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Assessment of the Physical Properties of Inclusion Complexes of Forchlorfenuron and γ-Cyclodextrin Derivatives and Their Promotion of Plant Growth

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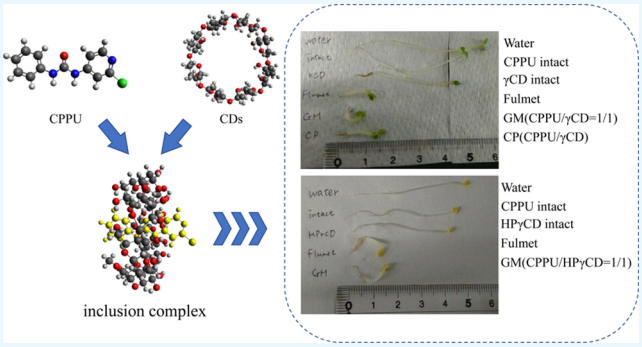
ABSTRACT: The current study prepared solid dispersions of forchlorfenuron (CPPU) and γ-cyclodextrin (γCD) or CPPU and 2-hydroxypropyl-γ-cyclodextrin (HPγCD) via cogrinding and coprecipitation to assess their physicochemical properties and their effect on plant growth. According to phase solubility diagrams, both CPPU/γCD and CPPU/HPγCD formed an inclusion complex at a molar ratio of 1/1. According to differential scanning calorimetry and powder X-ray diffraction, a ground mixture (GM) of CPPU and γCD (molar ratio = 1/1), a GM of CPPU and HPγCD (molar ratio = 1/1), and a coprecipitate (CP) of CPPU and γCD (molar ratio = 1/1) formed an inclusion complex. According to $^{1}H-^{1}H$ nuclear Overhauser effect spectroscopy NMR spectroscopy of the GMs and CP, the aromatic rings of the CPPU molecule are presumably included in CD from the wider to the narrower rim of its ring. Cultivation of broccoli sprouts with the GMs and CP resulted in no differences in the length of sprouts in comparison to a commercial preparation (Fulmet).



1. INTRODUCTION

Ingredients of agricultural chemicals that promote plant growth and development do so by promoting longer stems, promoting flowering, preventing fruits from dropping, and producing parthenocarpic fruits. Plant growth and development agents can artificially control the growth and development of agricultural crops, and they can be used to improve quality and reduce yield or reduce fruiting. Forchlorfenuron (CPPU) in particular is known to be a compound that promotes lateral growth and fruit enlargement (Figure 1A).[1] CPPU, a phenylurea synthetic plant hormone, acts in trace amounts on agricultural crops; CPPU leaves little residue and has little effect on animals and the environment.[2] CPPU is used to enlarge grapefruits, which it accomplishes by promoting cell division, further extending the period of cell division and increasing the total number of cells.[3] Commercially available preparations containing CPPU are dissolved using organic solvents and surfactants, and flammability and toxicity are a concern. Because CPPU is packaged in a glass bottle, a simple method of preparation is required because of the difficulty of handling during distribution or after use, and CPPU has to be diluted when used. If a naturally tolerated CPPU could be developed without the need for an organic solvent, it could be easily distributed. Moreover, convenience would be increased if CPPU were dissolved at the time of use. However, CPPU is poorly soluble in water (solubility: 0.11 mg/mL, 25 °C), so it would need pharmaceutical functionality to easily dissolve in water.

Cyclodextrin (CD) has a cyclic structure containing D-glucopyranose units linked by α (1 → 4) glycosidic bonds, and CD is classified as αCD, βCD, or γCD based on the number of glucopyranose units it contains (Figure 1B).

Facilitating the formation of inclusion complexes with various drug molecules can improve drug solubility and stability. CDs form inclusion complexes via hydrophobic interactions.[4] Piperine, the pungent component of black pepper, is unstable to light and poorly water-soluble, but its stability to light can be improved by its inclusion in γCD.[5] Voriconazole, an antifungal drug, is an oral preparation because of its poor solubility in water. Patients who have difficulty taking medication orally need to receive injections, and a study has found that inclusion complexes with CD improve the solubility of itraconazole, leading to the development of injections.[6] CD inclusion complexes are prepared using a variety of techniques, including coprecipitation,[7] kneading,[8] freeze-drying,[9] and cogrinding.[10] A study has reported that coprecipitation causes caffeic acid (CA) and γCD to form inclusion complexes, thus improving the dissolution of CA.[11] A solid dispersion of a cyclic polysaccharide and CD could be prepared. If CPPU could be encouraged to form an inclusion complex with CD, then the solubility of CPPU would improve, resulting in enhanced functionality. A study reported that

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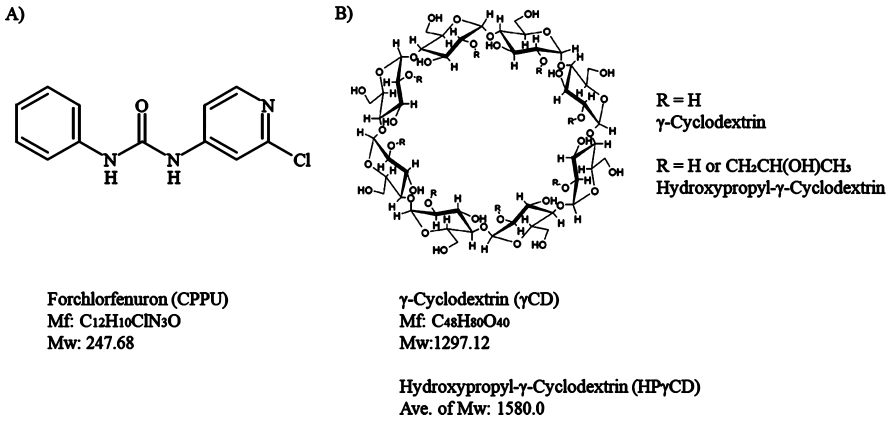

Figure 1. Chemical structures: (A) CPPU and (B) γCD and HPγCD.

CPPU forms a polymer with βCD.[12] However, γCD and γCD derivatives have a cavity with a larger diameter than that of βCD, and formation of CPPU and γCD complexes has not been reported. In addition, no study has evaluated how complex formation by CPPU and CD affects the solubility of CPPU. Therefore, the current study prepared inclusion complexes of CPPU and γCD or 2-hydroxypropyl-γ-CD (HPγCD) to assess their physicochemical properties, molecular interaction, solubility, and promotion of plant growth.

2. RESULTS AND DISCUSSION

2.1. Phase Solubility Studies. Solubility testing was performed to determine the molar ratio of CPPU and CD in inclusion complexes and to determine their stability constants in an aqueous solution. The results indicated that the solubility of CPPU increased linearly with γCD and HPγCD, producing a B_I and an A_L type of phase solubility diagram as described by Higuchi et al (Figure 2).[13] The stability constant (K_s) for

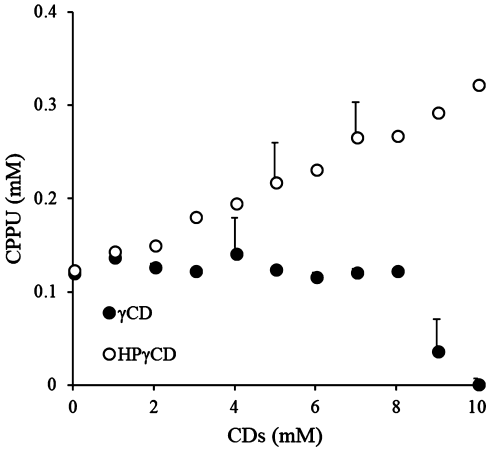

Figure 2. Phase solubility diagrams of CPPU/CDs. The results are expressed as the mean ± SD (n = 3).

HPγCD was 154.5 M^{-1}. The complexation efficiency (CE) for HPγCD was 0.0201. γCD produced a type B_I phase solubility diagram, and K_s and CE could not be calculated. An A_L type of diagram typically indicates that a complex is formed at a molar ratio of 1/1, so HPγCD forms a complex at a molar ratio of 1/1 in solution.

2.2. ^{1}H Nuclear Magnetic Resonance Analysis. The results of differential scanning calorimetry (DSC) and powder X-ray diffraction (PXRD) patterns suggest molecular interactions in both the ground mixture (GM) and coprecipitate (CP). ^{1}H nuclear magnetic resonance (NMR) spectroscopy was performed to investigate the molar ratio of inclusion in the CP. ^{1}H NMR spectra of CPPU, γCD, and CP are shown in Figure 3. In CPPU, two signals due to NH group hydrogens

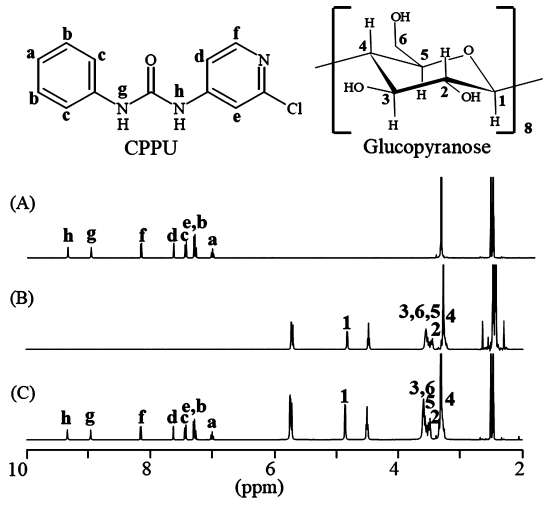

Figure 3. Measurement of ^{1}H NMR spectra of (A) intact CPPU, (B) γCD, and (C) CP of CPPU and γCD.

were evident at around 8.93−9.31 ppm. In γCD, signals due to hydrogen and hydroxyl groups of the glucose unit were evident. In CP, signals due to CPPU and γCD were, respectively, confirmed. A signal due to the NH group hydrogen of CPPU was evident at around 8.93−9.31 ppm, and this signal was produced by 1 proton, so there was 0.15 of a proton per hydrogen atom of CPPU in the CP. The signal due to hydrogen number 1 in the glucose unit of γCD was produced by 1 proton, so there was 0.125 of a proton per hydrogen atom of γCD. When the molar ratio of inclusion of the CP was calculated using the formula, the molar ratio of inclusion complex formation by γCD and CPPU was 1/1 when there was 0.15 of a CPPU molecule per 1 molecule of γCD.[14]

2.3. Differential Scanning Calorimetry. According to phase solubility diagrams, both CPPU/γCD and CPPU/HPγCD apparently form a complex at a molar ratio of 1/1 in an aqueous solution. A study by Shiozawa et al. reported that CA forms inclusion complexes with CD as a result of

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cogrinding or coprecipitation.[15] When a guest molecule ceases to melt or its peak shifts as a result of inclusion complex formation, changes in its thermal behavior become evident.[16] Accordingly, the current study performed DSC to examine the thermal behavior of the sample prepared by cogrinding or coprecipitation. In DSC, intact CPPU produced an endothermic peak because of melting at 175 °C (Figure 4A). A physical

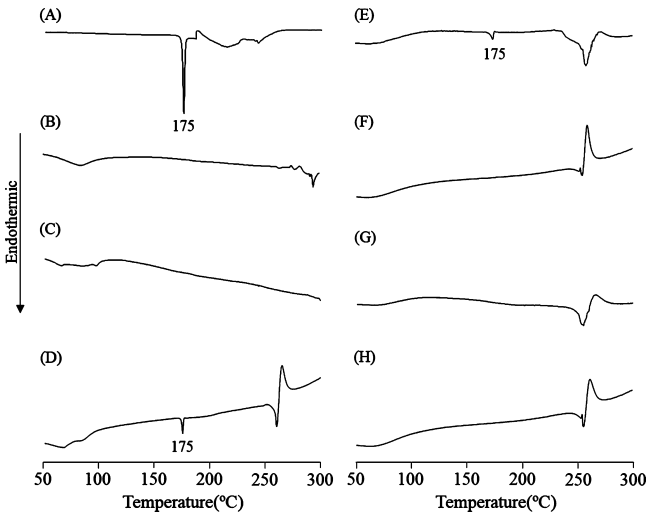

Figure 4. DSC curves of CPPU/CD systems: (A) intact CPPU, (B) γCD, (C) HPγCD, (D) PM of CPPU and γCD (1/1), (E) PM of CPPU and HPγCD (1/1), (F) GM of CPPU and γCD (1/1), (G) GM of CPPU and HPγCD (1/1), and (H) CP of CPPU and γCD (1/1).

mixture (PM) of CPPU and γCD (1/1) and a PM of CPPU and HPγCD (1/1) had an endothermic peak because of the melting of CPPU at 175 °C, so CPPU crystals were presumably present (Figure 4D,E). Interestingly, the endothermic peak due to CPPU was not evident in the GM of CPPU and γCD (1/1), the GM of CPPU and HPγCD (1/1), or the CP of CPPU and γCD (Figure 4F−H). When a drug is included in CD, an endothermic peak produced by melting of that drug disappears.[17] This suggests that CPPU, γCD, and HPγCD molecules formed inclusion complexes, causing the endothermic peak due to CPPU to disappear.

2.4. Powder X-ray Diffraction. The results of DSC suggested that the GM of CPPU and γCD (1/1), the GM of CPPU and HPγCD (1/1), and the CP of CPPU and γCD formed an inclusion complex. Thus, the crystalline state of the GMs and CP was examined using PXRD. Intact CPPU produced diffraction peaks because of CPPU at 2θ = 23.9° and 27.4°, and γCD produced a diffraction peak because of γCD at 2θ = 11.9° (Figure 5A,B). In contrast, HPγCD alone produced a halo pattern (Figure 4C). In addition, ground CPPU did not produce a halo pattern (not shown data). A PM of CPPU and γCD (1/1) and a PM of CPPU and HPγCD (1/1) produced diffraction peaks because of CPPU at 2θ = 23.9° and 27.4° and a diffraction peak because of γCD at 2θ = 11.9° (Figure 5D,E). In contrast, a GM of CPPU and γCD (molar ratio = 1/1) and a GM of CPPU and HPγCD (molar ratio = 1/1) produced no peaks but they did produce a halo pattern (Figure 5F,G). Cogrinding disrupts the structures of crystals, and the amorphous structure that results can produce a halo pattern in PXRD.[18] According to a previous study, supplying mechanical energy can facilitate the formation of amorphous

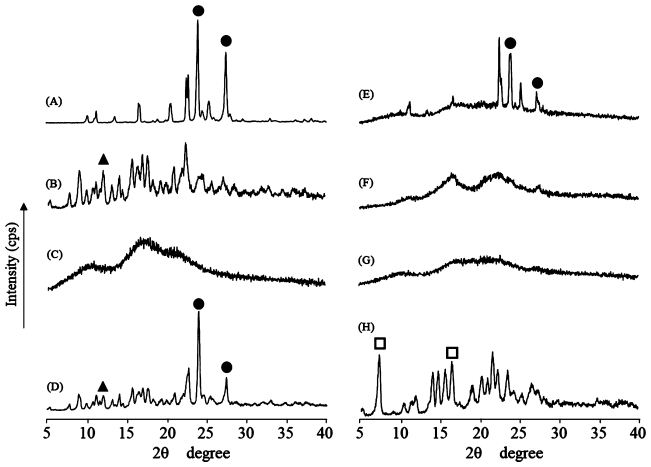

Figure 5. PXRD patterns of CPPU/CDs: (A) intact CPPU, (B) γCD, (C) HPγCD, (D) PM of CPPU and γCD (1/1), (E) PM of CPPU and HPγCD (1/1), (F) GM of CPPU and γCD (1/1), (G) GM of CPPU and HPγCD (1/1), and (H) CP of CPPU and γCD (1/1). (●) intact CPPU, (▲) γCD intact, (□) New peak.

inclusion complexes.[19] Diffraction peaks due to CPPU and due to γCD disappeared with the CP of CPPU and γCD, and new diffraction peaks appeared at 2θ = 7.4° and 16.1° (Figure 5H). However, new peaks (2θ = 7.5°, 12.0°, and 16.5°) are produced, and presumably, these peaks are specific to inclusion complexes of γCD and a guest molecule.[20] This suggests that the GM of CPPU and γCD (1/1), the GM of CPPU and HPγCD (1/1), and the CP of CPPU and γCD all formed inclusion complexes.

2.5. Near Infrared Absorption Spectrometry. PXRD patterns and results of DSC suggested that inclusion complexes are formed by the GM of CPPU and γCD (1/1), the GM of CPPU and HPγCD (1/1), and the CP of CPPU and γCD (1/1). Near infrared (NIR) absorption spectrometry was performed to investigate molecular interaction in detail. In NIR spectra, a peak due to the −OH groups of CD is produced at around 6660−7140 cm^{-1}.[21] A peak due to the −CH groups of CD is produced at around 8800 cm^{-1}.[22] Peaks due to the −NH groups are produced at around 6800 and 9000 cm^{-1}.

NIR spectra revealed that CPPU alone produced a peak because of its −CH groups at around 8800 cm^{-1} and a peak at around 9000 cm^{-1} because of its −NH groups. γCD alone produced a peak because of its −OH groups at around 7000 cm^{-1} (Figure 6). Second-derivative spectra revealed that the GM of CPPU and γCD (1/1) produced a shift in the peak because of the −CH groups of CPPU at around 8800 cm^{-1} and it produced a shift in the peak because of the −NH groups of CPPU at around 9000 cm^{-1} (Figure 6A,B). In addition, the peak due to the −NH groups of CPPU produced at around 6800 cm^{-1} and the peak due to the −OH groups of γCD produced at around 7000 cm^{-1} broadened (Figure 6C,D). A study has reported that broadening or shifting of a peak in an NIR spectrum is caused by molecular interaction of the functional groups of drugs.[23] Accordingly, molecular interaction presumably occurred between the −CH groups and −NH groups of CPPU and between the −OH groups of γCD in the GM of CPPU and γCD (1/1) and the CP of CPPU and γCD.

HPγCD alone produced a peak because of its −OH groups at around 7000 cm^{-1}. According to the second-derivative

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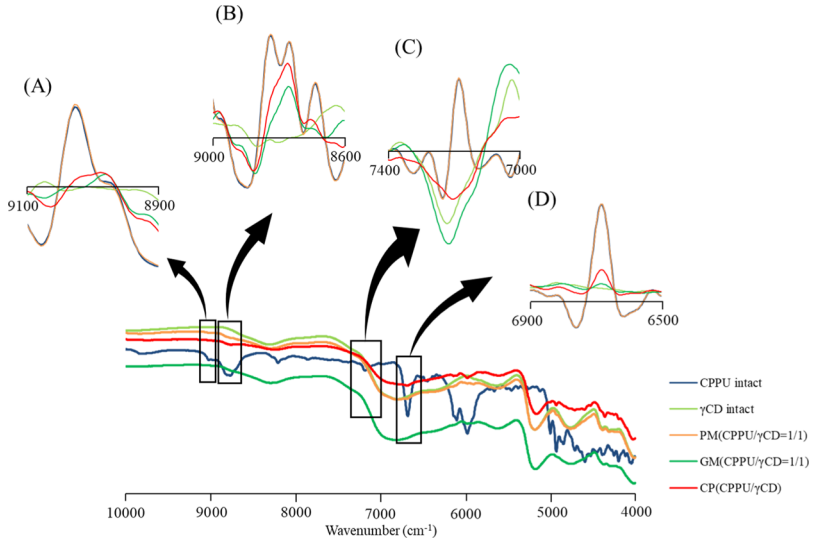

Figure 6. NIR spectra of CPPU/γCD systems: (A) amino group, (B) alkyl group, (C) hydroxy group, and (D) amino group.

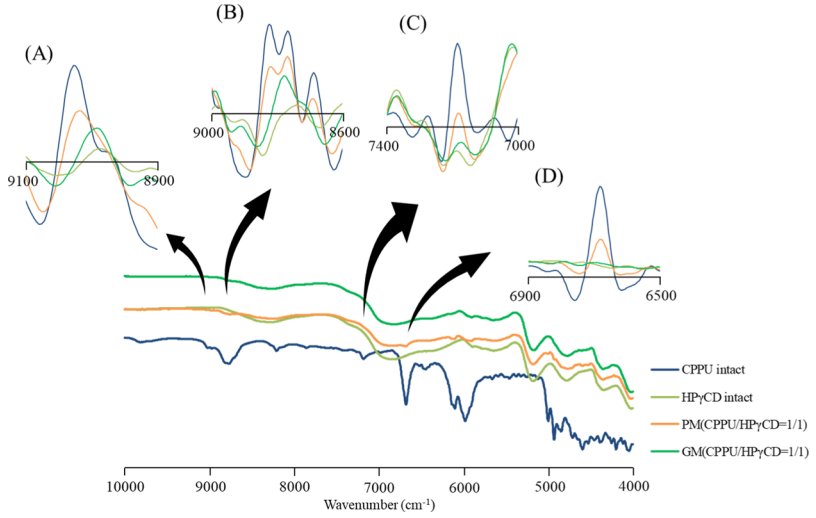

Figure 7. NIR spectra of CPPU/HPγCD systems (A) amino group, (B) alkyl group, (C) hydroxy group, (D) amino group.

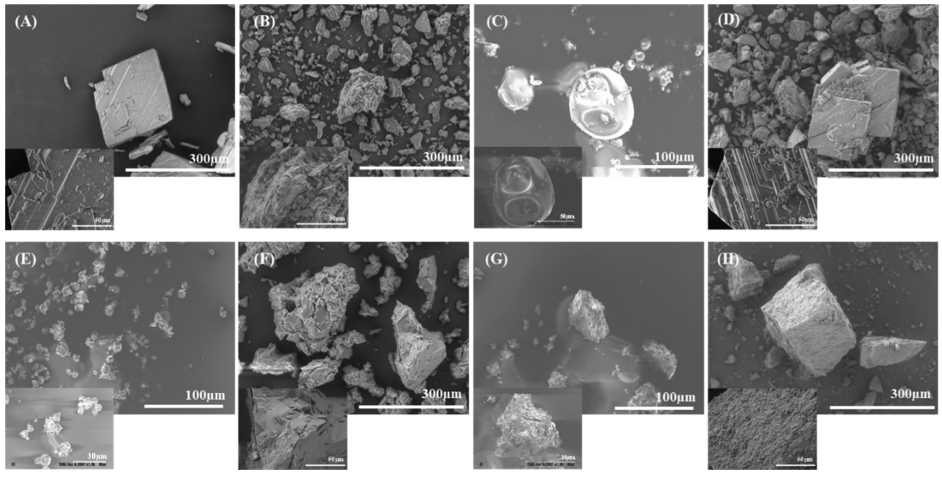

Figure 8. SEM image of CPPU/CDs systems: (A) intact CPPU, (B) γCD, (C) HPγCD, (D) PM of CPPU and γCD (1/1), (E) PM of CPPU and HPγCD (1/1), (F) GM of CPPU and γCD (1/1), (G) GM of CPPU and HPγCD (1/1), and (H) CP of CPPU and γCD.

spectra, the peak due to the −CH groups of CPPU produced at around 8800 cm^{-1} broadened and the peak due to the −NH groups of CPPU produced at around 9000 cm^{-1} shifted for the GM of CPPU and HPγCD (1/1) (Figure 7A,B). Moreover,

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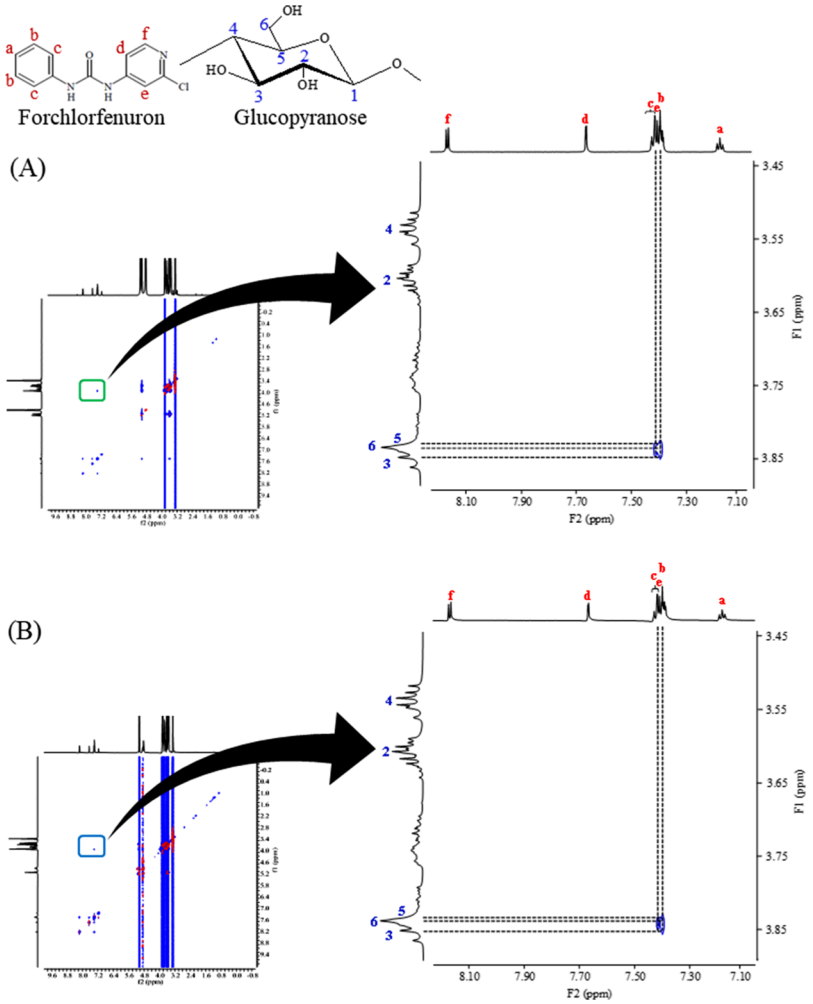

Figure 9. $^{1}H-^{1}H$ NOESY NMR spectra of CPPU/γCD systems: (A) GM of CPPU and γCD (1/1) and (B) CP of CPPU and γCD.

the peak due to the −NH groups of CPPU produced at around 6800 cm^{-1} and the peak due to the −OH groups of HPγCD produced at around 7000 cm^{-1} broadened (Figure 7C,D). These findings presumably indicate that there is molecular interaction between the −CH groups and −NH groups of CPPU and the −OH groups of HPγCD in the GM of CPPU and HPγCD (1/1) as well.

2.6. Scanning Electron Microscopy Imaging. Scanning electron microscopy (SEM) imaging was performed to observe the surface and morphology of each sample (Figure 8). The surface of CPPU was smooth, and the particle size of CPPU was around 200 μm. The surface of γCD was smooth, and irregularly shaped particles were evident. The particle size was around 75 μm. Smooth spherical particles were evident on the surface of HPγCD. The size of the particles was around 40 μm. In the PM of CPPU and γCD (1/1), CPPU and γCD particles were evident. In the PM of CPPU and HPγCD (1/1), particles of CPPU and HPγCD were evident. In the GM of CPPU and γCD (1/1), the particle surface was coarse, agglomerated particles of small fragments were evident, and many particles 150 μm or smaller in size were evident. In the GM of CPPU and HPγCD (1/1), fine particles of 5 μm or less aggregated, and a rough surface was evident. Cubic grains in which fine particles aggregated were evident in the CP of CPPU and γCD. According to a previous study, such findings are due to the formation of an inclusion complex.[24] The current findings suggest that cogrinding and coprecipitation resulted in the formation of an inclusion complex and that this influenced the particle diameter and particle surface.

2.7. Measurement of $^{1}H-^{1}H$ NOESY NMR Spectra. Spatial interactions between a guest molecule and the CD cavity can be ascertained with $^{1}H-^{1}H$ nuclear Overhauser effect spectroscopy (NOESY) NMR spectroscopy, so the positioning of the guest molecule within the inclusion complex was predicted using $^{1}H-^{1}H$ NOESY NMR spectroscopy.[25] In the GM of CPPU and γCD (1/1), the H-3 proton (3.85 ppm), H-5 proton (3.83 ppm), and H-6 proton (3.83 ppm) in the CD cavity and the H-b proton (7.37 ppm) and H-c proton (7.42 ppm) in the aromatic ring of CPPU produced cross peaks (Figure 9A). The H-3 proton is typically located in the wider rim of the ring of CD, and the H-6 proton is typically located in the narrower rim of that ring.[26] Cross peaks indicate that protons are less than 4 Å apart, and a more intense peak indicates that protons are closer together.[27] In the CP of CPPU and γCD (1/1), the H-3 proton (3.85 ppm), H-5 proton (3.83 ppm), and H-6 proton (3.83 ppm) in the CD cavity and the H-b proton (7.37 ppm) and H-c proton (7.42 ppm) in the aromatic ring of CPPU similarly produced cross peaks (Figure 9B). In the GM of CPPU and HPγCD (1/1), the H-3 proton (3.77 ppm), H-5 proton (3.55 ppm), and H-6 proton (3.59, 3.65 ppm) in the CD cavity and the H-b proton (7.08 ppm) and H-c proton (7.14, 7.08 ppm) in the aromatic

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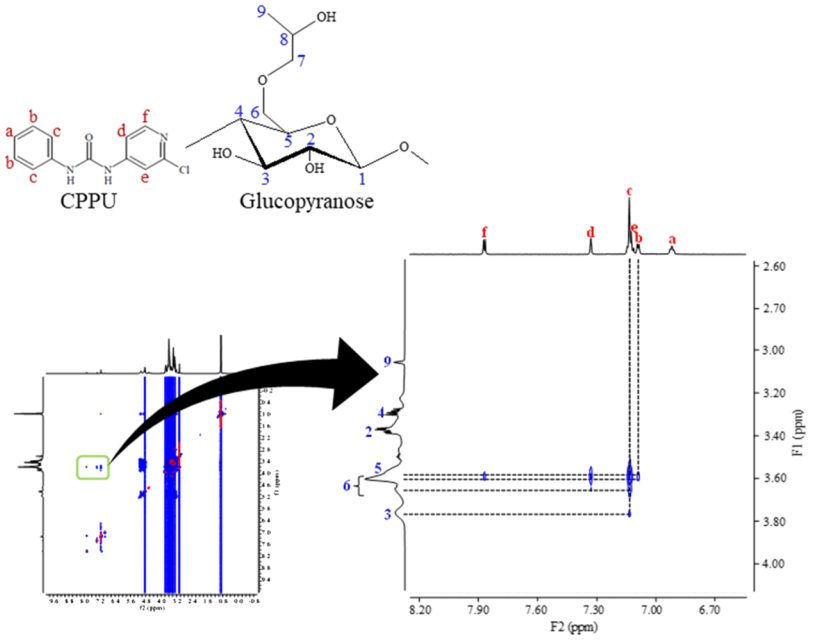
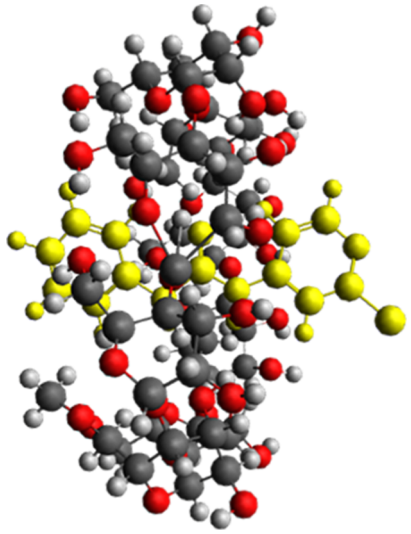


Figure 10. $^{1}H-^{1}H$ NOESY NMR spectra of GM (CPPU/HPγCD = 1/1) systems.

ring of CPPU produced cross peaks (Figure 10). Intense cross peaks were particularly produced by the H-b and H-c protons of the aromatic ring of CPPU and the H-6 proton in the cavity of HPγCD. Thus, the aromatic ring of the CPPU molecule appears to be oriented from the wider to the narrower rim of the ring of γCD and HPγCD (Scheme 1).

Scheme 1. Structural View of a CPPU/CD Complex



2.8. Dissolution Profile. Results thus far suggested that an inclusion complex is formed in a solid state. Accordingly, a dissolution test was performed to ascertain whether the formation of CPPU/γCD and CPPU/HPγCD inclusion complexes resulted in changes in the dissolution of CPPU. The samples used in the test were intact CPPU, the PM of CPPU and γCD (1/1), the PM of CPPU and HPγCD (1/1), the GM of CPPU and γCD (1/1), the GM of CPPU and HPγCD (1/1), and the CP of CPPU and γCD (1/1) (Figure 11). The results indicated that the rate of dissolution of intact CPPU was 2.6% 5 min after the start of the test. The rate of

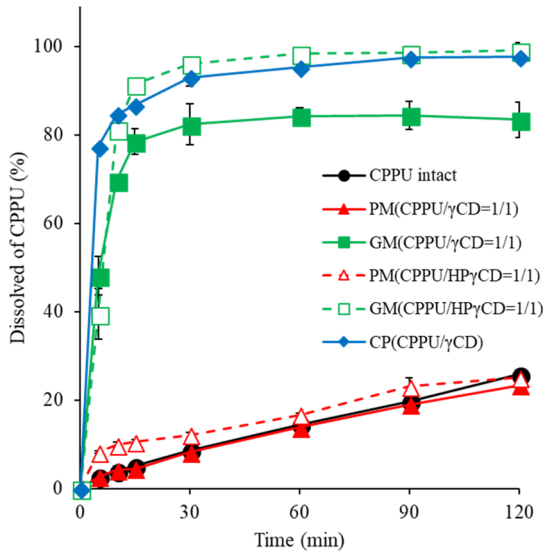


Figure 11. Dissolution profiles of CPPU/CD systems. The results are expressed as the mean ± SD (n = 3).

dissolution for the PM of CPPU and γCD (1/1) was 2.6% and that for the PM of CPPU and HPγCD (1/1) was 8.3%. In CPPU alone, the PM of CPPU and γCD (1/1), and the PM of CPPU and HPγCD (1/1), the dissolution rate gradually increased after 5 min and was about 20% at 120 min. The rate of dissolution of CPPU 5 min after the start of the test was 48.2% for the GM of CPPU and γCD (1/1), 39.5% for the GM of CPPU and HPγCD (1/1), and 77.3% for the CP of CPPU and γCD. In contrast, the dissolution rate increased after 30 min for the GM of CPPU and γCD (1/1), the GM of CPPU and HPγCD (1/1), and the CP of CPPU and γCD. The rate of dissolution of CPPU after 120 min was approximately 80% for the GM of CPPU and γCD (1/1), approximately 98% for the GM of CPPU and HPγCD (1/1), and approximately 97% for the CP of CPPU and γCD. In the initial 5 min, the dissolution rate of CPPU in the GM of CPPU and γCD (1/1) and the

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GM of CPPU and HPγCD (1/1) was lower than that in the CP of CPPU and γCD. This suggests that the difference in the crystallinity of the composite due to the difference in the method of preparation affects the wettability of the powder and is reflected in its dissolution properties. In addition, the GM of CPPU and HPγCD (1/1) had the highest dissolution rate of CPPU at 120 min. On the basis of solubility phase diagrams, the concentration of dissolved CPPU was linear and depended on the amount of HPγCD added. The CE was 0.0201. Because CE and guest molecules are generally correlated, the CE for the CPPU/HPγCD system could presumably be calculated CE and would indicate a high level of dissolution.

Dissolution of CPPU in the GMs and CP improved in comparison to that of intact CPPU and the PM. According to previous studies, a solid dispersion of a drug and CD can display an increased rate of dissolution via two mechanisms: disruption of the arrangement of molecules of a drug as a result of it becoming amorphous and the formation of an inclusion complex.[28,29] PXRD patterns revealed that CPPU and γCD in the GM and CPPU and HPγCD in the GM became amorphous and formed an inclusion complex. The aromatic rings of CPPU are hydrophobic, and the NIR spectra revealed molecular interaction between CPPU and CD. Moreover, the NMR spectra revealed that CPPU is included in the cavity of γCD or HPγCD in solution, so the improved dissolution of CPPU is the result of multiple factors (e.g., CPPU becoming amorphous and its formation of an inclusion complex). Nevertheless, solubility after 5 min differed as a result of differences in the method of preparation and the type of CD. This is presumably due to differences in molecular interaction in the solid dispersion during PXRD, DSC, and NIR spectroscopy. The above findings suggest that preparing a solid dispersion by cogrinding or coprecipitating CPPU and γCD or HPγCD can help to improve the solubility of CPPU. A CP could not be prepared with HPγCD, so this issue is a topic for future study.

2.9. Experimental Cultivation of Broccoli Sprouts. To determine the extent to which CPPU promoted plant growth as a result of the formation of CPPU/γCD and CPPU/HPγCD inclusion complexes, broccoli sprouts were cultivated using intact CPPU, intact γCD, intact HPγCD, the GM of CPPU and γCD (1/1), the GM of CPPU and HPγCD (1/1), the CP of CPPU and γCD, and a commercial preparation of CPPU (Fulmet). Sprouts in the control group were cultivated using distilled water. The sprout length did not differ between the control group and intact CPPU, γCD, and HPγCD (Figure 12A). The GM of CPPU and γCD (1/1), the GM of CPPU and HPγCD (1/1), and the CP of CPPU and γCD resulted in growth equivalent to that produced by Fulmet, although that growth was inferior to the growth produced by the control group. The sprout weight did not differ between the control group and intact CPPU, γCD, and HPγCD (Figure 12B). The GM of CPPU and γCD (1/1), the GM of CPPU and HPγCD (1/1), and the CP of CPPU and γCD resulted in growth equivalent to that produced by Fulmet. The sprout thickness did not differ between the control group and intact CPPU, γCD, and HPγCD (Figure 12C). The GM of CPPU and γCD (1/1), the GM of CPPU and HPγCD (1/1), and the CP of CPPU and γCD resulted in growth equivalent to that produced by Fulmet. A texture test of sprout thickness was performed on day 5 of cultivation, and hardness was calculated based on texture profile analysis (Figure 13). The results revealed no differences among the control group, intact CPPU, intact γCD,

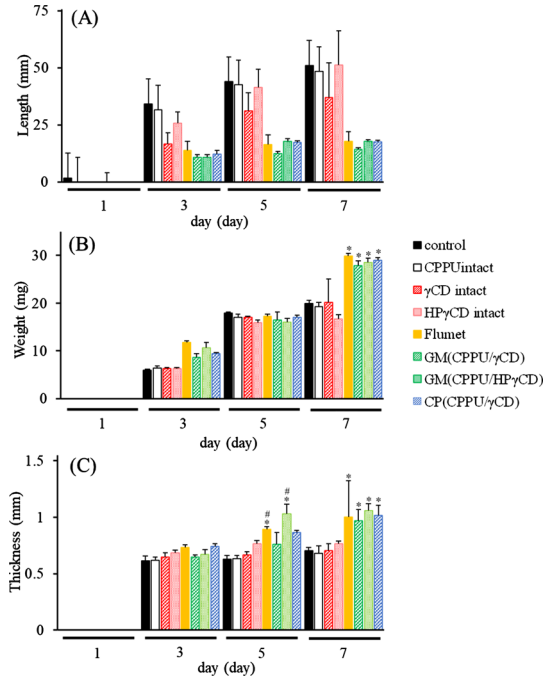

Figure 12. Effect of CPPU on broccoli sprouts over a period of 7 days. (A) Stem length, (B) weight of the broccoli sprout, and (C) stem thickness. The results are expressed as the mean ± SD (n = 20). *: $p < 0.05$ vs control, #: $p < 0.05$ vs GM of CPPU and γCD (1/1), †: $p < 0.05$ vs GM of CPPU and HPγCD (1/1) (*Tukey test*).

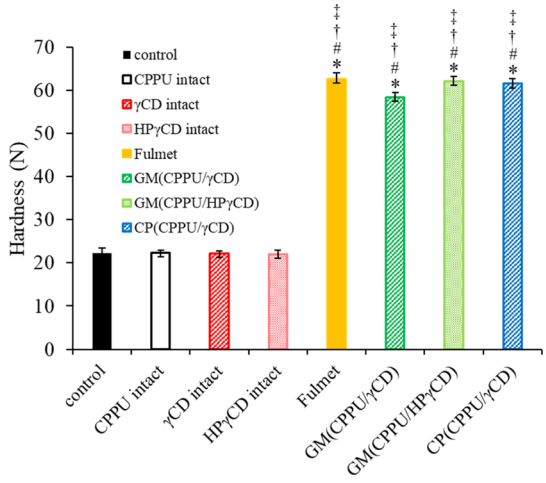

Figure 13. Results of a texture test on day 5. *: $p < 0.05$ vs control, #: $p < 0.05$ vs intact CPPU, †: $p < 0.05$ vs γCD, ‡: $p < 0.05$ vs HPγCD (*Tukey test*). Values are expressed as the mean ± SD (n = 5).

and intact HPγCD (no significant differences). Fulmet and the GM of CPPU and γCD (1/1), the GM of CPPU and HPγCD (1/1), and the CP of CPPU/γCD resulted in sprouts with equivalent hardness. Those sprouts had about 3 times the hardness of sprouts produced by the control group (significant difference; p 0.01) (Figure 14). These findings suggest that CPPU formed inclusion complexes with γCD and HPγCD, thus improving the solubility of CPPU. Nonetheless, the action of CPPU to promote cell division was equivalent to the action of Fulmet.

The commercial preparation (Fulmet) requires complicated preparation because it is in a liquid state. However, inclusion complexes were used to produce preparations in the current

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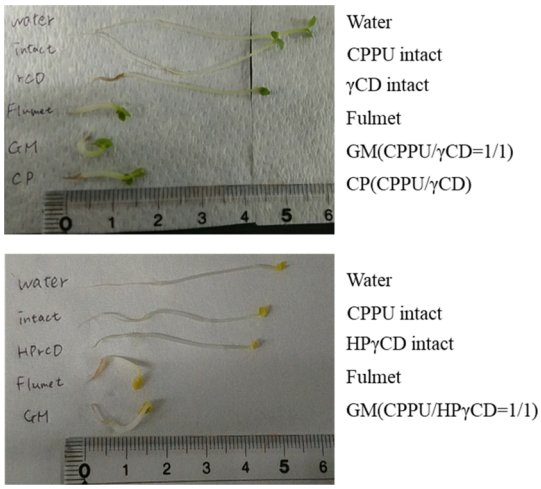

Figure 14. Image of each sample on day 5.

study. CPPU in Fulmet has increased water solubility because it has been dissolved in alcohol. Thus, this solution of CPPU in alcohol was assumed to result in greater plant growth effect than CPPU alone. However, preparations containing CPPU/CD complexes resulted in plant growth equivalent to that of Fulmet. Therefore, the solubility of CPPU is presumably one of the factors accounting for the fact that preparations containing CPPU/CD complexes resulted in greater plant growth than CPPU alone. These preparations are readily soluble in water and they do not require an organic solvent or surfactant as is found in commercial preparations (like Fulmet). In this study, inclusion of CPPU in CD improved the solubility of CPPU. Fulmet is a solution containing CPPU dissolved in alcohol, but use of CPPU/CD complexes allows commercial production of CPPU in a solid state. This would presumably reduce the burden of distribution. Moreover, use of CPPU/CD complexes allows CPPU to be highly active at a low concentration, so CPPU can be incorporated in commercial preparations at a low cost. Thus, new preparations can be developed for the benefit of farmers.

3. CONCLUSIONS

Phase solubility diagrams revealed that CPPU/γCD and CPPU/HPγCD formed an inclusion complex at a molar ratio of 1/1. The results of DSC, PXRD patterns, NIR spectra, and the results of a dissolution test of a GM and CP of CPPU and γCD or HPγCD revealed that CPPU and HPγCD formed inclusion complexes at a molar ratio of 1/1. The rate of dissolution of CPPU improved as a result of complex formation (CPPU/γCD and CPPU/HPγCD). In addition, the GM of CPPU and γCD, the GM of CPPU and HPγCD, and the CP of CPPU and γCD resulted in plant growth comparable to that as a result of a commercial CPPU preparation. The use of CPPU as a promoter of plant growth would presumably increase as a result of improving the solubility of complexes in the GM of CPPU and γCD, the GM of CPPU and HPγCD, and the CP of CPPU and γCD.

4. MATERIALS AND METHODS

4.1. Materials. *4.1.1. Chemicals.* CPPU was a bulk powder purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). γCD was donated by Cyclo Chem Co., Ltd. (Tokyo, Japan) and was used after storage at a temperature of 40 °C and a relative humidity of 82% for 7 days. HPγCD was a bulk powder purchased from Sigma-Aldrich Co., Ltd. (Tokyo, Japan). HPγCD was used with a molar substitution of 0.6. Fulmet, which is a commercial preparation of CPPU, was from Kyowa Hakko Bio Co., Ltd. (Tokyo). All other chemicals and solvents were of analytical grade and were purchased from Wako Pure Chemical Industries Co., Ltd.

4.1.2. Preparation of PM and Ground Mixtures. Each PM was prepared by weighing CPPU and CD to a molar ratio 1/1 and mixing the two with a vortex mixer for 1 min. Each GM was prepared by placing a PM (1 g) in an aluminum pan and grinding the mixture for 60 min using a vibrating rod mill (TI-500ET, CMT Co., Fukushima, Japan).

4.1.3. Preparation of a CP. A CP was prepared by dropwise addition of 0.07 mol/mL γCD solution to 0.2 mol/mL CPPU in acetone. The solution was stirred for 24 h at room temperature and then allowed to stand at room temperature for 24 h. The sample was filtered with a filter paper. The precipitate was washed with 3 mL of acetone and dried at room temperature for 24 h. In addition, attempts were made to prepare a CP using HPγCD, but those attempts were unsuccessful.

4.2. Methods. *4.2.1. Phase Solubility Studies.* Phase solubility studies were performed according to the method reported by Higuchi and Connors.[13] A supersaturated amount of CPPU (30 mg) was added to an aqueous solution (10 mL) of γCD (0−10 mM) or HPγCD (0−15 mM), and the mixture was shaken at 25 ± 0.5 °C and 200 rpm for 48 h to obtain a suspension. The suspension was filtered through a 0.45 μm membrane filter. Quantitation of CPPU was performed with high-performance liquid chromatography (LC-20A, Shimadzu Co., Ltd, Kyoto) using an Inertsil ODS-3 packed column (φ5 μm, 4.6 mm × 150 mm, GL Sciences, Tokyo). The samples were measured at a wavelength of 263 nm. The sample injection volume was 30 μL and the column temperature was 40 °C. A mobile phase of distilled water/acetonitrile (1/1) was used, and the CPPU retention time was 5 min at a flow rate of 1 mL/min. The apparent stability constant (K_s) of a CPPU/CD inclusion complex was calculated using eq 1 from the slope of the phase solubility diagram and the solubility (S_0) of CPPU in the absence of CD. In addition, the CEs of CPPU and CD were calculated using eq 2.

$$K_s = \text{slope}/S_0(1 - \text{slope}) \quad (1)$$

$$\text{CE} = \text{slope}/(1 - \text{slope}) \quad (2)$$

4.2.2. Measurement of ^{1}H NMR Spectra. NMR spectra were obtained using a Varian NMR System 400 MHz (Agilent Technologies, Tokyo). Dimethyl sulfoxide-d_6 was used as a solvent, and the measurement was performed with a pulse width of 90°, a delay time of 6.4 μs, a scan time of 3.723 s, and 128 integration steps at 26 °C.

4.2.3. Differential Scanning Calorimetry. A differential scanning calorimeter (Thermo plus EVO, Rigaku, Tokyo) was used. All samples were weighed (2 mg) and heated at a scanning rate of 5.0 °C/min with a nitrogen flow rate of 60 mL/min. Aluminum pans and lids were used for all samples.

4.2.4. Powder X-ray Diffraction. PXRD was performed using an X-ray diffractometer (MiniFlex II, Rigaku) with Cu radiation, a scan range of 2θ = 5−40°, and a scan rate of 4°/min. The intensities of diffraction were measured with a NaI scintillation counter coupled to a discriminator.

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4.2.5. NIR Absorption Spectrometry. Each sample was scanned with a Fourier-transform NIR spectrometer (Büchi N-500: Nippon Büchi, Tokyo) with a measurement wavelength of 10 000−4000 cm^{-1}, a measuring time of 8 s, a measuring temperature of 25 °C, and a cell with a 1 nm optical path length.

4.2.6. SEM Imaging. The SEM images were obtained with a scanning electron microscope (S3000N, Hitachi High-Technologies Corporation, Tokyo) at an acceleration voltage of 10 kV. The samples were mounted on aluminum stubs that were then coated with a thin layer of gold for 70 s to make them electrically conductive.

4.2.7. $^1H-^1H$ NOESY NMR Spectroscopy. Two-dimensional NOESY NMR spectroscopy and selective one-dimensional NMR spectroscopy were performed using a NMR spectrometer (Varian NMR System 700NB, Agilent) with a cold probe operating at 699.6 MHz. D_2O/CD_3OD (1/1) was used as a solvent. The measurement conditions were as follows: a pulse width of 90°, a relaxation time of 500 ms, a scanning time of 0.500 s, a fixed delay of 1.500 s, and a temperature of 25 °C.

4.2.8. Dissolution Profile. The dissolution profile of samples was determined using a dissolution apparatus (NTR 593, Toyama Sangyo) with 900 mL (37 7 0.5 °C) of distilled water that was stirred at 50 rpm using the paddle method described in the Seventeen Edition of the Japanese Pharmacopoeia. CPPU was weighed accurately to 30 mg and placed in the paddle. Dissolution sample (10 mL) was collected at 5, 10, 15, 30, 60, 90, and 120 min through a 0.45 μm membrane filter. Quantitation was performed in the same manner as in the phase solubility studies.

4.2.9. Experimental Cultivation of Broccoli Sprouts. To evaluate the equivalence of the promotion of plant growth by CPPU, cultivation experiments were conducted using a broccoli sprout cultivation kit (Greenfield Project Co., Ltd., Kumamoto). Five hundred seeds were planted in pots and the seedlings were placed in a dark place with a temperature of 25 °C and a humidity of 50%. The length, weight, and thickness of broccoli sprouts were measured every 2 d. On the fifth day, the texture of the plant was evaluated using a texture tester (Nippon Measurement System Co., Ltd., Nara). Measurement was performed at a test speed of 120 mm/min, an upper load limit of 100.00 N, and a rupture detection of 25%; measurements were made continuously.

4.2.10. Statistical Analysis. Data are expressed as the mean ± standard deviation (SD). The groups were compared using one-way analysis of variance followed by Tukey's test for multiple comparison. $p < 0.01$ was considered statistically significant.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CPPU, 1-(2-chloro-4-pyridyl)-3-phenylurea; γCD, γ-cyclodextrin; HPγCD, 2-hydroxypropy-γ-cyclodextrin; PM, physical mixture; GM, ground mixture; CP, coprecipitate; DSC, differential scanning calorimetry; PXRD, powder X-ray diffraction; NIR, near infrared; SEM, scanning electron microscopy; NMR, ^1H nuclear magnetic resonance

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