Enhancement of Water Solubility of Fullerene by Cogrinding with Mixture of Cycloamyloses, Novel Cyclic α-1,4-Glucans, *via* Solid–Solid Mechanochemical Reaction

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Improvement of solubility for fullerene (C_{60}) was studied by cogrinding with cycloamyloses using a ball mill in the solid state. Cycloamylose is a novel cyclic α-1,4-glucan produced from synthetic amylose by enzymatic reaction. Although sample solutions showed a pale yellow for the initial period of cogrinding with cycloamyloses and C_{60}, the color varied to brown after 48 h. Subsequently, the solubility of C_{60} was improved markedly to 560 (μg/ml) at 96 h. From powder X-ray diffraction analysis, the peak intensity of crystalline C_{60} decreased as the cogrinding time was extended. The UV–VIS absorption spectrum of C_{60} shows absorption bands at 262 and 340 nm in water with cycloamyloses, and 258 and 328 nm in *n*-hexane. These results suggested that C_{60} molecules were dispersed into cycloamyloses micellar system and the red-shift of the UV–VIS spectra was due to an intermolecular interaction between C_{60} and cycloamyloses.

Key words cycloamylose; fullerene; solubilization; cogrinding; mechanochemical

Cycloamylose is a α-1,4 cyclic glucan produced from amylose by D-enzyme which catalyzes an intramolecular transglycosylation reaction as well as cyclodextrin glucanotransferase and amylomaltase.[1—3] So far, a number of investigations have been conducted on the improvement of physicochemical property of various drugs by complexation with cyclodextrins, a typical cyclic glucan consisting of 6, 7 and 8 glucopyranose units.[4—6] Although cycloamylose should have similar basic structure to that of cyclodextrin, the degree of polymerization of cycloamyloses is higher and ranges from 17 to several hundred. In addition, it should be noted that cycloamylose shows much higher solubility into water and less aging, that is, coprecipitation due to aggregation, than cyclodextrin, in spite of their high molecular weight.[7] However, little is known about the physicochemical interaction between cycloamyloses and chemical compounds except for the results of our primitive study.[8]

Fullerenes have attracted much attention for their unique cage-like shape and biological activities such as HIV-1 protease inhibition, photodynamic tumor necrosis and action as an artificial vector for gene transfection.[9—12] On the other hand, solubilization of fullerenes into water also has been investigated extensively, since their applicability was strictly limited due to poor solubility to polar solvent. In addition to developing chemically modified hydrophilic fullerene derivatives, fullerene (C_{60}) has been solubilized into water combined with β-cyclodextrin,[13] γ-cyclodextrin,[14,15] polyvinylpyrrolidone[16] and fluoroalkyl oligomer.[17] Complexation between C_{60} and each solubilization agent was usually performed in the aqueous solution or organic solvent *i.e*. *via* solid–liquid reaction. Braun *et al*. have reported, however, that C_{60} is concentrated considerably higher when cogrinding with crystalline γ-cyclodextrin than with reflux boiling in γ-cyclodextrin solutions.[18]

In this paper, we describe the improvement of the solubility of C_{60} by cogrinding with cycloamyloses in the solid state. The molecular state of C_{60} in the complex obtained was evaluated using powder X-ray diffraction (XRD), UV–VIS spectroscopy and dynamic light scattering measurement.

Experimental

Materials Cycloamyloses were provided by Ezaki Glico Co., Ltd. (Osaka, Japan). Fullerene (C_{60}) was obtained from Frontier Carbon Co. (Tokyo, Japan). All other chemicals and solvents were reagent grade purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and used without further purification.

High-Performance Anion-Exchange Chromatography (HPAEC) HPAEC was carried out with a Dionex DX-500 system with a pulsed amperometric detector (ED-50, Dionex Co., Sunnyvale, CA). An anion exchange column (Carbopac PA-1, 4.0 mm i.d.×250 mm, Dionex) was used with sodium nitrate and sodium hydroxide solution as the mobile phase using a gradient system according to Koizumi *et al*. with minor modification.[19]

Particle Size Analysis A powder sample was dispersed or dissolved in water and sonicated for 1 min and passed through a 0.1 μm membrane filter (Millipore) before measurement. Particle size was determined by the dynamic light scattering method using a Nicomp 380ZLS® (Particle Sizing Systems, Santa Barbara, CA, U.S.A.) at 25 °C with a DPSS laser (532 nm).

Preparation of Grinding Mixture A ground mixture (GM) of C_{60} and cycloamyloses was prepared using a Desktop Ball Mill (V-1M, Irie Shokai Co., Ltd., Japan). In a typical experiment, 2.5 mg of C_{60} and 12.5 mg of cycloamyloses was put into a 5.0 ml shade glass vial with glass balls (diameter: 5 mm) and coground at 150 rpm at room temperature.

Determination of Solubilized Fullerene C_{60} After cogrinding, 2.5 ml of distilled water was put into the glass vial and incubated with gentle shaking for 1 h at room temperature. The suspension was then centrifuged at 25 °C and 3000 rpm for 1 h and the clear supernatant solution was passed through a membrane (0.45 μm, HLC-DISK 3, Kanto Chemical Co., Inc., Japan). The solution of cycloamyloses with solubilized C_{60} was subjected to UV–VIS spectroscopy (V-550, JASCO Co., Japan) and HPLC analysis (LC-2000, JASCO Co., Japan). The HPLC conditions were as follows: mobile phase, toluene–acetonitrile mixture (45 : 55); column; Inertsil ODS-2 (4.6 mm i.d.×250 mm, GL Science Co., Inc., Japan); flow rate, 1.0 ml/min; column temperature, ambient; detector, UV at 325 nm.[20]

Powder X-Ray Diffractometry (XRD) Powder XRD was carried out with a Rigaku Geigerflex Rad-II (Tokyo, Japan). Measurements were performed at 35 kV voltage, 25 mA current and a scanning speed of 6 °/min with a Cu$K\alpha$ radiation source.

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Results and Discussion

Properties of Cycloamyloses Cycloamylose is produced from synthetic amylose by enzymatic reaction (Fig. 1). The minimum degree of polymerization (DP) of cycloamylose produced by amylomaltase was 22, whereas the minimum DP of cycloamylose produced by potato D-enzyme was 17.[2)]

Figure 2 shows the HPAEC elution profile of cycloamyloses produced by amylomaltase. Cyclic glucan with DP22 are pointed out with arrows and the number above each peak refers to its DP. It was confirmed that the DP of cycloamyloses used in this study ranged from 22 to around 60 (the corresponding molecular weights were 3567 to 9729). Each cycloamylose might be eluted sequentially in agreement with its DP. This suggested that each cycloamylose existed as a homologous structure in aqueous solution.

There have been some papers on the physicochemical property of each cycloamylose, sometimes called large-ring cyclodextrin, with DP ranging from 9 to 31. Koizumi *et al.* recently demonstrated that the chemical shifts of all glucose carbons were practically unvarying for DP9 and DP31 in ^{13}C-NMR measurements.[19)] In addition, we have reported the solubilities, specific rotations and surface tensions of large-ring cyclodextrins with DP ranging from 10 to 17. Although complex formation of cycloamylose with various compounds was expected to improve the physicochemical property of those guest compounds, no surface activity was observed in each large-ring cyclodextrin, and it was still unknown whether larger ring cyclodextrin had surface activity.[21)] In this paper, we evaluated the physicochemical property of cycloamyloses and its solubilizing ability as a mixture.

The particle size distribution of cycloamyloses solution was measured by the dynamic light scattering method. The mean particle size was 3.5 nm as shown in Fig. 3, when measuring the 2.0% cycloamyloses solution. According to Saenger's research, the diameter of cycloamylose with DP26 was estimated as approximately 20 Å by calculation from crystallographic data.[22,23)] Hence, it was suggested that this measurement was performed reasonably and cycloamyloses were presented as a molecular colloid in aqueous solution. The mean particle size and distribution of cycloamyloses did not change for one week, so the colloidal solution of cycloamyloses would be stable at least for a week at ambient temperature. This indicated that a reduction in mean particle size due to chemical degradation did not occur, and neither did an increase in particle size by aggregation of cycloamyloses.

Solubilization of C_{60} by Cogrinding The solubilization of C_{60} was studied by cogrinding with cycloamyloses using a ball mill *via* solid–solid reaction. Figure 4 shows the correlation between cogrinding time and the concentration of solubilized C_{60}. Although sample solutions appeared pale yellow for the initial period of cogrinding with cycloamyloses and C_{60}, the color varied to brown after 48 h. Subsequently, the solubility of C_{60} was improved markedly to 560 (μg/ml) at 96 h, while the concentration of cycloamyloses was 5.0 (mg/ml). In the preliminary study using an agate mortar and pestle, C_{60} was solubilized to 30 (μg/ml) maximally in optimum conditions. Thus, ball milling may be a suitable grinding method for the solubilization of C_{60} by cogrinding with cycloamyloses. After that, the C_{60} solubilized with cycloamyloses was stable for one month at room temperature.

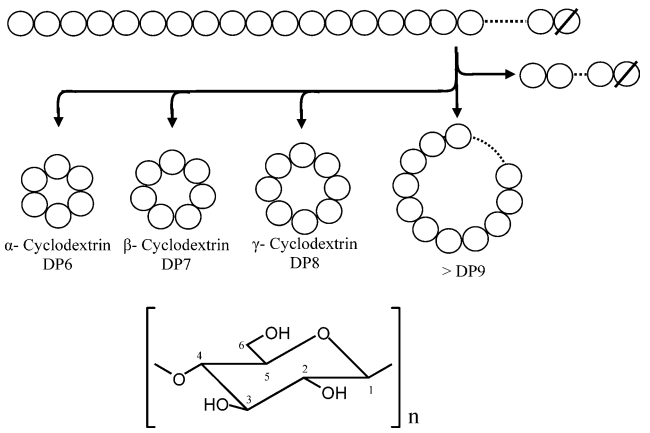

Fig. 1. Schematic Diagram of the Cyclization Reaction of Amylomaltase with Amylose

○: Glucosyl residue (chemical structure also indicated), ∅: glucosyl residue with a reduction end.[3)]

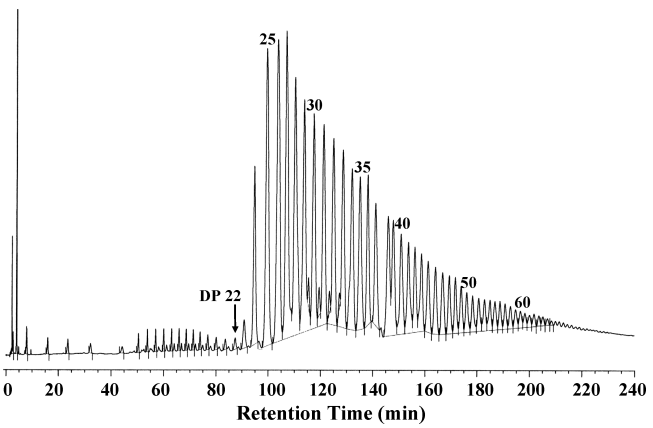

Fig. 2. HPAEC Elution Profile of Cycloamyloses Produced by the Action of Amylomaltase on Synthetic Amylose

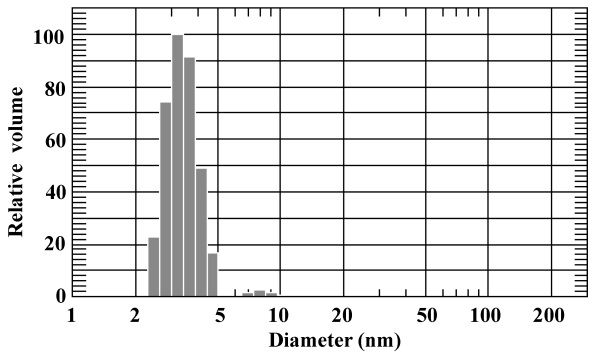

Fig. 3. Particle Size Distribution of Cycloamyloses Measured by Using the Dynamic Light Scattering Method

The weight ratio of ground mixture strongly affected the solubilization of C_{60} (Fig. 5). The solubilized amount of C_{60} was significantly reduced as the content of cycloamyloses decreased in the weight ratio, varying from 5 : 1 to 1 : 1 (cycloamyloses : C_{60}). Mechanochemical reaction might be unreached to the end-point in the 2 : 1 and 1 : 1 system at this period. In the 5 : 1 cycloamyloses–C_{60} system, 5.0 mg cycloamyloses were allowed to solubilize 0.56 mg C_{60} in 1 ml water approximately, thereby a 10-fold amount of cycloamy-

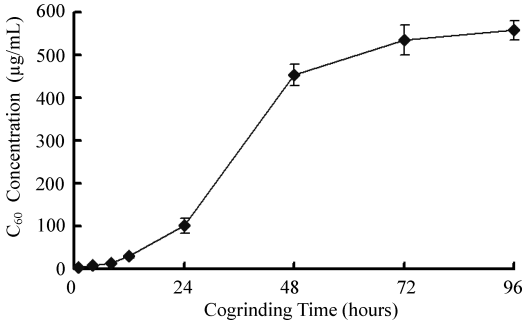

Fig. 4. Relationship between the Amount of Solubilized Fullerene C_{60} into Water and the Cogrinding Time

Cogrinding of cycloamyloses and fullerene (C_{60}) was performed at the weight ratio 5 : 1.

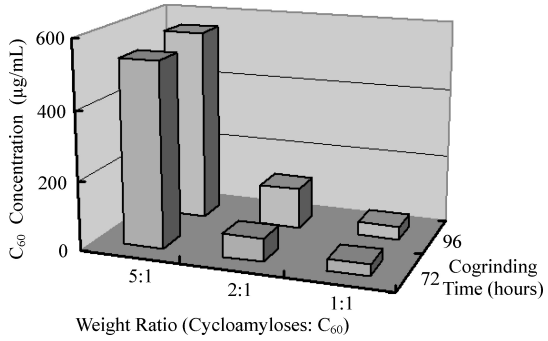

Fig. 5. Effect of the Sample Ratio in the Glass Pot of the Ball Mill on the Amount of Solubilized Fullerene into Water

Each point indicates the mean±S.D. of 3 experiments.

Table 1. Comparison of the Solubilized Amount of Fullerene (C_{60}) Prepared by Various Methods with Solubilization Agents

Solubilization agent	Solubility of C_{60} (μg/ml)	Concentration of solubilization agents (mg/ml)	Preparation method	Ref.
γ-Cyclodextrin	110	8.43	Cogrinding	18
δ-Cyclodextrin	110	1.4	Cogrinding	24
Fluoroalkyl oligomer	100	1000	Solvent	17
PVP	400	50	Evaporation	16
Cycloamyloses	560	5.0	Cogrinding	—

loses should interact with C_{60} in practice. In addition, given that the mean molecular weight of cycloamyloses is 6500, the stoichiometry was calculated as 1 : 1 (molecular weight of C_{60} is 720). Table 1 summarizes the solubility of C_{60} as improved by various methods. The solubility of C_{60} with cycloamyloses is greater than those prepared by other methods.

Evaluation of Molecular State of C_{60} in Solid and Liquid States In order to study the molecular state of C_{60} in the solid state, powder XRD was employed for a ground mixture of C_{60} and cycloamyloses. Although cycloamyloses had no crystalline structure, diffraction peaks of crystalline C_{60} were observed at 2θ, equal to 10.8, 17.7 and 20.8°. New diffraction peaks implying the formation of crystalline complex did not appear. The peak intensity assigned to crystalline C_{60} decreased as the cogrinding time was extended (Fig. 6). This suggested that the crystalline structure of C_{60} was disrupted, and then C_{60} molecules were substantially dispersed into amorphous cycloamyloses in the solid state. Accordingly, mechanochemical effect which induced interaction between cycloamylose and C_{60} molecule was observed during the cogrinding process.

To investigate the molecular state of C_{60} in the liquid state, particle size distribution was analyzed using the dynamic light scattering method. The mean particle size was about 50 nm when measuring the aqueous solution of a ground mixture consisting of C_{60} and cycloamyloses (Fig. 7). Accordingly, C_{60} molecules could be dispersed into water with cycloamyloses as the colloidal solution.

Figure 8 shows the UV–VIS absorption spectrum of C_{60} in the cycloamyloses solution, which was almost same as that in the polyvinylpyrrolidone (PVP) solution.[16] The absorption bands were observed at 264 and 340 nm in water and 258 and 328 nm in *n*-hexane. Thus, cycloamyloses provided a hydrophobic circumstance which accommodated the C_{60} molecule, possibly like a synthetic polymer such as PVP. In addition, these results suggested that C_{60} molecules existed in a cycloamyloses micellar system and the red-shift of the

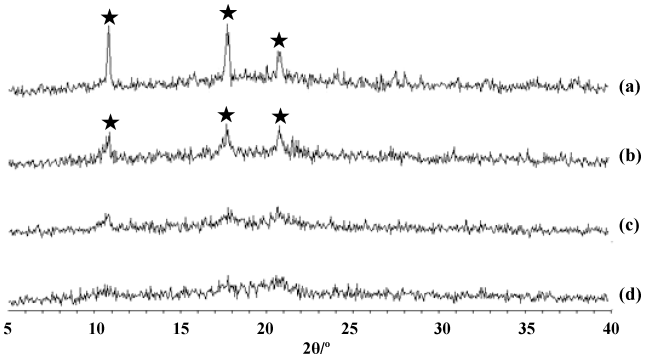

Fig. 6. Change in the Powder X-Ray Diffraction Pattern of Ground Mixture of Cycloamyloses and Fullerene (C_{60}) at the Weight Ratio 5 : 1 on Grinding

Grinding time: (a) 0; (b) 1; (c) 24; (d) 48 h.

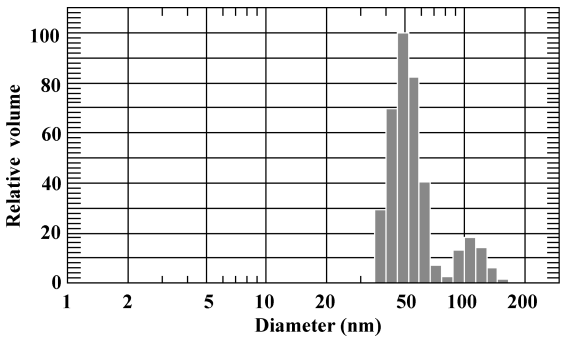

Fig. 7. Particle Size Distribution of Colloidal Solution Consisting of Cycloamyloses and Fullerene (C_{60})

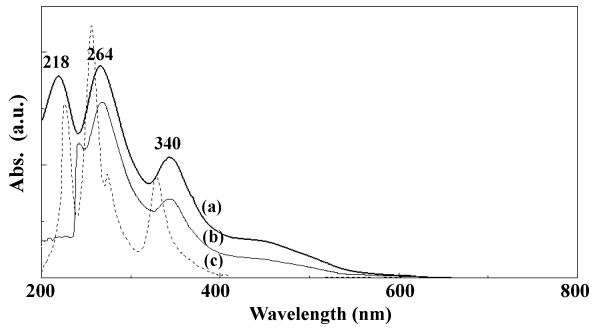

Fig. 8. UV–VIS Spectra of Fullerene (C_{60}) in Water with Cycloamyloses (a) and PVP[18] (b), and in *n*-Hexane (c)

UV–VIS spectra was due to an intermolecular interaction between C_{60} and cycloamyloses.

Conclusion

We found in this study that the cogrinding method with cycloamyloses had some advantages compared to the solubilizing methods previously reported, for following reasons: the cogrinding method with cycloamyloses (i) showed superior solubilization for C_{60}, (ii) was done without organic solvent, and (iii) was demonstrated using a natural product. Further study is expected to elucidate the mechanism in the solubilization process and the micellar system, and apply it to various compounds including medicinal substances. In any case, a new member has been added to the family of solubilization agents for poorly water-soluble compounds.

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