JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Improvement of Antifungal Activity of 10-Undecyn-1-ol by Inclusion Complexation with Cyclodextrin Derivatives

TZE LOON NEOH,* TAKAHIRO TANIMOTO, SHUICHI IKEFUJI, HIDEFUMI YOSHII, AND TAKESHI FURUTA

Department of Biotechnology, Tottori University, 4-101 Koyamacho-minami, Tottori, 680-8552, Japan

The inclusion complexation behavior between 10-undecyn-1-ol and cyclodextrin (CD) derivatives, namely, randomly methylated β-CD (RM-β-CD) and hydroxypropyl-β-CD (HP-β-CD), was studied in terms of solubility improvement, apparent stability constant, and the inclusion ratios of the resultant inclusion complexes. The aqueous solubility of 10-undecyn-1-ol was greatly improved through complexation with the CD derivatives. RM-β-CD is comparatively more efficient in solubilizing 10-undecyn-1-ol with an apparent stability constant outstripping that of HP-β-CD by about an order of magnitude. Comparative in vitro evaluations of the growth inhibition effects of inclusion complex solutions toward *Rosellinia necatrix*, a phytopathogenic fungus, were performed. In comparison with the positive control, appreciable improvements of the antifungal activity of 10-undecyn-1-ol through the addition of CD derivatives were observed visually. The improvement was evaluated in terms of area covered by the mycelia of *Rosellinia necatrix* and their growth rate. RM-β-CD was proven to be more effective compared to HP-β-CD with regard to the reduction of both fungal mycelium-covered area and growth rate constant, presumably owing to greater solubility enhancement by RM-β-CD and thus the bioavailability of 10-undecyn-1-ol. Inclusion complexation of 10-undecyn-1-ol with CD derivatives suggests a potential means for production of an environmentally friendly 10-undecyn-1-ol-based fungicide to counteract *R. necatrix*.

KEYWORDS: Cyclodextrin derivative; 10-undecyn-1-ol; inclusion complex; antifungal activity; *Rosellinia necatix*

INTRODUCTION

Various derivatives of neem tree or *Azadirachta indica* have potential use in toiletries, pharmaceuticals, the manufacture of agricultural implements and furniture, cattle and poultry feeds, nitrification of soils for various agricultural crops, and pest control (*1*). It is well-known for its pesticidal properties (*2, 3*) and has been used for centuries for medicinal and pest-management purposes (*4*). Results of storage tests mostly indicate that the leaf powder, the seed oil, and all kinds of extracts do indeed have a negative effect on seed-eating insects (*5*). Neem leaf extract was also studied by Govindachari et al. (*6*) for its antifungal activity by spore germination inhibition against *Fusarium oxysporum* and *Colletotrichum lindermuthianum*. The authors correlated the antifungal activity of neem leaf extract to the high concentration of a compound called 10-undecyn-1-ol. 10-Undecyn-1-ol is a chemical substance poorly soluble in water. By increasing its aqueous solubility, it is reasonable that its bioavailability will concurrently be enhanced.

According to Schena et al. (*7*), the white root rot caused by the fungus *Rosellinia necatrix* Prill. (anamorph: *Demathophora necatrix* Hartig) is destructive to many fruit tree species including almond, peach, plum, apple, pear, olive, cherry, and avocado. In Japan, this fungal disease, which spreads rapidly and is very difficult to prevent, has done great damage to commercially grown grapevines, apple and pear trees, and other crops (*8*). In orchards in Japan, 50–100 L of fungicide (active ingredient: fluazinam; concentration = 790 μg/g) are drenched on a single fruit tree at least every other year to control the disease (*9*). However, this use of enormous amounts of fungicides has raised concerns about soil pollution.

Cyclodextrins (CDs) are a family of cyclic oligosaccharides with torus-like molecular structures. The naturally existing CDs are α-, β-, and γ-CD, which consist of six, seven, and eight α-1,4-glycosidic bonded D-glucopyranosyl units, respectively. CDs, owing to their unique physicochemical properties, serve particularly well as carrier molecules of lipophilic organic and inorganic compounds via encapsulation of these compounds within the central cavities, providing shield from the polar forces of aqueous solutions. However, the applicability of natural CDs has been constrained by their relatively low aqueous solubility. As a result, chemical substitutions at the hydroxyl groups with functional groups such as methyl and hydroxypropyl groups

* Corresponding author (telephone +81-857-31-5272; fax +81-857-31-0881; e-mail neoh@bio.tottori-u.ac.jp).

10.1021/jf0731898 CCC: $40.75
Published on Web 05/03/2008

have been introduced, giving rise to various CD derivatives that excel in properties such as encapsulation characteristics and solubility. A number of published works showed that both dissolution properties and consequently microbiological activities of econazole, an antifungal agent with very low water solubility of about 7.86 μM at 25 °C, can be improved by complexation, in particular, with β-CD (*10, 11*). Bioavailability enhancement of drugs by CDs was also discussed in several reviews (*12–15*). As improvement of bioactivity of sparingly soluble drugs by CDs has usually been related to the enhancement of their water solubility in the presence of CD, alkylated and particularly methylated CDs, which were demonstrated to be often more effective as solubilizing and complexing agents than parent CDs (*16, 17*), would reasonably be expected to performed better. Ahmed et al. (*18*) compared the effect of natural CDs (α-CD and β-CD) and heptakis(2,6-di-*O*-methyl)-β-CD (DM-β-CD) on the antimycotic activity of clotrimazole and reported that the solid inclusion complex of clotrimazole with DM-β-CD showed better enhancement of antimycotic activity of clotrimazole as compared with α-CD and β-CD.

In view of the advantages offered by both the CD derivatives and 10-undecyn-1-ol, and the potential benefits their inclusion complex might deliver toward the environment as a fungicide, we have employed CD derivatives to improve the aqueous solubility of 10-undecyn-1-ol to enhance its antifungal property. This paper reports the preparation and characterization of inclusion complexes formed between 10-undecyn-1-ol and CD derivatives, namely, randomly methylated β-CD (RM-β-CD) and 2-hydroxypropyl-β-CD (HP-β-CD). We are especially interested in the solubilization effects of the CD derivatives on 10-undecyn-1-ol, the binding abilities between the host and guest molecules, and the inclusion ratios of the resultant inclusion complexes. In addition, the effect of host−guest complexation on the growth of *R. necatrix* was also investigated to prove the improved antifungal activity by CD derivatives. We also compared the growth inhibition effect between both CD derivatives. These studies are intended to provide fundamental information for production of an environmentally friendly yet effective 10-undecyn-1-ol-based fungicide against *R. necatrix*.

MATERIALS AND METHODS

Materials. Standard grade randomly methylated β-CD (RM-β-CD) with a degree of substitution (DS) of 1.6–1.9 per anhydro glucose unit and 2-hydroxypropyl-β-CD (HP-β-CD) with DS of 0.6–0.9 per anhydro glucose unit from Wacker-Chemie GmbH (Munich, Germany) were purchased from Cyclochem Co., Ltd. (Kobe, Japan). All CD derivatives were dried in vacuo at 90 °C for 24 h prior to use. 10-Undecyn-1-ol (97%) was purchased from GFS Chemicals Inc. (Powell, OH), Frowncide SC from ISK Biosciences K. K. (Tokyo, Japan), chloroform from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and methyl myristate (>98%) from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Potato dextrose agar (PDA) from Daigo, Nihon Pharmaceutical Co., Ltd. (Tokyo, Japan), was purchased from Wako Pure Chemical Industries, Ltd. Distilled water was used throughout the entire study. Unless otherwise stated, all of the chemicals were of analytical reagent grade and were used as received.

Solubility of 10-Undecyn-1-ol in CD Derivative Solutions. CD derivatives (RM-β-CD and HP-β-CD) were weighed and added with distilled water to obtained concentrations of 5, 10, 15, 20, 25, 30, 40, 60, and 80 mM. Solubility studies were performed as described by Higuchi and Connors (*19*). Ten milliliters each of the prepared CD solutions and distilled water were transferred into vials (24 mm in diameter × 50 mm in height) by using a micropipet, and each treatment was prepared in duplicate. Subsequently, an excessive amount of 10-undecyn-1-ol (≈350 μL) was added. These vials were shaken at 180 rpm in the BR-15LF bioshaker (Taitec Corp., Saitama, Japan) at 25 °C for 5 days to attain equilibrium. After shaking, the saturated solutions of 10-undecyn-1-ol were centrifuged at 3000 rpm for 5 min. The supernatant liquid of undissolved 10-undecyn-1-ol was first removed using a micropipet. Four milliliters of the aqueous phase was transferred into a screw-capped test tube and added with 2 mL of chloroform containing methyl myristate (0.05% v/v) as internal standard for solvent extraction. Extraction was performed at 90 °C for 20 min with intermittent vigorous vortexing of the mixtures. The mixtures were centrifuged at 3000 rpm for 15 min after extraction.

The concentration of 10-undecyn-1-ol in chloroform was quantified by gas chromatography. Briefly, two microliters of the chloroform portion of each sample was injected in duplicate into the Shimadzu GC-14A gas chromatograph (Shimadzu Corp., Kyoto, Japan) installed with a flame ionization detector (FID) and a 2 m long × 3.2 mm i.d. glass column packed with 5% Advance-DS on Shinchrom A, 80/100 mesh (Shimadzu Corp.). Injection port, column, and detector temperatures were set constant at 180, 150, and 200 °C, respectively. Nitrogen was flowed as carrier gas at a constant flow rate of 50 mL/min.

Determination of Stability Constant *K′* by Solubility Method. This method was first reported by Higuchi and Connors (*19*) and later applied by several authors (*20–22*) to study the phase solubility of host−guest systems of CDs and various guest molecules. The apparent 1:1 stability constants, K' (mM^{-1}), of 10-undecyn-1-ol/CD derivative systems were calculated using the equation

$$K' = \frac{\text{slope}}{C_0(1 - \text{slope})} \quad (1)$$

where K' (mM^{-1}) denotes the apparent stability constant, slope denotes the inclination of the linear correlation in the phase solubility diagram, and C_0 (mM) denotes the equilibrium solubility of 10-undecyn-1-ol in the absence of CD derivatives, which is obtainable from the phase solubility diagram as the intercept at *y*-axis. In other words, the C_0 value represents the saturation concentration of 10-undecyn-1-ol in distilled water.

Preparation of Inclusion Complex Powder. One gram of CD derivative was dissolved into 1 mL of distilled water in a vial (24 mm in diameter × 50 mm in height). 10-Undecyn-1-ol was then added in an amount of from 0.1 to 5 molar times with respect to the amount of CD derivative. This molar ratio of 10-undecyn-1-ol/CD derivative is hereafter denoted "molar ratio during complexation". The vials were capped and shaken at 150 rpm in the BR-15LF bioshaker (Taitec Corp.) at 25 °C for 12 h. The solutions were then lyophilized for 12 h and further dried in vacuo at 90 °C for 24 h. The inclusion complexes collected were pounded into powdery form. For quantification of 10-undecyn-1-ol encapsulated in CD derivative, 0.1 g of the inclusion complex powder was added with 2 mL of methyl myristate-containing chloroform (0.05% v/v) and 4 mL of distilled water to extract the encapsulated 10-undecyn-1-ol. The methods of extraction and gas chromatography were identical to those explained in the solubility study. The molar ratio of 10-undecyn-1-ol to CD derivative in the inclusion complex powder was defined as "inclusion ratio" in the unit of moles of 10-undecyn-1-ol per mole of CD derivative.

Preparation of Inclusion Complex Solution. Aqueous mixtures of 10-undecyn-1-ol and CD derivative were prepared in a 1 mL volumetric flask. CD derivative was first dissolved into a small amount of sterilized water in the volumetric flask into which respective prescribed volumes of 52, 103, and 155 μL of 10-undecyn-1-ol were subsequently added. Each of the prescribed volume was determined on the basis of the respective final 10-undecyn-1-ol concentrations of 5, 10, and 15% v/v in the inclusion complex solutions. The amounts of CD derivative used were computed in such a way that the molar ratios were obtained at 0.1, 0.2, 0.3, 0.4, and 0.5 mol CD derivative/mol 10-undecyn-1-ol. To avoid confusion with the inclusion ratios of inclusion complex powders, the content of CD derivative in the inclusion complex solutions was identified in terms of molar ratio of CD derivative to 10-undecyn-1-ol. The mixtures were finally topped up to a final volume of 1 mL with distilled water. The aqueous mixtures were subjected to 4 h of ultrasonication in the Bransonic Ultrasonic Cleaner (1510J-MT, Branson Ultrasonic Corp., Danbury, CT) and followed by shaking in the BR-15LF bioshaker at 180 rpm, 25 °C, for 20 h.

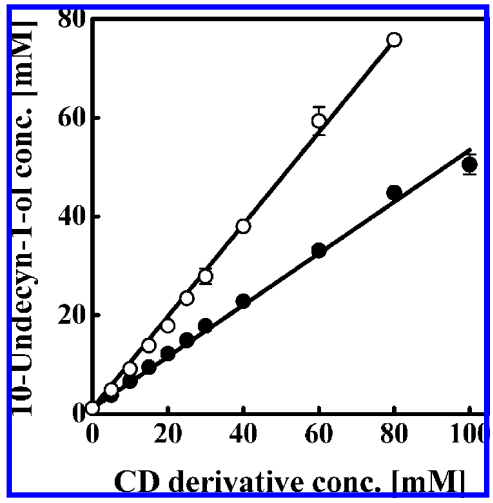

Figure 1. Phase solubility diagrams of the 10-undecyn-1-ol/RM-β-CD system (open symbol) and 10-undecyn-1-ol/HP-β-CD system (solid symbol) in water at 25 °C.

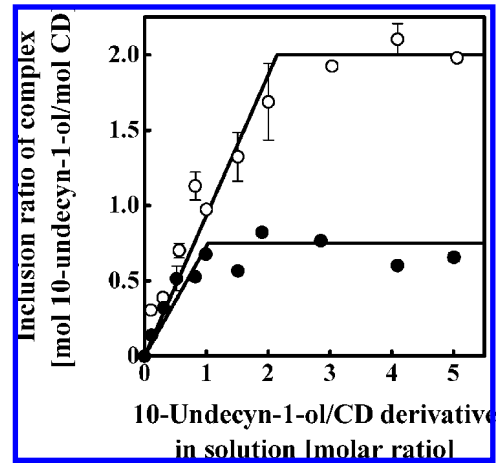

Figure 2. Inclusion ratios of the 10-undecyn-1-ol/RM-β-CD inclusion complex (open symbol) and 10-undecyn-1-ol/HP-β-CD inclusion complex (solid symbol) obtained at various 10-undecyn-1-ol/CD derivative ratios in solution during preparation of inclusion complex powders.

Growth Inhibition Effect of 10-Undecyn-1-ol on *R. necatrix*. PDA plates were prepared from a PDA medium with a concentration of 39 g/L using plastic Petri dishes that measured 9 cm in diameter. Each PDA plate contained approximately 20 mL of PDA medium. *R. necatrix* was kindly provided by Dr. Fumitoshi Yasuda of Tottori Horticultural Experiment Station (Tottori, Japan). The culture solution of roughly 2-week-incubated *R. necatrix* (5 mg of fungal mycelium/mL) of 200 μL was spread on each PDA plate. The ADVANTEC 5A filter papers (Toyo Roshi Kaisha, Ltd., Tokyo, Japan), which measured 2.5 cm in diameter, were placed on the center of the plates, and 200 μL of either sterilized water or the previously prepared inclusion complex solutions was soaked onto the filter papers. As such, all of the treatments could basically be classified into three main groups based on the final added amount of 10-undecyn-1-ol, namely 10, 20, and 30 μL. After that, the plates were covered, sealed with parafilm, and incubated in a 25 °C incubator (MIR-253, Sanyo Electric Biomedical Co., Ltd., Tokyo, Japan). Each treatment was prepared in triplicate. In a preliminary study, 10 μL of 10-undecyn-1-ol, which weighed approximately 8.5 mg, was compared against an equivalent weight of fluazinam [3-chloro-*N*-(3-chloro-5-trifluoromethyl-2-pyridyl)-α,α,α-trifluoro-2,6-dinitro-*p*-toluidine]. Similarly, each treatment was prepared in triplicate. All of the PDA plates were incubated to the longest of 13 days. During the incubation period, digital images of the PDA plates were taken with the Olympus C-3040 Zoom digital camera (Olympus Corp., Tokyo, Japan) for image processing and analysis using *Scion Image* software (release Alpha 4.0.3.2, Scion Corp., Frederick, MD).

RESULTS AND DISCUSSION

Phase Solubility. Phase solubility studies of 10-undecyn-1-ol in binary systems with CD derivatives were performed to obtain information on the stability of the inclusion complexes in aqueous phase. **Figure 1** shows the phase solubility diagrams of 10-undecyn-1-ol at 25 °C in aqueous RM-β-CD and HP-β-CD. 10-Undecyn-1-ol is sparingly soluble in water with a solubility C_0 of 1.14 ± 0.02 mM. The solubility of 10-undecyn-1-ol increased with increasing concentration of CD derivatives. As compared to the solubility of 10-undecyn-1-ol in distilled water in the absence of CD derivatives, linear increases up to 68- and 47-fold in 10-undecyn-1-ol solubility were achieved in the respective tested concentration ranges of RM-β-CD (80 mM) and HP-β-CD (100 mM), with no solubility limits observed. Solubility enhancement by CDs, both native and derivative, at the highest 152-fold for natamycin (*23*), 5-fold for norflurazon (*24*), 4.7-fold for iprodione (*25*), and 4-fold for permethrin (*26*), has been reported. The increase in solubility may partly be attributed to the complex formation with the CD derivatives. Comparing the two CD derivatives, RM-β-CD was apparently more effective in solubilizing 10-undecyn-1-ol. As shown in **Figure 1**, the correlations were strictly linear showing formation of inclusion complexes of constant compositions, which are commonly classified as the "A_L" type isotherms. From the results, it is known that both inclusion complexes were still soluble within the range of concentrations studied as no plateau was observable in the isotherms. Szejtli (*21*) reported a similar solubility isotherm with an unlimited linear solubility increase in an aqueous system comprising dimethyl-β-CD and methyltestosterone.

The 1:1 apparent stability constants K' of these inclusion complexes were calculated from the phase solubility diagrams using eq 1. The computed values were 10.7 and 1.03 mM^{-1} for RM-β-CD and HP-β-CD, respectively. The high apparent stability constants suggest strong affinities between 10-undecyn-1-ol and the CD derivatives in aqueous phase and the high stability of the complexes. Higher K' value for 10-undecyn-1-ol/RM-β-CD system suggested a comparatively more readily occurring and stable complexation in the system. Besides hydrophobicity, the inclusion complexation and thus the K' value may also be affected by some other factors, for instance, the steric conformation of the guest compound. The apparent stability constant of the diclofop-methyl/RM-β-CD complex was 4.74 mM^{-1} (*27*), only around two-fifths that of the 10-undecyn-1-ol/RM-β-CD complex regardless of the higher hydrophobicity of diclofop-methyl (water solubility = 8.8×10^{-3} mM at pH 7 and 25 °C) relative to that of 10-undecyn-1-ol. The high K' values calculated in this study may presumably be related to the elongated molecular structure of 10-undecyn-1-ol, which eases its interaction with the CD cavity.

Formation of Inclusion Complex Powder. Figure 2 shows the relationship between inclusion ratios of inclusion complex powders and the molar ratio of 10-undecyn-1-ol to CD derivatives in solution during complexation. At low molar ratios, the inclusion ratios increased in response to the increase of 10-undecyn-1-ol to CD derivative ratio in solution during complexation. The inclusion ratios increased up to certain values, after which leveling off occurred despite the continual rise in the molar ratio during complexation. Maximum inclusion ratios were achieved at molar ratios during complexation of 2 and 1 for RM-β-CD and HP-β-CD, respectively.

The maximum inclusion ratios were 1.85 and 0.65 mol 10-undecyn-1-ol/mol CD derivative for inclusion complexes from

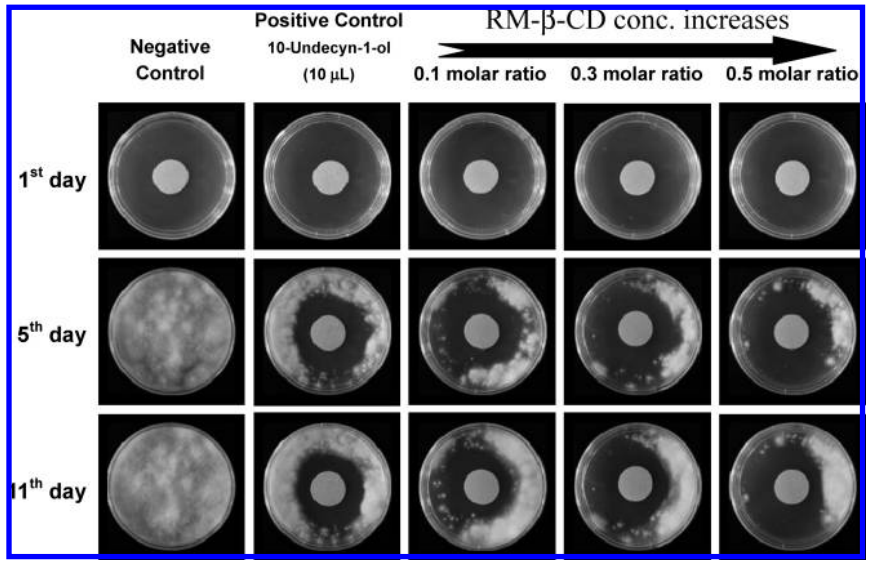

Figure 3. Improvement of growth inhibition effect of 10-undecyn-1-ol on *Rosellinia necatrix* by the increase of RM-β-CD concentration in inclusion complex solution.

RM-β-CD and HP-β-CD, respectively. A single molecule of RM-β-CD was found to be capable of encapsulating a maximum number of two 10-undecyn-1-ol molecules. Meanwhile, HP-β-CD was able to include only one 10-undecyn-1-ol molecule to the maximum with more than one-fourth of the encapsulant molecules existing in noncomplexed form. In agreement with the solubility phase diagram, HP-β-CD was capable of solubilizing 10-undecyn-1-ol only up to three-fourths of a mole of its own amount.

In the system of 10-undecyn-1-ol and RM-β-CD, irrespective of the fact that the encapsulant was able to solubilize an equimolar amount of 10-udecyn-1-ol in aqueous phase, an inclusion complex of 1:2 host to guest ratio was obtained. Although the mechanism is still unclear, the possible reason that brought up this rather unexpected result is postulated to be that the inclusion of the second 10-undecyn-1-ol molecule into the cavity preresided by another 10-undecyn-1-ol molecule somehow takes place during the lyophilization process.

Effect of 10-Undecyn-1-ol on mycelial growth of *R. necatrix*. In the preliminary study, 10-undecyn-1-ol was compared against fluazinam—the active ingredient in a fungicide with the trade name of Frowncide SC—which is currently in use to counteract the white root rot disease. The results indicated that 10-undecyn-1-ol was equally as effective as fluazinam (data not shown). However, as a naturally occurring compound, 10-undecyn-1-ol provides a more preferable choice for practical use as fewer burdens will be caused to the environment, especially in terms of pollution.

Figure 3 illustrates the inhibition effect of 10-undecyn-1-ol and its inclusion complex solutions with RM-β-CD on the mycelial growth of *R. necatrix*. The leftmost column is the negative control treatment, which contained no 10-undecyn-1-ol, whereas the second column from the left was the positive control treated with 10 μL of 10-undecyn-1-ol concentrate. The rest are those treated with 10-undecyn-1-ol/RM-β-CD inclusion complex solutions with molar ratios of RM-β-CD to 10-undecyn-1-ol of 0.1, 0.3, and 0.5 arranged in ascending order from the left. Except for the negative control, all of the treatments involved the addition of an identical amount of 10 μL of 10-undecyn-1-ol.

In the negative control, the fungus was visible with the naked eye covering the whole surface of PDA medium upon the fifth day of incubation. Although growth of the fungus could be observed since then, changes were trivial. In the positive control, the fungus also started to be visible from the fifth day of incubation. However, its growth was limited only to the edge of the plates. With time, the fungus grew radially inward and on the 11th day, a clear fungus-free circle could be observed in the center of the plates. Treatment with aqueous mixtures of RM-β-CD and 10-undecyn-1-ol at molar ratios of 0.1 produced sounder retardation effect on the growth as the fungus was observed only at one side of the edge. As the molar ratios rose higher to 0.3 and 0.5, the mycelial growth of *R. necatrix* was further retarded. The increase in the amount of dissolved RM-β-CD improved the inhibition effect of 10-undecyn-1-ol on mycelial growth of the fungus.

A combination of digital image analysis and fitting of Weibull's equation to the empirical data was employed to enable numerical analysis of the inhibition effect of 10-undecyn-1-ol. The mycelium-covered area in the PDA plate S was defined as

$$S = S_0 - (S_1 + S_p) \quad (2)$$

where S_0 (cm^2) is the surface area of the PDA medium, S_1 (cm^2) the non-mycelium-covered area, and S_p (cm^2) the surface area covered by the filter paper that measures 2.5 cm in diameter. The dimensionless relative mycelium-covered area A, given by normalization against the surface area of the PDA medium (S/S_0), was plotted against the incubation time for subsequent numerical analyses.

Weibull's equation was fitted to the calculated data of relative mycelium-covered area against the incubation time

$$A = A_{max}[1 - \exp(-[kt]^n)] \quad (3)$$

where A_{max} is the maximum relative mycelium-covered area, k (day^{-1}) the growth rate constant, t (days) the incubation time, and n the growth mechanism parameter, which gave satisfactory fitting at a fixed value of 2.

Figure 4, panels **a**, **b**, and **c** present the proliferation curves of *R. necatrix* under the respective treatments of 10, 20, and 30 μL of 10-undecyn-1-ol with different contents of RM-β-CD. Growth inhibition effect of 10-undecyn-1-ol was observed to have improved with the increase in 10-undecyn-1-ol concentration in the inclusion complex solutions. Besides, the increase of RM-β-CD concentration from 0 to 0.5 mol/mol 10-undecyn-

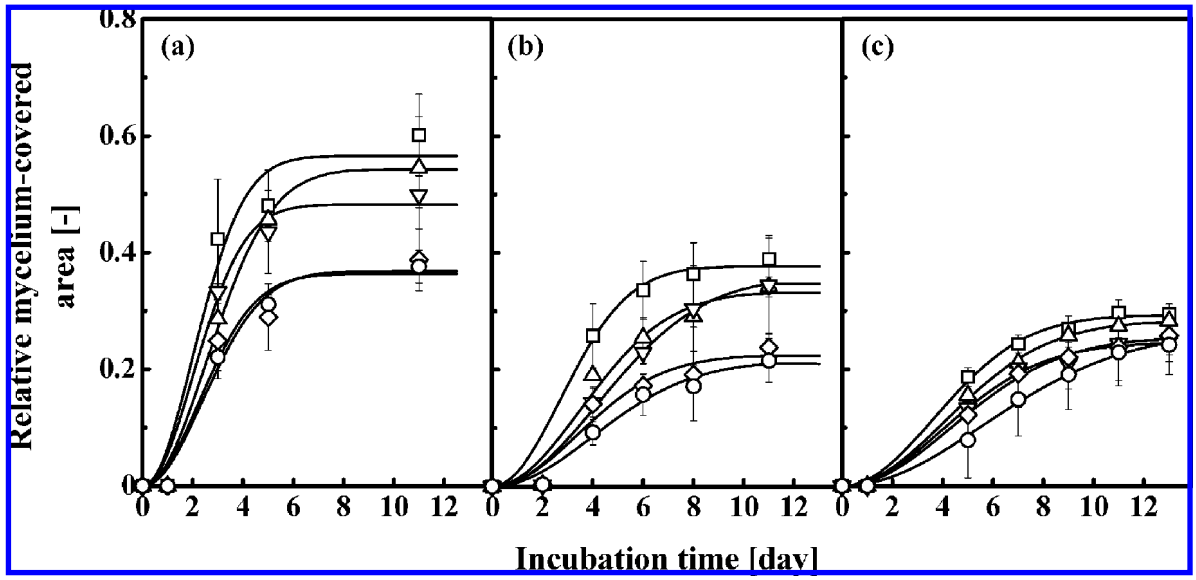

Figure 4. Proliferation curves of *Rosellinia necatrix* under treatments of various concentrations of 10-undecyn-1-ol and RM-β-CD in the inclusion complex solutions. The amount of 10-undecyn-1-ol was varied from (**a**) 10 μL to (**b**) 20 μL to (**c**) 30 μL. The molar ratios of RM-β-CD to 10-undecyn-1-ol in the inclusion complex solutions were 0 (□), 0.2 (△), 0.3 (▽), 0.4 (◇), and 0.5 (○). Incubation was carried out at 25 °C. The error bars represent the standard deviations of the triplicate PDA plates for each treatment.

Table 1. Maximum Relative Mycelium-Covered Area A_{max} as a Function of the RM-β-CD to 10-Undecyn-1-ol Ratio of the Inclusion Complex Solutions Used in Treatments of 10, 20, and 30 μL of 10-Undecyn-1-ol

	A_{max}		
RM-β-CD/10-undecyn-1-ol (molar ratio)	10 μL	20 μL	30 μL
0	0.56	0.39	0.29
0.1	0.48	0.35	0.41
0.2	0.54	0.32	0.28
0.3	0.48	0.34	0.25
0.4	0.37	0.22	0.25
0.5	0.37	0.21	0.25

1-ol was also observed to have improved the growth inhibition effect. A similar result was reported with iprodione in that its complexation with β-CD resulted in a >2-fold increase in antifungal activity (*25*). However, there was also a study that reported only sustainment of the in vitro antifungal activity for inclusion-complexed natamycin despite the resulting solubility enhancement (*23*). In the treatment with 20 μL of 10-undecyn-1-ol, the relative mycelium-covered area was reduced the greatest by half by the addition of RM-β-CD compared to the positive control sample. The maximum relative mycelium-covered area A_{max} of each treatment is summarized in **Table 1** to give a clearer picture of the effect of RM-β-CD in inhibiting the growth of *R. necatrix*. A_{max} decreased linearly with increasing molar ratio of the inclusion complex solutions. In the case of 10 μL of 10-undecyn-1-ol, a maximum of 35% reduction in A_{max} was achieved, whereas an A_{max} reduction of up to 50% was achieved in the treatment of 20 μL of 10-undecyn-1-ol. However, as for the case of 30 μL of 10-undecyn-1-ol, the weakening of the inhibition effect was observed probably due to the limitation in Petri dish size where the decrease of A_{max} values with the addition of RM-β-CD was less significant. The growth rate constants of *R. necatrix* in the positive control treatments with only 10-undecyn-1-ol were denoted k_0. The relative values of growth rate constants to those of the corresponding positive control samples (k/k_0) are presented as a function of the molar ratio of inclusion complex solution in **Figure 5**. Besides reducing mycelial coverage, RM-β-CD also managed to slow the growth of *R. necatrix* with regard to the

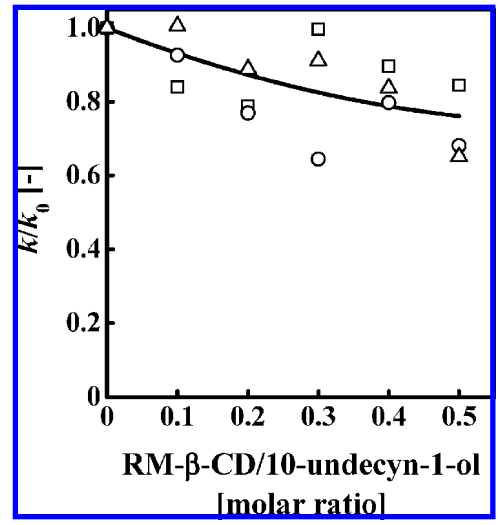

Figure 5. Relative growth rate constant k/k_0 as a function of the RM-β-CD to 10-undecyn-1-ol ratio of the inclusion complex solutions used in treatments of 10 μL (□), 20 μL (○), and 30 μL (△) of 10-undecyn-1-ol.

positive control treatments. Similarly, the addition of HP-β-CD also revealed improvement in growth inhibition effect of 10-undecyn-1-ol (data not shown). However, HP-β-CD was generally less effective than RM-β-CD in terms of both A_{max} reduction and relative growth rate constant. This could plausibly be due to the lower aqueous solubility of HP-β-CD.

The strengthening of growth inhibition effect by CD derivatives could possibly be explained by the improved bioavailability of 10-undecyn-1-ol caused by the improvement of its water solubility by the presence of CD derivatives. With the solubility being an important factor that influences the foliar translocation and penetration of systemic herbicides and fungicides, the inclusion complex of isoproturon, a herbicide, with β-CD has been prepared and characterized (*28*). There are also several studies being conducted on the basis of the improvement effect on the bioavailability of poorly soluble bioactive compounds through solubility enhancement by inclusion complexation with CDs (*29–32*). According to Loftsson and Masson (*33*), CDs, in

general, enhance topical drug delivery by increasing the drug availability at lipophilic biological barrier surface. At the surface, the drug molecules partition from the CD cavity into the lipophilic barrier. Athanassiou et al. (*34*) also discussed several mechanisms that might be responsible for the increased antimicrobial activity of β-lactam via complexation with CD. The mechanisms proposed include (a) protection of drug molecules due to inclusion complexation, (b) increase of diffusion rates of complexed drug molecules through the outer membrane of bacteria, and (c) destabilization of the outer membrane of bacteria by CD, which eventually leads to an enhancement of cell membrane permeability and, thus, diffusion rates of various drugs.

An important conclusion that can be drawn from the present study is that the complexation of 10-undecyn-1-ol with CD derivatives promoted the mycelial growth retardation effect of 10-undecyn-1-ol on *R. necatrix*. Nonetheless, the mechanisms of action are still unclear, and a detailed study would be necessary to elucidate the entire process. Inclusion complexation of 10-undecyn-1-ol with CD derivatives provides a potential means through which not only could the fungicidal actions of 10-undecyn-1-ol be improved but also the environmental burdens of synthetic agrochemicals could be alleviated.

ACKNOWLEDGMENT

We are grateful to Dr. Fumitoshi Yasuda of Tottori Horticultural Experiment Station for the gift of *Rosellinia necatrix*.

LITERATURE CITED

(1) Koul, O.; Isman, M. B.; Ketkar, C. M. Properties and uses of neem, *Azadirachta indica*. *Can. J. Bot.* **1990**, *68*, 1–11.
(2) Schmutterer, H. Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. *Annu. Rev. Entomol.* **1990**, *35*, 271–297.
(3) Liu, Y.; Chen, G. S.; Chen, Y.; Lin, J. Inclusion complexes of azadiractin with native and methylated cyclodextrins: solubilization and binding ability. *Bioorg. Med. Chem.* **2005**, *13*, 4037–4042.
(4) Kaushik, N.; Vir, S. Variations in fatty acid composition of neem seeds collected from the Rajasthan state of India. *Biochem. Soc. Trans.* **2000**, *28*, 880–882.
(5) Boeke, S. J.; Boersma, M. G.; Alink, G. M.; van Loon, J. J. A.; van Huis, A.; Dicke, M.; Rietjens, I. M. C. M. Safety evaluation of neem (*Azadirachta indica*) derived pesticides. *J. Ethnopharmacol.* **2004**, *94*, 25–41.
(6) Govindachari, T. R.; Suresh, G.; Masilamani, S. Antifungal activity of Azadirachta indica leaf hexane extract. *Fitoterapia* **1999**, *4*, 417–420.
(7) Schena, L.; Nigro, F.; Ippolito, A. Identification and detection of *Rosellinia necatrix* by conventional and real-time Scorpion-PCR. *Eur. J. Plant Pathol.* **2002**, *108*, 355–366.
(8) Kanda, S.; Ishiguro, M.; Kano, S.; Aimi, T.; Kitamoto, Y.; Morinaga, T. Heterothallic life cycle in the white root rot fungus *Rosellinia necatrix*. *Mycoscience* **2003**, *44*, 389–395.
(9) Kanadani, G.; Date, H.; Nasu, H. Effect of fluazinam soil-drench on white root rot of grapevine. *Ann. Phytopathol. Soc. Jpn.* **1998**, *64*, 139–141.
(10) Mura, P.; Liguori, A.; Bramanti, G.; Campisi, E.; Faggi, E. Improvement of dissolution properties and microbiological activity of miconazole and econazole by cyclodextrin complexation. *Eur. J. Pharm. Biopharm.* **1992**, *3*, 119–123.
(11) Pedersen, M.; Edelsten, M.; Nielsen, V. F.; Scarpellini, A.; Skytte, S.; Slot, C. Formation and antimycotic effect of cyclodextrin inclusion complexes of econazole and miconazole. *Int. J. Pharm.* **1993**, *90*, 247–254.
(12) Szejtli, J. Cyclodextrin complexed generic drugs are generally not bio-equivalent with the reference products: therefore the increase in number of marketed drug/cyclodextrin formulations is so slow. *J. Incl. Phenom. Macrocycl. Chem.* **2005**, *52*, 1–11.
(13) Martin Del Valle, E. M. Cyclodextrins and their uses: a review. *Process Biochem.* **2004**, *39*, 1033–1046.
(14) Singh, M.; Sharma, R.; Banerjee, U. C. Biotechnological applications of cyclodextrins. *Biotechnol. Adv.* **2002**, *20*, 341–359.
(15) Chaubal, M. V. Application of formulation technologies in lead candidate selection and optimization. *Drug Discovery Today* **2004**, *9*, 603–609.
(16) Uekama, K.; Irie, T. Pharmaceutical applications of methylated cyclodextrin derivatives. In *Cyclodextrins and their Industrial Uses*; Duchêne, D., Ed.; Editions De Santé: Paris, France, 1987; pp 393–439.
(17) Müller, B. W.; Brauns, U.; Backensfeld, T. Cyclodextrin derivates for solubilization, stabilization and absorption of drugs. In *Proceedings of the Fourth International Symposium on Cyclodextrin*; Huber, D., Szejtli, T., Eds.; Munich, Germany, Kluwer Academic Publishers: Dordrecht, The Netherlands, 1988; pp 369–382.
(18) Ahmed, M. O.; El-Gibaly, I.; Ahmed, S. M. Effect of cyclodextrins on the physicochemical properties and antimycotic activity of clotrimazole. *Int. J. Pharm.* **1998**, *171*, 111–121.
(19) Higuchi, T.; Connors, K. Phase solubility techniques. In *Advances in Analytical Chemistry Instrumentation*; Reilly, C. N., Ed.; Wiley Interscience: New York, 1965; Vol. 4, pp 117–212.
(20) Tomono, K.; Goto, H.; Suzuki, T.; Ueda, H.; Nagai, T.; Watanabe, J. Interaction of iodine with 2-hydroxypropyl-α-cyclodextrin and its bactericidal activity. *Drug Dev. Ind. Pharm.* **2002**, *28*, 1303–1309.
(21) Szejtli, J. Cyclodextrins in pharmaceuticals. In *Cyclodextrin Technology*; Davies, J. E. D., Atwood, J. L., Iwamoto, T., Li, N. N., Lipkowski, J.; MacNicol, D. D., Saenger, W., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1988; pp 186–293.
(22) Linares, M.; Bertorello, M. D.; Longhi, M. Solubility of a isoxazolyl-naphthoquinone by complexation with hydroxypropyl-β-cyclodextrin. In *Proceedings of the Ninth International Symposium on Cyclodextrins*; Torres Labandeira, J. J., Vila-Jato, J. L., Eds.; Santiago de Compostela, Spain, Kluwer Academic Publishers: Dordrecht, The Netherlands, 1998; pp 207–210.
(23) Koontz, J. L.; Marcy, J. E. Formation of natamycin:cyclodextrin inclusion complexes and their characterization. *J. Agric. Food Chem.* **2003**, *51*, 7106–7110.
(24) Villaverde, J.; Morillo, E.; Pérez-Martínez, J. I.; Ginés, J. M.; Maqueda, C. Preparation and characterization of inclusion complex of norflurazon and β-cyclodextrin to improve herbicide formulations. *J. Agric. Food Chem.* **2004**, *52*, 864–869.
(25) Zhu, X. L.; Wang, H. B.; Chen, Q.; Yang, W. C.; Yang, G. F. Preparation and characterization of inclusion complex of iprodione and β-cyclodextrin to improve fungicidal activity. *J. Agric. Food Chem.* **2007**, *55*, 3535–3539.
(26) Yang, G. F.; Wang, H. B.; Yang, W. C.; Gao, D.; Zhan, C. G. Bioactive permethrin/β-cyclodextrin inclusion complex. *J. Phys. Chem. B* **2006**, *110*, 7044–7048.
(27) Cai, X. Y.; Liu, W. P.; Chen, S. W. Environmental effects of inclusion complexation between methylated β-cyclodextrin and diclofop-methyl. *J. Agric. Food Chem.* **2005**, *53*, 6744–6749.
(28) Manolikar, M. K.; Sawant, M. R. Study of solubility of isoproturon by its complexation with β-cyclodextrin. *Chemosphere* **2003**, *51*, 811–816.
(29) Lezcano, M.; Al-Soufi, W.; Novo, M.; Rodríguez-Núñez, E.; Tato, J. V. Complexation of several benzimidazole-type fungicides with α- and β-cyclodextrins. *J. Agric. Food Chem.* **2002**, *50*, 108–112.
(30) Lezcano, M.; Novo, M.; Al-Soufi, W.; Rodríguez-Núñez, E.; Tato, J. V. Complexation of several fungicides with β-cyclodextrin: determination of the association constants and isolation of the solid complexes. *J. Agric. Food Chem.* **2003**, *51*, 5036–5040.

(31) Consonni, R.; Recca, T.; Dettori, M. A.; Fabbri, D.; Delogu, G. Structural characterization of imazalil/β-cyclodextrin inclusion complex. *J. Agric. Food Chem.* **2004**, *52*, 1590–1593.

(32) Schirra, M.; Delogu, G.; Cabras, P.; Angioni, A.; D'hallewin, G.; Veyrat, A.; Marcos, J. F.; Candelas, L. G. Complexation of imazalil with β-cyclodextrin, residue uptake, persistence, and activity against *Penicillium* decay in citrus fruit following postharvest dip treatments. *J. Agric. Food Chem.* **2002**, *50*, 6790–6797.

(33) Loftsson, T.; Masson, M. Cyclodextrins in topical drug formulations: theory and practice. *Int. J. Pharm.* **2001**, *225*, 15–30.

(34) Athanassiou, G.; Michaleas, S.; Lada-Chitiroglou, E.; Tsitsa, T.; Antoniadou-Vyza, E. Antimicrobial activity of β-lactam antibiotics against clinical pathogens after molecular inclusion in several cyclodextrins. A novel approach to bacterial resistance. *J. Pharm. Pharmacol.* **2003**, *55*, 291–300.

Received for review October 31, 2007. Revised manuscript received January 21, 2008. Accepted January 24, 2008. This study is financially supported by a Grant-in-Aid for Scientific Research (c) (17580110). T.L.N. is indebted to the Japanese government for a Monbukagakusho Scholarship.

JF0731898