These facts would enable POs to be used for many kinds of food without changing taste. Moreover, POs could be used for a reduction of the problem of insolubilized calcium phosphate on the efficiency in the food industry[28,29]

Potato starch is produced in large scale in Japan (about 250,000 tons/year).[101] The author succeeded in finding the POs in the potato processing waste. It was considered that POs are very useful for food

or drink as a inhibitor of calcium phosphate precipitate and as an accelerator of calcium absorption. Furthermore, as the POs are presence in a manufacturing waste, low cost production of POs is anticipated. Production of POs in industrial scale is becoming possible even at present. Reduction of waste by utilization of by-products is one of the biggest subject in today's agriculture for protection of global environment.[102)]

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Appendix

List of published papers

(1) Kamasaka, H.; Uchida, M.; Kusaka, K.; Yamamoto, K.; Yoshikawa, K.; Okada, S.; Ichikawa, T.

"Inhibitory Effect of Phosphorylated Oligosaccharides Prepared from Potato Starch on the Formation of Calcium Phosphate"

Biosci. Biotech. Biochem., **59**, 1412-1416 (1995)

(2) Kamasaka, H.; To-o, K.; Kusaka, K.; Kuriki, T.; Kometani, T.; Hayashi, H.; Okada, S.

"The Structures of Phosphoryl Oligosaccharides Prepared from Potato Starch"

Biosci. Biotech. Biochem., **61**, 238-244 (1997)

(3) Kamasaka, H,; To-o, K.; Uchida, M.; Kusaka, K.; Kuriki, T.; Kometani, T.; Hayashi, H.; Okada, S.; Ichikawa, T.

"Studies of Phosphoryl Oligosaccharides Prepared from Potato Starch (Proceedings of the Symposium on Amylase and Related Enzymes, 1996)"

J. Appl. Glycosci., **44**, 253-261 (1997).

(4) Kamasaka, H.; To-o, K.; Kusaka, K.; Kuriki, T.; Kometani, T.; Okada, S.

"Action pattern of Neopullulanase on Phosphoryl Oligosaccharides Prepared from Potato Starch"

J. Appl. Glycosci., **44**,(1997). (in press)

(5) Kamasaka, H.; To-o, K.; Kusaka, K.; Kuriki, T.; Kometani, T.; Okada, S.

"Effect of Phosphoryl Oligosaccharides on Iron Solubility under Neutral Conditions"

Biosci. Biotech. Biochem., **61**,1209-1210 (1997).

(6) Kamasaka, H.; To-o, K.; Kusaka, K.; Kuriki, T.; Kometani, T.; Okada, S.

"A Way of Enhancing the Inhibitory Effect of Phosphoryl Oligosaccharides on the Formation of Calcium Phosphate Precipitate by Using the Coupling Reaction of Cyclomaltodextrin glucanotransferase"

J. Appl. Glycosci., **44**,(1997). (in press)

(7) Kamasaka, H.; Kusaka, K.; To-o, K.; Kuriki, T.; Kometani, T.; Okada, S.

"Inhibitory Effect on the Formation of Calcium Phosphate Precipitate by the Conjugates of Ovalbumin and Phosphoryl Oligosaccharides"

J. Appl. Glycosci., **44**,(1997). (in press)