PREPARATION OF CYCLOSOPHORAOSE-A AND ITS COMPLEX-FORMING ABILITY

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ABSTRACT. Cyclosophoraoses (CySs) are unbranched cyclic (1→2)-β-D-glucans produced by many strains of *Agrobacterium* and *Rhizobium*. Pure CyS-A, the group member having the smallest molecular size (degree of polymerization 17), was efficiently prepared by liquid chromatography using charcoal and ODS columns from the culture fluid of the mutant strain RA-12 from *R. phaseoli* AHU 1133. The complex-forming ability of CyS-A was estimated from its enhancement of the solubilities of slightly soluble guest molecules in water using methods [I], [II], and [III]. In [I], an aqueous solution of CyS-A was shaken with the guest molecule, while, in [II], it was shaken with an acetone solution of the guest compound. In method [III], freeze-dried CyS-A powder was stirred with an acetone solution of the guest compound. The CyS-A cavity is thought to be able to accommodate three-dimensionally extended guest molecules, *e.g.*, indomethacin. Method [II] was the best for obtaining CyS-A inclusion complexes, while method [III] would be recommended if the guest molecule is labile in the presence of water. Crystalline CyS-A inclusion complexes have not been obtained, but CyS-A complexes are expected to greatly enhance the solubilities of slightly soluble or insoluble guest molecules in water, because CyS-A is much more soluble than β-cyclodextrin. Method [II] or [III] may afford a useful means of obtaining oily drug, *e.g.*, vitamin E and K_1, in an amorphous state.

INTRODUCTION

Many strains of *Agrobacterium* and *Rhizobium* both intracellularly[1,2] and extracellularly[3-6] produce unique polysaccharides, cyclic (1→2)-β-D-glucans, which have been shown to be unbranched, cyclic molecules varying in size from 17 to at least 24 residues. Koizumi *et al.*[7] isolated eight pure cyclic (1→2)-β-D-glucans (cyclosophoraose (CyS) A-H) produced by *A. radiobacter* IFO 12664 and determined the degree of polymerization (DP) of each CyS by high-performance liquid chromatography (HPLC) of its partial hydrolyzate. To obtain a mutant strain having CySs hyperproductivity but not producing acidic polysaccharide, Higashiura *et al.*[8] attempted mutagenation of *R. phaseoli* AHU 1133 which predominantly

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produces CyS-A[9,10]. The isolated mutant strain RA-12 was found to produce 4.6-fold more CySs than the parent strain and no acidic polysaccharide. Liquid chromatography on a charcoal column instead of the conventional method involving repeated precipitation with ethanol was used to prepare CySs from the culture fluid.

The present work was conducted to find whether this new cyclic glucan can function as a host of an inclusion complex. We first developed an efficient method for isolating CyS-A, the member of the CyS group having the smallest molecular size (DP 17), from the culture fluid of *R. phaseoli* RA-12, and then studied its complex-forming ability with various drugs.

MATERIALS AND METHODS

Materials

Propericiazine, mp 116-117°C, and nitrazepam, mp 227-228°C (dec.), were supplied by Shionogi & Co., Ltd., (Osaka, Japan). Reserpine, 99%, mp 250°C (dec.) was from Aldrich (Milwaukee MI, U.S.A.). The other drugs used were of the reagent grade: indomethacin, mp 155-161°C; fluorescein, mp 314-316°C; ergosterol, mp 168°C; vitamin D_3, mp 84-87°C; vitamin K_3, mp 105-107°C; vitamin E and K_1, both oils. β-Cyclodextrin was used after recrystallization from water, $[\alpha]_D^{18}$ +165.5°. All other materials were of analytical grade. Water used in the solubility studies was deionized and distilled. Eluents for HPLC were filtered through a 0.45-μm membrane filter and degassed.

Microorganism and Medium

Rhizobium phaseoli RA-12[8] and the medium described by Amemura *et al.*[4] were used to produce CySs.

General Methods

Melting points were measured with a micro melting point apparatus (Yanagimoto, Kyoto, Japan) and are uncorrected. The pH measurements were carried out on a pH meter M-8 (Horiba, Kyoto, Japan). Optical rotations were determined with a JASCO DIP-4 digital polarimeter (JASCO, Tokyo, Japan). Lyophilization was carried out with a freeze dryer FD-1 (Tokyo Rika, Tokyo, Japan). A UVIDEC-610C double beam spectrophotometer (JASCO) was used for the determination of absorbances. HPLC analyses were performed using a Twincle pump, a VL-611 variable-loop injector, and a variable-wavelength ultraviolet (UV) detector UVIDEC-100 III (all from JASCO) and a refractive index (RI) detector SE-31 (Showa Denko, Tokyo, Japan). The columns used were a μBondapak C_{18} (300×3.9 mm i.d.) (Waters Assoc., Milford, MA, U.S.A.), a Finepak SIL C_{18} (250×4.6 mm i.d.)(JASCO), a YMC-Pack A-212(C_8)(150×6 mm i.d.) and a YMC-Pack AL-312(C_{18})(150×6 mm i.d.)(both from Yamamura Chemical, Kyoto, Japan). Preparative chromatography was carried out using a mini micro pump KHD-W-104 (Kyowa Seimitsu, Tokyo, Japan) with a Waters RI R-403 detector. A column packed with

LiChroprep RP-18 (25-40 μm, 300×30 mm i.d., Merck) was used.

Production and Isolation of CyS-A

The medium (30 L) in a 50-L jar fermentor was inoculated with 1.5 L of preculture. The culture was shaken at 300 rpm for 3 days at 30°C on a rotary shaker. CyS-A was isolated from the culture fluid as shown in Chart 1.

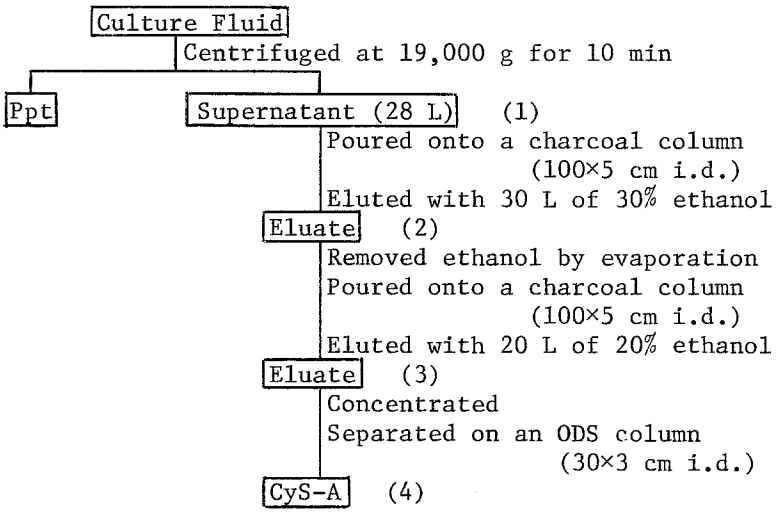

Chart 1. Isolation of CyS-A from the culture fluid of *Rhizobium phaseoli* RA-12.

The yields of CySs in the four steps were: (1) 31.6 g, (2) 29.1 g, (3) 23.1 g, (4) 12.7 g.

Solubility Studies

CyS-A was brought into contact with the drugs shown in TABLE II by methods [I], [II], and [III].

Method [I]. Pure CyS-A was dissolved in water to obtain 2.5×10^{-3}-8×10^{-2} M solutions. To each aqueous solution in a vial was added a large excess of the drug to be tested. The vials were tightly sealed and vigorously shaken on a shaker for 24 h at 30°C to allow equilibration to be attained. The unstable vitamin D_3, E, and K_1 were treated at 20°C for 72 h in the dark. After equilibration, the aqueous solution was filtered through a 0.2-μm membrane filter.

Method [II]. Instead of using the solid drug, 1 mL of 1×10^{-2} M drug solution in acetone was shaken with 1 mL of CyS-A aqueous solution. After equilibration, the mixture was evaporated, lyophilized, and dissolved in 1 mL of water, and then the insoluble drug was removed by filtration with a 0.2-μm membrane filter.

Method [III]. One mL of CyS-A aqueous solution, concentration of which varies from 2.5×10^{-3} M to 1×10^{-2} M, was lyophilized in a flask. Next, 2×10^{-3} M of the drug in 5 mL of acetone was added and the mixture in the stoppered flask was stirred for 24 h in an incubator SHR-200 M (Sanyo, Osaka, Japan) at 30°C. Vitamin D_3, E, and K_1 were treated at 20°C. After equilibration, the mixture was filtrated to remove the drug remaining in the acetone. The residue was dissolved in water and lyophilized. The resulting powder was dissolved in 1 mL of water and sent through a 0.2-μm membrane filter.

Determination of the Amount of Drug in the Aqueous Solution. The amount of the drug in the filtrate obtained by method [I], [II] or [III] was determined by UV spectrophotometry or HPLC. CyS-A has a λmax at about 250 nm and affects drug determination by spectrophotometry up to 300 nm. Fluorescein was assayed at 491 nm after ionization by dilution of the filtrate with a borate buffer (pH 9.0). Indomethacin could be assayed at 320 nm after dilution with water and HPLC was also applicable. The other drugs were analyzed by HPLC under the conditions shown in TABLE I.

TABLE I CONDITIONS OF DRUG DETERMINATION BY HPLC

Drug	Column	Eluent	Flow Rate (mL/min)	Detection at (nm)	Retention Time(min)
Indomethacin	Finepak SIL C_{18}	CH_3OH-0.05%CH_3COOH (70:30)	1.0	320	10.0
Ergosterol	Finepak SIL C_{18}	C_2H_5OH-H_2O (90:10)	1.0	265	11.0
Vitamin D_3	Finepak SIL C_{18}	CH_3OH-H_2O (90:10)	1.5	265	12.3
Vitamin E	Finepak SIL C_{18}	CH_3OH-H_2O (95:5)	1.0	292	10.5
Vitamin K_1	Finepak SIL C_{18}	CH_3OH-H_2O (95:5)	1.5	249	11.0
Vitamin K_3	Finepak SIL C_{18}	CH_3OH-H_2O (55:45)	1.0	250	13.0
Nitrazepam	Finepak SIL C_{18}	CH_3OH-H_2O (55:45)	1.0	310	10.5
Propericiazine	YMC-Pack A-212(C_8)	C_2H_5OH-H_2O (83:17)	1.0	272	11.5
Reserpine	YMC-Pack A-212(C_8)	C_2H_5OH-H_2O (55:45)	1.0	267	12.5

A μBondapak C_{18} column can be used instead of a Finepak SIL C_{18} column. HPLC analyses of CyS-A and acetone were carried out with a YMC-Pack AL-312 (C_{18}) column/methanol-water (2:98) system, with an RI detector for CyS-A and a UV detector set at 265 nm for acetone.

RESULTS AND DISCUSSION

Preparation of CyS-A

CySs was separated from the culture fluid of *R. phaseoli* RA-12 according to the method of Higashiura *et al.*[8]. The culture fluid was centrifuged to remove cells and other insoluble materials, the resulting supernatant was poured onto a charcoal column, and after washing the column with water, adsorbed CySs were desorbed with 30% ethanol. As this fraction still contained impurities, as shown in Figure 1-(2), a charcoal column was used again to remove them. The components in the fraction were re-adsorbed on a charcoal column and CySs were desorbed with 20% ethanol. The eluate contained mainly CyS-A together with a small amount of CyS-B and CyS-C. CyS-A was efficiently isolated from the CySs mixture by preparative HPLC on an ODS column with water. Chromatograms of components in the initial supernatant (1) and the eluates from two charcoal columns (2), (3) and one ODS column (4) are shown in Figure 1.

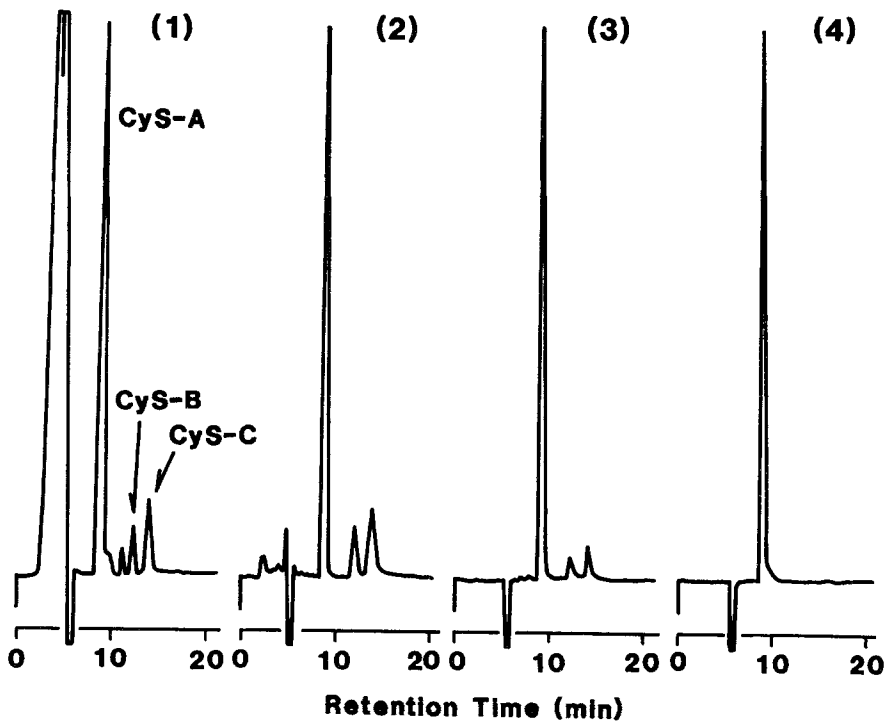

Figure 1. Chromatograms of components in four purification steps. Chromatographic conditions: column, YMC-Pack AL-312 ODS (150×6 mm i.d.); eluent, methanol-water (6:94); flow rate, 0.7 mL/min; detector, RI at 1×10^{-5} RI units full-scale; temperature, ambient.

Properties of CyS-A

CyS-A shows a specific rotation of -13.00° – -13.50° (c = 14, H_2O) and is much more soluble in water than the most soluble cyclodextrin (CyD, γ): 1 g of CyS-A dissolves in about 0.6 mL of water at 25°C. Because of increasing viscosity, a more accurate estimation of solubility was difficult. The pH of 10^{-2} M aqueous solution of CyS-A is 6.0 ± 0.3. CyS-A

has a molecular weight of 2754 (17 glucose units) with a cavity diameter of *ca.* 9 Å and depth of 15 Å (*cf.* the sizes of γ-CyD, 8.5 Å and 7 Å), according to CPK models. The molecule is shaped like a distorted ring which is flexible.

Complex-Forming Ability of CyS-A

The CyS-A complex-forming ability was estimated from the enhancement of the solubility of slightly soluble guest molecules in water by their interaction with CyS-A (TABLE II). By the usual solubility method (method [I]), CyS-A could enhance the solubilities of only three drugs, indomethacin, fluorescein, and propericiazine, within 24 h. Consequently, three-dimensionally extended guest molecules like these three compounds were assumed to fit the CyS-A cavity. CPK models suggest that the whole molecule of indomethacin can penetrate into the cavity.

TABLE II COMPLEX-FORMING ABILITY OF CyS-A WITH VARIOUS GUEST MOLECULES

	[I]	[II]	[III]
Indomethacin	+	+	+
Fluorescein	+	+	+
Propericiazine	+	+	+
Ergosterol	±	+	+
Vitamin D_3	±	+	+
Vitamin E	-	+	+
Vitamin K_1	-	+	+
Reserpine	-	+	+
Nitrazepam	-	-	+
Vitamin K_3	-	-	-

Figure 2 shows the solubility of indomethacin as a function of the CyS-A concentration and the system is compared with one containing β-CyD. The slopes of both isotherms are practically identical, and hence the stability of the CyS-A complex seems almost identical with that of the β-CyD complex, but CyS-A-induced solubilization is greater since CyS-A has a much higher solubility than β-CyD. Interaction between CyS-A and some drugs occurred very slowly. Vitamin D_3, which practically insoluble in water, had detectable solubility in water containing CyS-A after 72 h.

Method [II] is the best for obtaining CyS-A inclusion complexes. Equilibrium is attained relatively faster and the solubility of various kinds of drugs could be improved. For example, the solubility of vitamin K_1 in water is hardly measurable, but a concentration of 0.2 mg/mL was reached in water containing 7.5×10^{-3} M CyS-A (Figure 3).

Both the solubility curves obtained for the indomethacin/CyS-A system (Figure 2) and the vitamin K_1/CyS-A system (Figure 3) showed a Bs type phase diagram[11], (in the solubility diagram of the indomethacin/CyS-A system, the plateau region is expected to obtain by the use of an adequate amount of indomethacin), and precipitation of solid complex in the higher concentration range of CyS-A was observed.

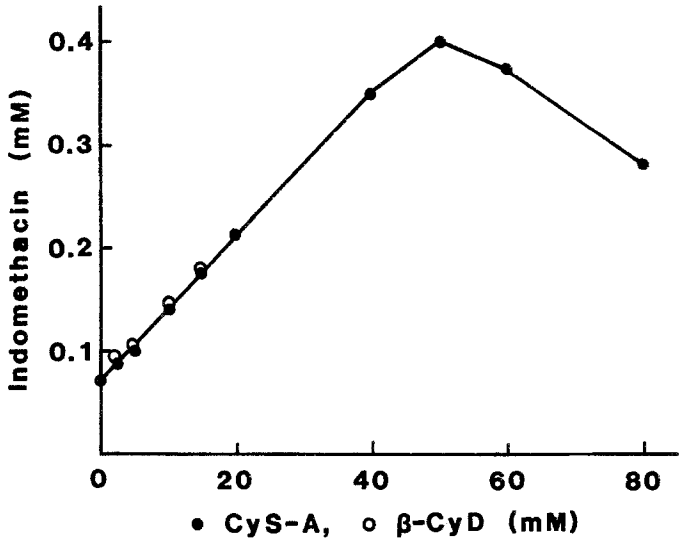

Figure 2. Solubility increase of indomethacin in cyclosophoraose-A and β-cyclodextrin solutions at 30°C (method [I]).

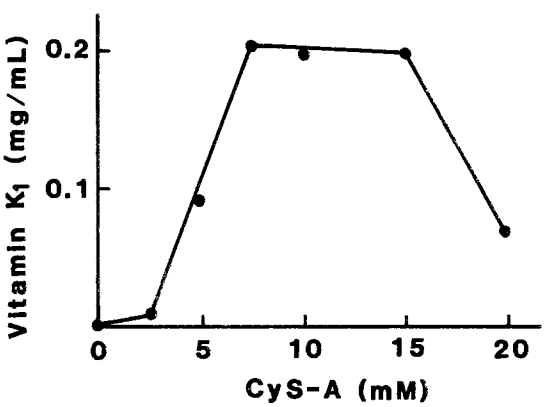

Figure 3. Solubility increase of vitamin K_1 in cyclosophoraose-A solutions at 20°C (method [II]).

Method [III] leads to interaction between CyS-A and more drugs than method [I]. In the case of vitamin D_3, the solubility in 7.5×10^{-3} M CyS-A solution increased up to 2.3×10^{-2} mg/mL after 24 h and this concentration was maintained. When method [II] was used, the solubility rose only to 1.2×10^{-2} mg/mL after 24 h and then decreased, and in method [I], vitamin D_3 was barely detected after 72 h (Figure 4). Moreover, the concentration of vitamin D_3 in aqueous solution prepared for HPLC decreased with time in method [II], but did not change for a relatively long time in method [III] (Figure 5). These phenomena seem to arise from the lability of vitamin D_3 in water and the complex formed in method [III] is thought to have had a more stable structure. Although the reason is unknown, the solubility of nitrazepam could be enhanced only by method

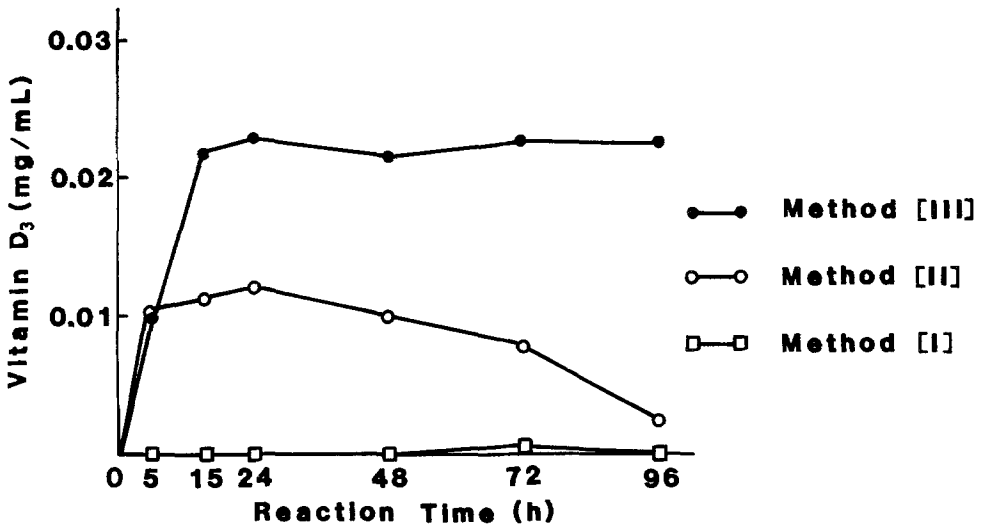

Figure 4. Solubility of vitamin D_3 in 7.5×10^{-3} M cyclosophoraose-A solution at 20°C in methods [I], [II], and [III] as a function of time.

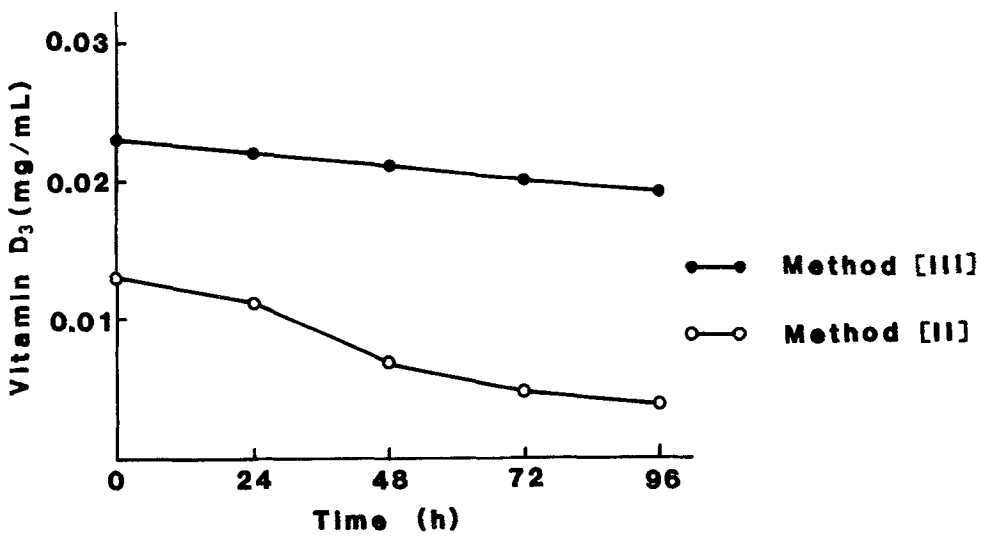

Figure 5. Change of vitamin D_3 concentration in the aqueous solution prepared by methods [II] and [III]. (reaction time:24 h)

[III].

CyS-A was expected to form a binary inclusion complex with acetone and also ternary complexes with acetone and the guest molecules examined, but no acetone peak was detected in chromatograms of lyophilized reaction mixtures. There was no acetone even in the sample prepared by stirring CyS-A with acetone or acetone solution of drugs, followed by filtration and drying in air.

In many cases examined, some precipitation of complexes was observ-

ed, but no crystalline complex was obtained.

Vitamin K_3 did not react with CyS-A in all three methods. Vitamin K_3 seems to be too small to fit the CyS-A cavity.

Our study on CyS-A complexes has just begun and thus no definite conclusions can be drawn from this study. Our work continues using different approaches.

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