

Preparation and Solubility of Phosphorylated β -Cyclodextrins¹

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ABSTRACT

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β -Cyclodextrin (CD) was phosphorylated with phosphoryl chloride in aqueous alkaline media at different temperatures and pH values. The phosphorylated cyclodextrin (PCD) were characterized by phosphorus contents and positions of substitution as determined by ³¹P-NMR spectroscopy. Reaction of CD with equimolar POCl₃ for 3 hr at pH 12 and 45°C yielded in a PCD with a phosphorus content of 5.67%. The ratio of mono- and diphosphate esters increased when the reaction temperature was raised from 25 to 60°C. The monoesterified phosphate groups were mainly

located at C-6 of the anhydroglucose units when the reaction pH was 11 or 12. Reactions at pH 10, however, led to a higher degree of substitution at C-2 than at C-6. Phosphorylation enhanced the water solubility of CD. Solubility of a PCD (5.65% phosphorus) was 35% at pH 8 and 25°C. Simultaneously, solubility of the PCD in 25% ethanol in water was much greater than unsubstituted CD (22.3 vs. 2.8%). The PCD enhanced the water solubility of nonpolar compounds, such as β -carotene.

Cyclodextrin (CD) has a truncated corn shape, and consists of 6, 7, or more α -1,4-linked glucopyranose units in cyclic fashion. Due to the nonpolar cavity of the CD molecule and its polar exterior surface, it can form inclusion complexes with a number of nonpolar compounds (Bender and Komiyama 1978, Szejtli 1988). Cyclodextrin can be used in a wide range of applications such as removal of undesirable compounds from foods and beverages (Shaw and Wilson 1985, Yu 1988, Wilson et al 1989, Su and Yang 1991, Smith et al 1995, Yen and Tsai 1995), inhibition of enzymatic browning and oxidation (Glenside et al 1990), microencapsulation of food flavors such as garlic oil (Song et al 1993), and aqueous solubilization of insoluble compounds (Hara and Hashimoto 1986).

Among the several CD types, β -CD composed of seven anhydroglucose units, is most available because of its high production yield. Low solubility of β -CD in water, however, is the major hurdle in its application. Various approaches have been used to increase the solubility of β -CD, including addition of urea (Pharr et al 1989) or metal salts (Buvári and Barcza 1989), and structural modifications. Methylated or carboxymethylated CD was reported to have enhanced water solubility (Szejtli 1984, 1988). So also does adding α -D-glucosyl or maltosyl side units at C-6 (Abe et al 1984, 1986).

Phosphorylation increased paste viscosity and clarity of wheat starch because the phosphate groups made the starch ionic (Lim and Seib 1993a). More recently, phosphorylated CD (PCD) was reported to be suitable for the use as a chiral separating agent in capillary electrophoresis (Szejtli 1995). But little is known about the phosphorylation reaction of CD and the physicochemical properties of the PCD.

In this study, β -CD was phosphorylated with POCl₃ in aqueous alkaline media in various reaction conditions. The reaction yield and phosphate ester locations were analyzed by using ³¹P-NMR spectroscopy. Solubility of the PCD was examined in water and several polar organic solvents.

MATERIALS AND METHODS

Crystalline β -CD was provided by Daesang Co., Ltd. (Seoul, Korea). Phosphorus oxychloride (POCl₃) and β -carotene were purchased from Hayashi Pure Chemicals (Osaka, Japan) and Sigma Chemical Co. (St. Louis, MO), respectively.

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Phosphorylation of β -CD

β -CD (40 g) was dispersed in water (40 mL), and adjusted to pH 12 by adding 20% aqueous NaOH solution. The solution was heated to 45°C and POCl₃ (5.4 g) was added to the solution dropwise for 20 min with vigorous stirring. Solution pH 12 was kept by adding the NaOH solution during the POCl₃ addition, and stirring was continued for 3 hr at 45°C. The reaction mixture was cooled to room temperature, and adjusted to pH 8.0 with 2N HCl. The solution was dialyzed (MW cut-off 1,000) for 30 hr at room temperature to remove inorganic salts, and then concentrated to a syrup by rotary vacuum evaporation. Absolute ethanol (200 mL) was slowly added to the syrup with vigorous stirring, and then kept at 4°C overnight. The amorphous precipitate was collected by vacuum filtration through a glass filter (0.45 μ m) and dried at 40°C overnight in a convection oven.

Alternatively, reaction pH and temperature were changed from 10 to 12, and from 25 to 60°C, respectively. Molar ratio of POCl₃ to β -CD in the reaction mixture was also changed from 0.1 to 1.5.

³¹P-NMR Spectroscopy

Phosphorylated β -CD (PCD, 30 mg) was dissolved in 0.02% aqueous sodium azide solution (1 mL), and 0.05% sodium pyrophosphate solution (100 μ L) was added to the solution as an internal reference. Ethylenediamine tetraacetic acid (EDTA, 4 mg) was added to the solution to improve resolution. After the EDTA was completely dissolved, the solution was adjusted to pH 8.0-8.5 by adding NaOH, and deuterium oxide (0.5 mL) was added for field-frequency lock. ³¹P-NMR spectra were obtained using an AMX-500 NMR spectrometer (Bruker Instruments, Germany) at 202 MHz. Flip angle and sweep width were 65° and 20,000 Hz, respectively. During the data acquisition, proton resonance was broad-band decoupled. All chemical shifts were recorded in ppm from an 85% H₃PO₄ external reference (0 ppm).

Total phosphorus content and the ratio of mono- and diphosphate esters were measured based on the peak area on the spectra by referring to the internal reference of sodium pyrophosphate (Lim et al 1994). Degree of substitution (DS) of the monoesterified phosphate groups was calculated from this equation (Paschall 1964):

$$DS \text{ (disodium salt)} = [162 \times \text{monoester P}(\%)] / [3100 - 124 \times \text{monoester P}(\%)]$$

Solubility of CD

PCD (1-2 g) was excessively added in water or various organic solvents (2 mL) to saturation, and the dispersion was stirred for 20 min. Organic solvents tested included ethanol, methanol, acetone, and acetonitrile. Aqueous alcohol solutions (25, 50, and 75%, v/v) were also tested. The saturated PCD dispersion was filtered through a glass filter (0.45 μ m) and then the total carbohydrate in the filtrate was measured using the phenol-sulfate method (Dubois et al 1956).

Solubility of PCD was determined in a wide range of temperature (15–75°C) and pH (4–12). The pH was adjusted NaOH or HCl solution. Effect of calcium ion in the solution (0.5% CaCl₂, w/v) on the water solubility of PCD at room temperature was also analyzed.

Inclusion Complex of PCD with β -Carotene

Solid β -carotene was slowly added with stirring to an aqueous solution (1 mL) of β -CD (5, 10, or 18 mg) or PCD (5–200 mg) at room temperature until excess solid β -carotene was observed for 5 min. The solution was centrifuged at 3,000 rpm for 30 min, and sediment was discarded. Acetone (10 mL) was added to supernatant, and the mixture was stirred for 30 min to extract the complexed carotene. The precipitated CD or PCD was removed by centrifugation at 3,000 rpm for 30 min, and the β -carotene content in the supernatant was determined by measuring the absorbance of the solution at 436 nm (AOAC 1995). Pure β -carotene solutions in acetone at different concentrations were used as the standards for the analysis.

RESULTS AND DISCUSSION

Characterization of ³¹P-NMR Spectrum

As Lim and Seib (1993b) reported ³¹P-NMR spectra of the phosphorylated wheat starches, phosphodiester signals (cross-linking) of PCD were located in a chemical shift range from -1.0 to 1.0 ppm (Fig. 1). Phosphomonoester signals (monosubstitution) appeared in a range from 4.0 to 6.0 ppm, depending on the esterification location in the anhydroglucose units. In the PCD spectrum, diester phosphorus signals were divided into two major groups at approximately -1 and 1 ppm, in accordance with literature (Lim and Seib 1993b). Perhaps one set results from C-6 to C-6 diesters, and the other from C-6 to C-3 and C-2 diesters. More research is needed to understand clearly how these signals are different.

Three intense signals in the monoester range represented the three different locations of monoestered phosphorus at an anhydroglucose units: C-2 (δ 4.3), C-3 (δ 4.7), and C-6 (δ 5.7) (Lim and Seib 1993b, Lim et al 1994). Sodium pyrophosphate used as an internal standard showed an intense phosphorus signal at -6.6 ppm, and residual orthophosphate salt appeared at \approx 3 ppm as reported by Lim et al (1994).

Phosphorylation in Different Reaction Conditions

Total phosphorus content in the PCD prepared at pH 12 varied from 1.20 to 5.65% by reaction temperature (25, 45 and 60°C). Reaction yield based on the total phosphorus content was highest at 45°C (Table I, Fig. 2), and increased gradually with reaction time (Table I). The recovered amount of PCD at the maximum phosphorylation (45°C, pH 12, 3 hr) was approximately equal to that of

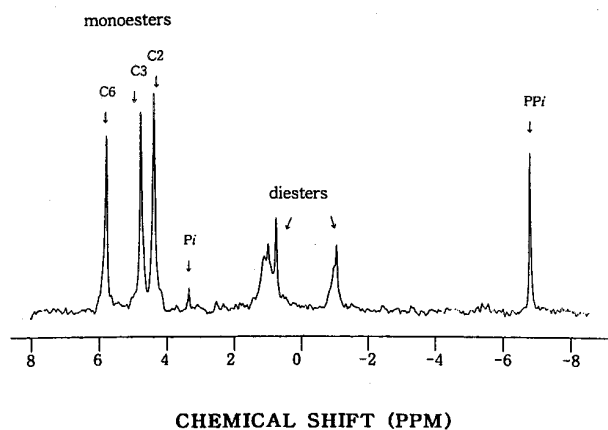


Fig. 1. ¹H-decoupled ³¹P nuclear magnetic resonance spectrum of phosphorylated β -cyclodextrin prepared at 60°C and pH 10. Pi and PPi indicate residual inorganic orthophosphate and pyrophosphate, respectively. C2, C3, and C6 indicate locations of phosphate monoesters.

the initial amount of CD, indicating that the majority of PCD was recovered from the reaction mixture. Under other reaction conditions with lower phosphorylation yield, PCD recovery was also slightly lower (data not shown).

CD and water competed for the reaction with the phosphorylation reagent (POCl₃). When the reaction temperature was 60°C, POCl₃ might have reacted more preferentially with water than when the temperature was lower, resulting in lower reaction yield for CD phosphorylation. At 60°C, however, monoesterification occurred at threefold greater yield than diesterification (Table I) whereas at 25°C, diester formation was more prevalent than monoesterification (0.8:1 P ratio). Under the reaction conditions for the maximum reaction yield (pH 12, 45°C), mono- and diesterifications occurred at similar rates (1.2:1.0).

Phosphorylation of β -CD with POCl₃ was catalyzed by alkali. At 45°C, total phosphorus content increased dramatically from 0.14 to 5.65% as the reaction pH increased from 10 to 12 (Table I). The ratio of mono- and diesterification was also changed with reaction pH and reaction period. Based on the results from the NMR spectra, at a low pH and early stage of the reaction, diester formation appeared more favored, but as the pH and reaction period increased monoester formation overpassed (Figs. 3 and 4). Therefore, for the preparation of a PCD with high level of monoestered phosphates, high reaction temperature and pH would be advisable when POCl₃ is used as phosphorylating agent.

TABLE I
Total Phosphorus (P) Content and Phosphorus Ratio of Monoesters and Diesters (Mono:Di) in Phosphorylated β -Cyclodextrins Prepared with POCl₃ (1:1 molar ratio) Under Various Reaction Conditions

Reaction			Total P Content (%)	Mono:Di	DS ^a
°C	pH	Time (hr)			
45	12	3	5.65	1.18:1	0.188
45	12	2	3.38	0.81:1	0.086
45	12	1	2.92	0.45:1	0.050
45	11	3	1.68	0.74:1	0.039
45	10	3	0.14	2.29:1	0.005
60	12	3	1.20	3.00:1	0.049
60	11	3	0.47	1.80:1	0.016
25	12	3	1.22	0.78:1	0.029
25	11	3	0.74	0.42:1	0.012

^a Degree of substitution of monoesters.

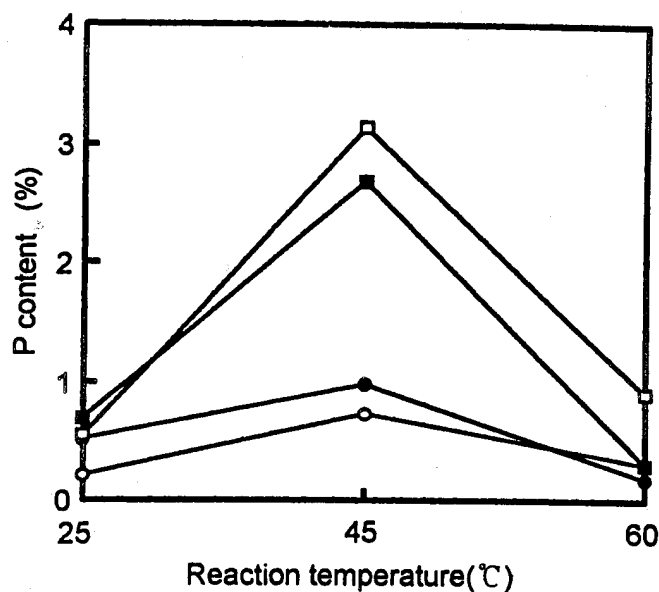


Fig. 2. Phosphorus content of monoesters and diesters in phosphorylated cyclodextrin reacted for 3 hr at different reaction temperatures and pH values. Monoesters at pH 11 and 12 (○, □, respectively); diesters at pH 11 and 12 (●, ■, respectively)

Location of Phosphate Monoesters

The reaction temperature and pH significantly changed the location of the monoestered phosphate groups. At 45°C and pH 12 where the degree of phosphorylation was maximum, monoesterification occurred more favorably on the primary hydroxyl group (C-6) than the secondary hydroxyl groups (C-2 and C-3) (Fig. 5). At pH 10, 11, and 12, the phosphorus ratios of monoesters on C-6, C-3, and C-2 were 0.5:0.7:1.0, 2.6:1.8:1.0, and 5.6:4.1:1.0, respectively. The C-2 hydroxyl groups became more reactive than the C-3 and C-6 when the reaction pH decreased to 10, although the phosphorylation yield was low.

Water Solubility

Water solubility of β -CD at room temperature (25°C) was 2.4%, which was slightly higher than the value (1.8%) reported by Chatjigakis et al (1992). Upon phosphorylation of β -CD, water solubility was significantly increased (Table II). The PCD reacted at 45°C and pH 10, 11, and 12 displayed solubilities of 5.3, 13.3, and 34.6%,

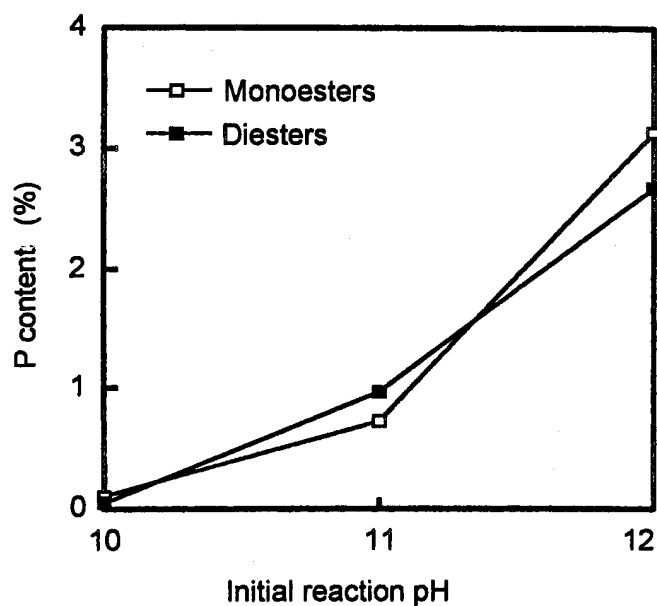


Fig. 3. Phosphorus content of monoesters and diesters in phosphorylated cyclodextrin reacted for 3 hr at different initial reaction pH values at 45°C.

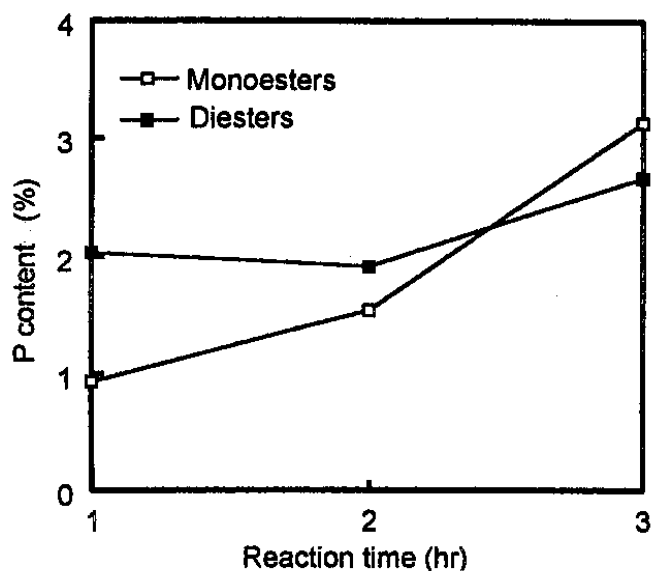


Fig. 4. Phosphorus content of monoesters and diesters in phosphorylated cyclodextrin at different reaction periods at 45°C and pH 12.

respectively. The saturated PCD solutions used for the solubility test had a range of pH 8.0–8.5. The increased solubility was attributed to the high ionic strength of the phosphate groups. When the reaction pH was 12 and time was 3 hr, respectively, the total phosphorus content in the PCD was slightly higher at the reaction temperature of 25°C than at 60°C (Table I). But the water solubility of the PCD appeared opposite (Table II). This was because the monoester phosphate content was greater at 60°C than at 25°C. The monoesterified phosphate groups provided the hydrophilic properties to the PCD, whereas diesterified groups could produce the opposite effect, because of the molecular weight increase.

Water solubility of PCD increased as temperature of the solution increased (15–75°C). The PCD prepared at 45°C and pH 12 showed 52.1% solubility at 75°C (Fig. 6). Unmodified CD also showed solubility increases with the solution temperature, but the difference in the solubility between CD and the PCD remained significant in the temperature range.

Water solubility of PCD increased significantly as the solution pH increased (Fig. 7), but the unmodified CD exhibited only a minor increase in solubility with pH increase. Deprotonation of the phosphate groups was facilitated by alkali, and it increased the ionic properties as well as water solubility of CD. But at a low pH such as 4.0 or 6.0, the PCD solubility dropped below a value of 15% because the phosphates had increased level of protonation (Fig. 7). Therefore, the PCD may not be as good a solubility enhancer in

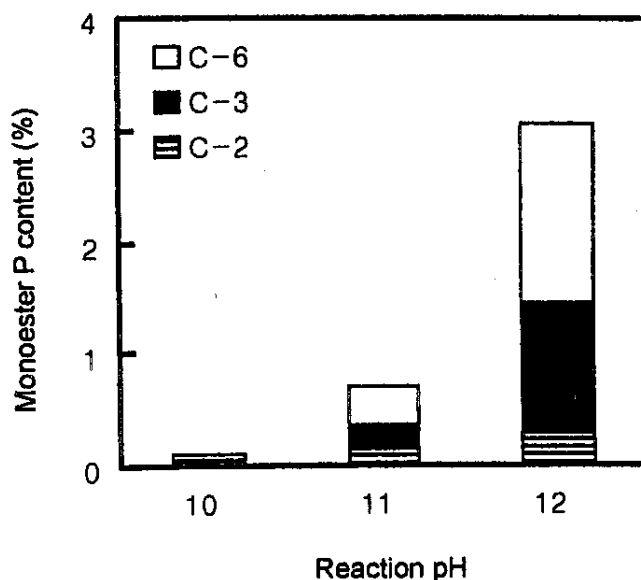


Fig. 5. Location of phosphate monoesters in phosphorylated cyclodextrin at different reaction pH values at 45°C.

TABLE II
Water Solubility of Phosphorylated β -Cyclodextrins Prepared Under Various Reaction Conditions with Equimolar Amounts of POCl_3

°C	pH	Time (hr)	Solubility ^a
25	12	3	15.88
	11	3	13.81
	10	3	13.48
45	9	3	5.39
	12	3	34.60
	12	2	32.26
60	12	1	28.32
	11	3	13.25
	10	3	5.31
	12	3	25.61
	11	3	18.60
	10	3	7.82
β -cyclodextrins			2.12

^a % (w/v) at 25°C.

acidic media as are the glycosyl modifiers (Abe et al 1984, 1986). Similar results were reported by Lim and Seib (1993a) with low DS wheat starch phosphates.

Solubility in Organic Solvents

PCD was insoluble in a pure solvent of alcohol, acetonitrile, or acetone (Table III) as was the unmodified CD. In aqueous ethanol, as the alcohol concentration was reduced, the solubility of PCD increased dramatically, whereas the solubility increase of CD was minor (Fig. 8). Consequently, in 50 and 25% ethanol solutions, the PCD was soluble at 12 and 22%, respectively, whereas β -CD showed only 2.3 and 2.8% solubilities, respectively. The PCD solubility was slightly reduced when methanol was used (Fig. 8). The high solubility of the PCD in alcohol solutions could be useful when applied in alcoholic media such as liquors and perfumes.

Solubility of PCD in Calcium Solution

The solubility of PCD was reduced by the presence of calcium ion in aqueous solution (Table IV). In 5% solutions of most of the

PCD products, calcium chloride addition (0.5%) resulted in precipitation of the PCD. Only the PCD prepared by the reaction at 45°C, pH 12 for 3 hr remained fully soluble. But the PCD reacted for 1 or 2 hr in the same conditions showed solubility decreases from 5 to 4.7% and 3.6%, respectively (Table IV). The ratio of mono- and diesters of phosphate groups had little influence on the solubility changes by calcium. The calcium effect on the solubility was not observed with unmodified β -CD. The divalent calcium ions could form salt bridges between two phosphate groups, resulting in similar effects of cross-linking. Therefore, the reduced mobility of PCD due to the intermolecular associations caused the decrease of water solubility. The same effects by calcium addition were also observed with phosphorylated wheat starches (Lim and Seib 1993a).

Inclusion Complex with β -Carotene

As the concentrations of unmodified β -CD increased from 5 to 18 mg/mL (maximum solubility), the β -carotene solubility in water also increased from 0.01 to 0.23 mg/mL. In the same concentration range, the PCD prepared at pH 12 and 45°C increased the carotene solubility at a similar rate. Furthermore, the solubility of

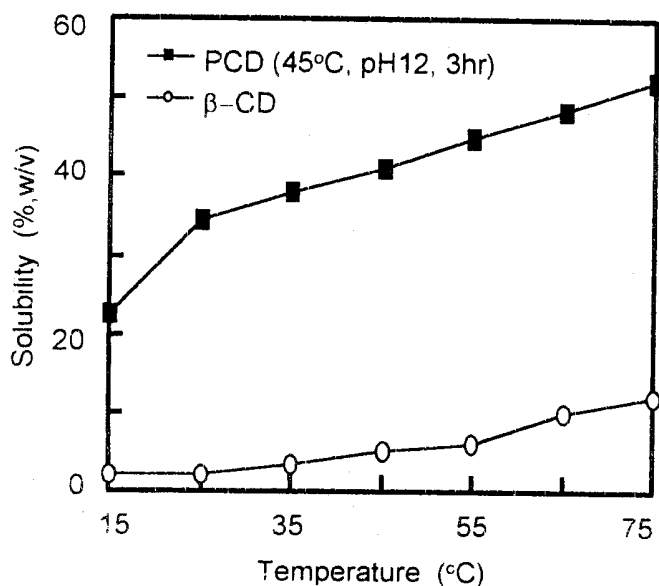


Fig. 6. Water solubility of unmodified and phosphorylated β -cyclodextrins at various temperatures.

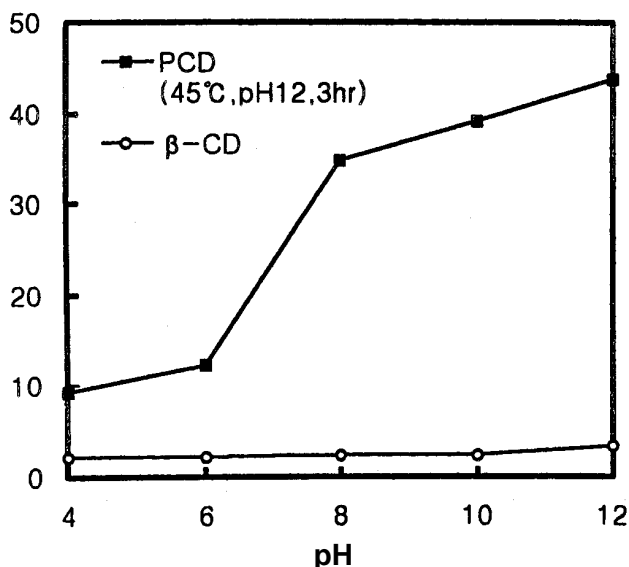


Fig. 7. Solubility of β -cyclodextrin and phosphorylated cyclodextrin at various pH values at 25°C.

TABLE III
Solubility of Phosphorylated β -Cyclodextrins (PCD) and β -Cyclodextrins (β -CD) in Organic Solvents^a

	EtOH	MeOH	Acetonitrile	Acetone
PCD ^b	0.00	0.03	0.00	0.05
β -CD	0.00	0.03	0.00	0.02

^a % (w/v) at 25°C.

^b Prepared at 45°C, pH 12, by 3 hr of reaction.

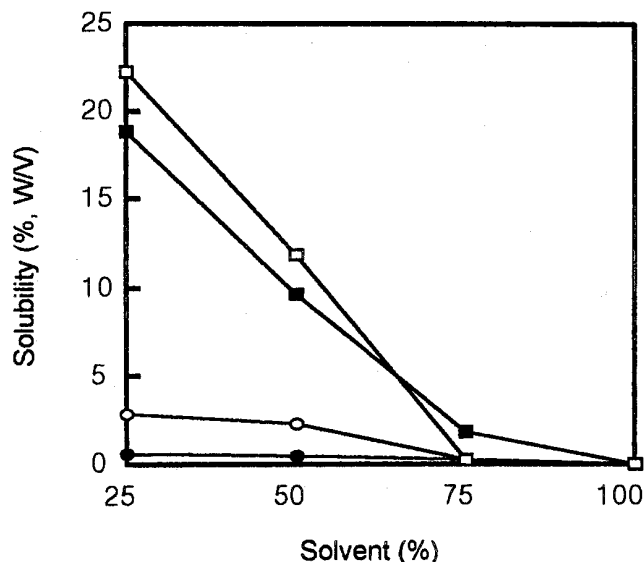


Fig. 8. Solubility of β -cyclodextrin (β -CD) and phosphorylated cyclodextrin (PCD) in aqueous alcohols at 25°C. β -CD in MeOH (\bullet); PCD (45°C, pH 12, 3hr) in MeOH (\blacksquare); β -CD in EtOH (\circ); PCD (45°C, pH 12, 3hr) in EtOH (\square).

TABLE IV
Effect of Calcium Addition (0.5% CaCl_2) on Water Solubility of Phosphorylated β -Cyclodextrins

°C	pH	Time (hr)	Total P(%)	Mono:Di ^a	Solubility (%) ^b	
					No Ca	With Ca
45	12	1	2.92	0.45:1	5.0	3.6
45	12	2	3.38	0.81:1	5.0	4.7
45	12	3	5.65	1.18:1	5.0	5.0
60	12	3	1.20	3.00:1	5.0	4.1

^a % (w/v) at 25°C.

^b Ratio of mono- and diphosphate esters.

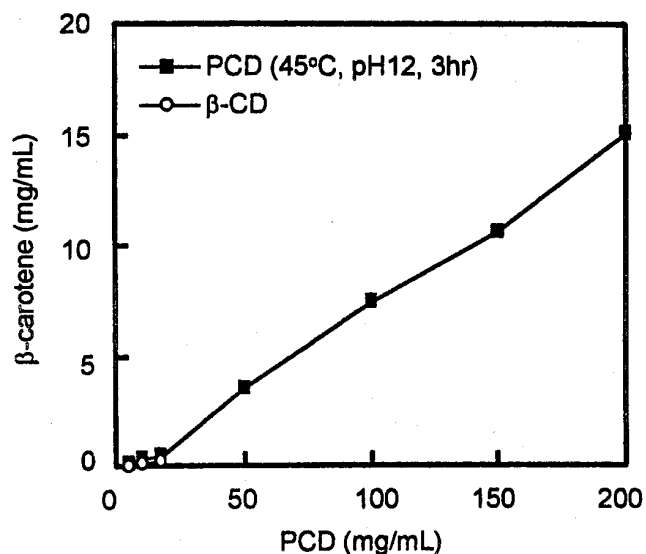


Fig. 9. Solubility of β -carotene in PCD solutions at 25°C.

the PCD as high as 200 mg/mL permitted the continuous increase of the dissolved amount of β -carotene in water (Fig. 9).

The slope in Fig. 9 indicates the binding ability of β -carotene to the PCD. At 100 mg/mL of PCD, β -carotene solubility reached 7.4 mg/mL. Supposing that each PCD molecule had one monoestered phosphate group, the calculated molecular weight of PCD (disodium salt) would be 1,259. Based on the molecular weight and the ratio of PCD and β -carotene (100 vs. 7.4 mg) in the solution, the molar ratio of PCD to β -carotene was 6:1, which was a much higher ratio when compared to the values for other guest compounds in the literature (Smith et al 1995, Yen and Tsai 1995). Perhaps β -carotene could form a complex with many PCD molecules along the axis of the linear hydrocarbon chain. More research is needed for the application of PCD.

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