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Inclusion Complex of Conjugated Linoleic Acid (CLA) with Cyclodextrins

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Conjugated linoleic acid (CLA) inclusion complexes with α-cyclodextrin (α-CD), *β*-cyclodextrin (*β*-CD), and *γ*-cyclodextrin (*γ*-CD) (designated CLA/CDs inclusion complexes) were prepared to determine the mole ratio of CLA complexed with CDs and the oxidative stability of CLA in the CLA/CDs inclusion complexes. When measured by GC, ^{1}H NMR, and T_1 value analyses, 1 mole of CLA was complexed with 5 mol of α-CD, 4 mol of *β*-CD, and 2 mol of *γ*-CD. The oxidation of CLA induced at 35 °C for 80 h was completely prevented by the formation of CLA/CDs inclusion complexes.

KEYWORDS: Conjugated linoleic acid (CLA); cyclodextrins (CDs); inclusion complexes of CLA with CDs; oxidation

INTRODUCTION

Conjugated linoleic acid (CLA) exhibits anticarcinogenic activity for several carcinogen-induced animal models (*1*−*6*) and human cancer cells (*7*−*10*) as well as other significant biological activities, such as immune stimulation (*11*, *12*), body fat reduction (*13*, *14*), and cholesterol reduction in blood (*15*). CLA is a collective term for several positional (9,11; 10,12) and geometric (*c*,*t*; *t*,*c*; *c*,*c*; and *t*,*t*) configurations of octadecadienoic acid (C18:2) with a conjugated double-bond system (*16*). CLA chemically synthesized from linoleic acid by alkaline isomerization contains approximately 47.2% *c*9,*t*11 CLA and 50.7% *t*10,*c*12 CLA isomers (*17*). The conjugated double-bond system of CLA might provide specific biological functions given for CLA, yet it is susceptible to oxygen, resulting in the oxidation of CLA.

In vitro, the free CLA is oxidized as rapidly as linoleic acid (*18*−*20*). However, the matrices containing esterified CLA in lipid fractions such as phospholipids and triglycerides are resistant to oxygen, relative to those containing unesterified CLA. For example, the oxidation of mammary gland tissues, containing esterified CLA from rats fed CLA, is slowed, relative to control, when evaluated by the thiobarbituric acid reactive substances (TBARS) assay (*3*). In addition, TBARS values in meat from pigs and chickens and in eggs from hens, fed substantial amounts of CLA, are also significantly reduced during storage at 4 °C, compared to those of control samples (*21*−*23*). These studies suggest that free CLA must be protected from oxidation for use in food as a fortifier or additive.

In our previous paper (*19*), oxidation of CLA microencapsulated with α-cyclodextrin (α-CD), *β*-cyclodextrin (*β*-CD), or *γ*-cyclodextrin (*γ*-CD) was greatly reduced, but the magnitude of the protective efficacy of the α-CD, *β*-CD, and *γ*-CD (designated CDs) was in the order α-CD > *β*-CD > *γ*-CD. The exact reason CDs exhibit such different protective effects has not been elucidated to date. In addition, no report for the formation of inclusion complexes of CLA with CDs is available in the literature, and thus the structure and relative orientation of the CLA within the cavity of CDs are unknown.

In the present study, the stoichiometry of the CLA complexed with CDs and the oxidative stability of CLA in CLA inclusion complexes with CDs (designated CLA/CDs inclusion complexes) were examined. Gas chromatographic (GC), ^{1}H NMR spectroscopic, and proton T_1 analyses were carried out to arrive at the stoichiometry. Headspace oxygen depletion was measured to evaluate the oxidation stability of CLA in CLA/CDs inclusion complexes.

MATERIALS AND METHODS

Materials. Linoleic acid (99%) was obtained from Nu Check PREP Inc. (Elysian, MN). CLA, composed of 47.2% *c*9,*t*11 CLA and 50.7% *t*10,*c*12 CLA, was synthesized from linoleic acid by alkaline isomerization at 180 °C (*17*). α-, *β*-, and *γ*-CDs were generously provided by Dr. Matsui (Shimane University, Japan). Deuterated dimethyl-d_6 sulfoxide (DMSO-d_6, 99.96 atom % D) and petroleum ether were obtained from Aldrich Chemical Co. (Milwaukee, WI). Serum brown bottles (50 mL), open-top aluminum seals, and silicone septa were obtained from Wheaton (Millville, NJ). Other chemicals used were of reagent grade.

Preparation of CLA/CDs Complexes. *β*-CD was used in the determination of optimal conditions for the preparation of CLA/CDs complexes, containing both adsorbed and included CLA. CLA (0.4 g;

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1.43 mmol) was dissolved in 96% aqueous ethanol (25.0 mL) in an Erlenmyer flask (200 mL) and then mixed with β-CD (2.0 g; 1.69 mmol) suspended in distilled water (25.0 mL, 40 °C). The temperature of the mixture was increased to proper temperatures (50, 70, and 90 °C) for 5 min and maintained for periods of time (0, 1, 2, and 4 h) to complex CLA with β-CD, followed by cooling to 25 °C for given durations (1, 2, 4, and 8 h). All processes were conducted under nitrogen during mixing with a magnetic stirrer bar. The CLA/β-CD complex was recovered by centrifugation (10000 rpm) at 4 °C for 10 min and used for the preparation of CLA/β-CD inclusion complex, which contains only CLA inserted into the cavity of β-CD. The optimal condition for the preparation of the CLA/β-CD complex determined was applied to the preparation of CLA/α-CD and CLA/γ-CD complexes.

Preparation of CLA/CDs Inclusion Complexes. CLA/CDs inclusion complexes were prepared by removing adsorbed CLA from the CLA/CDs complexes. The CLA/CD complex samples, suspended in appropriate solvents (petroleum ether, methanol, ethanol, and diethyl ether) at a ratio of 1.0 g of sample/20 mL, were vortexed for 10 s to remove the adsorbed CLA from the CLA/CDs complexes. The CLA/CDs inclusion complexes were recovered from the solvent by centrifugation (10000 rpm) at 4 °C for 10 min. These CLA-removing steps were repeated five times. CLA content in solvents was measured by GC as described below.

Determination of the Mole Ratio of CLA to CDs in CLA/CDs Inclusion Complexes. CLA/CDs inclusion complexes were prepared from 1.43 mmol of CLA and an appropriate amount of α-, β-, or γ-CD to obtain a 1:1, 1:2, 1:4, or 1:6 mole ratio of CLA/CDs. CLA was extracted from CLA/CDs inclusion complexes and suspended in hot water at a ratio of 1.0 g of sample/50 mL in a flat-bottom flask, by stirring with a magnetic stirrer bar for 1 min. The CLA amount in solvents was measured by GC as described below. For ^{1}H NMR and oxidative stability analyses, the inclusion complexes were prepared from the CLA/CDs complexes at a 1:6 mole ratio and then dried in a freeze-drier (Ilshin Co., Ltd., Seoul, Korea).

Determination of CLA Content by GC. CLA was recovered from the sample, containing 1 mg of heptadecanoic acid as an internal standard (IS), by removing solvents under vacuum or by extracting, three times, with hexane (3 volumes of the sample) according to the conventional method. The CLA (10−50 mg) in a screw-cap test tube (15 mL) was methylated with 1.0 N H_2SO_4/methanol (3 mL) in a water bath (55 °C) for 5 min (*24*). After purification, the content of CLA was analyzed by GC (Hewlett-Packard 5890, Avondale, PA) equipped with a flame ionization detector and a fused silica capillary column (Supelcowax-10, 60 m × 0.32 mm, i.d., 25 μm film thickness) (*16*). The oven temperature was increased from 180 to 200 °C at a rate of 2 °C/min and then held for 30 min. Temperatures of the injection port and detector were maintained at 240 and 260 °C, respectively. Nitrogen (99.9%) was used as a carrier gas with a flow rate of 2 mL/min. Response factors (RF) of *c*9,*t*11 CLA and *t*10,*c*12 CLA isomers were 1.17 and 1.18, respectively. Total CLA content in a sample was calculated as follows: total CLA (mg) = $A_s/A_i \times$ IS (mg) × RF_a, where A_s, A_i, and RF_a refer to the total area of *c*9,*t*11 CLA and *t*10,*c*12 CLA isomers, the area of IS, and the average response factor (1.175), respectively.

Headspace Oxygen Analysis for the Stability of CLA in CLA/CDs Inclusion Complexes. The CLA/CDs inclusion complex sample (equivalent to 100 mg of CLA) was added to a serum sample bottle (50 mL), containing a magnetic stirrer bar, and then capped with a Teflon-lined aluminum cap. The sample bottles were incubated in a shaking bath (35 °C, 250 rpm; Ilshin Co., Ltd., Seoul, Korea) for 80 h. The control bottle contained only a magnetic stirrer bar. A sample bottle containing only CLA or CD was also prepared. The headspace oxygen content in the sample bottles was measured by GC described by Kim et al. (*19*).

NMR Analysis. The CLA/CDs inclusion complexes, dissolved in DMSO-d_6, were sonicated in an Ultrasonic FS-28 sonicator (Fisher Scientific, Springfield, NJ) for 45 min to ensure the inclusion complex of CLA in CDs. High-resolution ^{1}H NMR spectra were determined at 35 °C and recorded in DMSO-d_6 on a Bruker AW-500 NMR spectrometer, operating at a base frequency of 500 MHz. An inversion recovery method including a 180−τ−90 pulse sequence was applied for determining the T_1 values. T_1 for each of the data was obtained according to the method described by Jyothirmayi et al. (*25*).

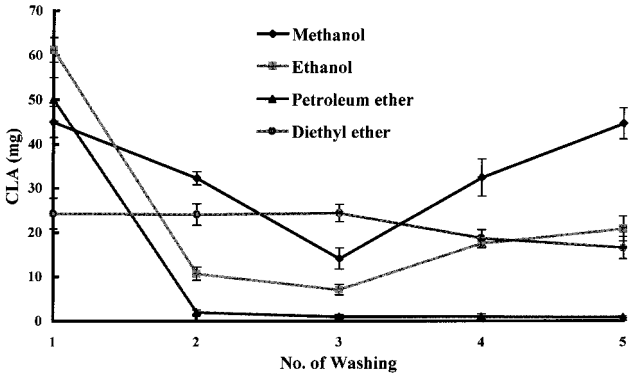

Figure 1. Effects of solvents on the removal of adsorbed CLA from CLA/β-CD complex, prepared with CLA (0.4 g) and β-CD (2.0 g) by the optimal condition for the preparation of CLA/β-CD complex. Each data point is the mean ± SD for three experimental data.

RESULTS

Inserting the CLA molecule into the cavity of CDs created CLA/CDs inclusion complexes. In the present study, the stoichiometry of CLA/CDs inclusion complexes was elucidated by GC, ^{1}H NMR, and T_1 value analyses, and the oxidative stability of CLA in the inclusion complexes was evaluated by GC analysis. Thus, this study consisted of three experiments. These included (1) the preparation of CLA/CDs inclusion complexes, (2) the determination of the mole ratio of CLA complexed with CDs, and (3) the determination of the oxidative stability of CLA in CLA/CDs inclusion complexes.

Preparation of CLA/CDs Inclusion Complexes. As seen in **Figure 1**, petroleum ether removed 50.1 mg of CLA from the CLA/β-CD complex by the first washing, but no further CLA was removed by increasing the number of washing times. Other solvents (methanol, ethanol, and diethyl ether), however, removed both adsorbed CLA and included CLA due to the increase in the amount of CLA in solvents by increasing the number of washing times. These results are in agreement with the results of Szejtli and Banky-Elod (*26*), who reported that petroleum ether was a suitable solvent for the removal of only the adsorbed linoleic acid from the complex of linoleic acid with amylose. Subsequently, petroleum ether was used to remove adsorbed CLA from the CLA/CDs complexes for the preparation of CLA/α-CD and CLA/γ-CD inclusion complexes.

Table 1 shows the effects of complexation temperatures on the formation of CLA/β-CD inclusion complex. When the complex was prepared at 70 °C, the composition of the CLA complexed with β-CD was 34.3%, whereas the composition of adsorbed and uncomplexed CLA was 12.6 and 53.1%, respectively. The effects of 50 and 90 °C on the complexation of CLA with β-CD were worse than that of 70 °C, indicating that the optimal complexation temperature under the test condition was 70 °C.

Table 2 shows effects of complexation durations (0, 1, 2, and 4 h) on the formation of CLA/β-CD inclusion complex at 70 °C. When the CLA/β-CD inclusion complex was prepared without holding the temperature at 70 °C, it contained 34.3% CLA in the cavity of β-CD. However, the composition of the complexed CLA was reduced to <29.2% by holding at 70 °C for >1 h, suggesting that it was not necessary to hold the temperature at 70 °C for the preparation of the CLA/β-CD

Table 1. Effects of Complexation Temperatures on the Formation of CLA/β-CD Inclusion Complex[a]

	distribution of CLA[b] (mg)			
mixing temp (°C)	uncomplexed	adsorbed	complexed	total
50	182.7 ± 4.0[c] (44.8)[d]	107.5 ± 8.7 (26.4)	117.6 ± 5.3[Ae] (28.8)	407.8 ± 6.4 (100)
70	211.2 ± 8.4 (53.1)	50.1 ± 4.9 (12.6)	136.4 ± 4.0[B] (34.3)	397.7 ± 5.9 (100)
90	161.5 ± 9.9 (40.8)	127.8 ± 4.9 (32.3)	106.8 ± 6.1[A] (26.9)	396.1 ± 7.6 (100)

[a] The temperature of the mixture of β-CD (2.0 g/25 mL distilled water, 40 °C) and CLA (0.4 g/25 mL of 96% aqueous ethanol) increased to given temperatures (50, 70, and 90 °C) for 5 min and then immediately cooled to 25 °C for 4 h. These processes were conducted under nitrogen during mixing with a magnetic stirrer bar. [b] Uncomplexed CLA, CLA retained in solution without forming complexation with β-CD; adsorbed CLA, CLA removed from the CLA/β-CD complexes by washing once with petroleum ether; complexed CLA, CLA inserted into the cavity of β-CD. [c] Mean ± SD of three experimental data. [d] The number in parentheses represents percentage of the given CLA amount against total CLA. [e] Means with different capital superscript letters in the same column represent a significant difference at $p < 0.05$ by Duncan's multiple test.

Table 2. Effects of Complexation Duration at 70 °C on the Formation of CLA/β-CD Inclusion Complex[a]

	distribution of CLA[b] (mg)			
heating duration (h)	uncomplexed	adsorbed	complexed	total
0	211.2 ± 8.4[c] (53.1)[d]	50.1 ± 4.9 (12.6)	136.4 ± 4.0[Ae] (34.3)	397.7 ± 5.9 (100)
1	173.7 ± 5.6 (43.7)	111.7 ± 5.4 (28.1)	112.0 ± 2.4[B] (28.2)	397.4 ± 4.5 (100)
2	184.4 ± 8.9 (46.6)	96.2 ± 5.5 (24.3)	115.5 ± 3.6[B] (29.1)	396.1 ± 6.3 (100)
4	217.1 ± 4.5 (54.5)	64.9 ± 4.0 (16.3)	116.1 ± 1.9[B] (29.2)	398.1 ± 3.6 (100)

[a] The temperature of the mixture of β-CD (2.0 g/25 mL distilled water, 40 °C) and CLA (0.4 g/25 mL of 96% aqueous ethanol) increased to 70 °C for 5 min and was maintained for the given durations, followed by cooling to 25 °C for 4 h. These processes were conducted under nitrogen during mixing with a magnetic stirrer bar. [b] Uncomplexed CLA, CLA retained in solution without forming complexation with β-CD; adsorbed CLA, CLA removed from the CLA/β-CD complexes by washing once with petroleum ether; complexed CLA, CLA inserted into the cavity of β-CD. [c] Mean ± SD of three experimental data. [d] The number in parentheses represents percentage of the given CLA amount against total CLA. [e] Means with different superscript capital letters in the same column represent a significant difference at $p < 0.05$ by Duncan's multiple test.

Table 3. Effects of Cooling Duration on the Formation of CLA/β-CD Inclusion Complex[a]

	distribution of CLA[b] (mg)			
cooling duration (h)	uncomplexed	adsorbed	complexed	total
1	171.8 ± 1.1[c] (43.4)[d]	141.4 ± 5.9 (35.8)	82.5 ± 2.0[Ae] (20.8)	395.7 ± 4.3 (100)
2	184.7 ± 5.4 (46.6)	106.6 ± 7.0 (26.9)	105.1 ± 2.6[B] (26.5)	396.4 ± 5.7 (100)
3	183.2 ± 5.7 (46.0)	105.9 ± 3.3 (26.6)	109.1 ± 9.1[B] (27.4)	398.2 ± 6.8 (100)
4	211.2 ± 8.4 (53.1)	50.1 ± 4.9 (12.6)	136.4 ± 4.0[C] (34.3)	397.7 ± 5.9 (100)
8	201.0 ± 6.1 (51.5)	59.3 ± 4.7 (15.2)	130.0 ± 5.3[C] (33.3)	390.3 ± 6.8 (100)

[a] The temperature of the mixture of β-CD (2.0 g/25 mL distilled water, 40 °C) and CLA (0.4 g/25 mL of 96% aqueous ethanol) increased to 70 °C for 5 min and immediately cooled to 25 °C for given durations. These processes were conducted under nitrogen during mixing with a magnetic stirrer bar. [b] Uncomplexed CLA, CLA retained in solution without forming complexation with β-CD; adsorbed CLA, CLA removed from the CLA/β-CD complexes by washing once with petroleum ether; complexed CLA, CLA inserted into the cavity of β-CD. [c] Mean ± SD of three experimental data. [d] The number in the parentheses represents percentage of the given CLA amount against total CLA. [e] Means with different superscript capital letters in the same column represent a significant difference at $p < 0.05$ by Duncan's multiple test.

complex. Similarly, the best cooling duration from 70 to 25 °C for the preparation of the CLA/β-CD complex was found to be 4 h, because the amount of CLA complexed with β-CD for 4 h was not significantly different from that for 8 h (**Table 3**).

Consequently, the CLA/β-CD inclusion complex was prepared by increasing the complexation temperature of the mixture of CLA and β-CD to 70 °C for 5 min and immediately decreasing the temperature to 25 °C for 4 h, followed by washing the complex once with petroleum ether. This condition was applied to prepare CLA/α-CD and CLA/γ-CD inclusion complexes.

Mole Ratio of CLA to CDs in CLA/CDs Inclusion Complexes. The T_1 value analysis of the groups of CLA in CLA/CDs inclusion complexes was studied to elucidate the molecular nature of CLA in the complexes (**Table 4**). It was found that the groups of CLA in the inclusion complexes with CDs showed lower T_1 values than those of uncomplexed CLA. The presence of uncomplexed glucose along with CLA did not reduce T_1 values of CLA (data not shown). The proton signal of the carboxyl group of CLA was broad at 11.3 ppm. In CLA complexed with α-CD and β-CD, the CH_2 (C2) showed maximum reduction in T_1 values, 56.4% in CLA/α-CD and 58.2% in CLA/β-CD, clearly indicating the insertion of a carboxyl end in the cavity of α- and β-CDs. In CLA/γ-CD inclusion complexes, however, the T_1 reduction of the CH_2 (C2) was only 36.7%, suggesting that the CH_2 was less affected by γ-CD than by α-CD or β-CD. T_1 reductions of the CH_3 of CLA in CLA/α-CD and CLA/β-CD were 53.6 and 41.3%, respectively, whereas the T_1 reduction of CLA/γ-CD was 28.1%. It could be concluded from these results that the complexation of CLA with α-CD and β-CD was quite different from that of CLA with γ-CD.

The conjugated diene of CLA in CLA/α-CD, CLA/β-CD, and CLA/γ-CD inclusion complexes showed about 51.4, 41.1, and 45.7% reductions in T_1, respectively, implying that the conjugated diene protruded within the cavity of CDs. T_1 values for all protons, except for the CH_2 (C8, C14), of CLA, were decreased by the formation of complex with CDs, although the magnitude of the reduction was different from the given proton of CLA in CLA/CDs inclusion complexes. The decreases in T_1

Table 4. Reduction in T_1 Values of Groups of CLA in CLA/CDs Inclusion Complexes

protons of allylic carbon of CLA	T_1 value		percentage of reduction in T_1
	free CLA	inclusion CLA	
	CLA/α-CD		
–CH═CH–CH═CH–	1.459, 6.045, 2.106, 1.754 (2.841)[a]	1.806, 1.033, 1.440, 1.244 (1.381)	51.4
–C2–H	2.770	1.209	56.4
–C8, –C14–H	–[b]	–	–
–C17–H	1.775	1.361	23.3
–C3–7, –C15, –C16–H[c]	2.094	1.751	16.4
–C18–H	2.782	1.291	53.6
	CLA/β-CD		
–CH═CH–CH═CH–	1.459, 6.045, 2.106, 1.754 (2.841)	1.547, 1.711, 1.669, 1.759 (1.672)	41.1
–C2–H	2.770	1.157	58.2
–C8, –C14–H	–	–	–
–C17–H	1.775	1.601	9.8
–C3–7, –C15, –C16–H[c]	2.094	1.477	29.5
–C18–H	2.782	1.633	41.3
	CLA/γ-CD		
–CH═CH–CH═CH–	1.459, 6.045, 2.106, 1.754 (2.841)	1.352, 1.848, 1.546, 1.427 (1.543)	45.7
–C2–H	2.770	1.753	36.7
–C8, –C14–H	–	–	–
–C17–H	1.775	0.773	56.5
–C3–7, –C15, –C16–H[c]	2.094	1.514	27.7
–C18–H	2.782	2.001	28.1

[a] The number in the parentheses represents an average of four T_1 values of the conjugated diene protons. [b] Not detected. [c] An average T_1 value of protons of C3–7, C15, and C16.

for CLA/α-CD and CLA/β-CD were similar and found to be CH_2 (C2) > CH_3 (C18) > CH═CH–CH═CH > CH_2 (C17) > CH_2 (C3–7, C15, C16) for CLA/α-CD and only the order of CH_2 (C3–7, C15, C16) > CH_2 (C17) was only switched for CLA/β-CD. Similarly for CLA/γ-CD, the decrease was in the order of CH_2 (C17) > CH═CH–CH═CH > CH_2 (C2) > CH_3 (C18) > CH_2 (C3–7, C15, C16). It is clear from T_1 values that CD complexed with CLA through carboxyl and methyl ends, centered on the bending portion (conjugated diene) of CLA, but did not complex with the CH_2 (C8 and C14). It is also shown that the CLA complexation pattern with γ-CD was quite different from that with α-CD and β-CD. Nevertheless, these results did not reveal any information about the stoichiometry of CLA complexed with CDs.

The stoichiometry of the mole ratio of CLA complexed with CDs in CLA/CDs inclusion complexes was elucidated by ^{1}H NMR analysis. The ^{1}H NMR spectra of CLA, β-CD, and the CLA/β-CD inclusion complex prepared at a 1:6 mole ratio, dissolved in DMSO-d_6, are shown in **Figure 2**. Proton signals of the methyl, conjugated diene, and carboxyl groups of CLA appeared at 0.85–0.88, 5.23–6.29, and 11.30 ppm (broad), respectively, whereas proton signals of β-CD appeared at 3.28–5.69 ppm. As compared to uncomplexed CLA, no significant chemical shift of the protons of CLA/CDs inclusion complexes was found, indicating that most of the proton signals of CLA and CDs were not affected by the complexation. However, a significant difference was observed in the relative proton area ratio of α-D-glucopyranose C1–H to conjugated diene C9–H of the CLA/CDs (**Table 5**). The relative area ratios were 29.1 for CLA/α-CD, 28.0 for CLA/β-CD, and 16.6 for CLA/γ-CD, indicating that number of moles of CDs complexed with one CLA molecule were 5 in CLA/α-CD, 4 in CLA/β-CD, and 2 in CLA/γ-CD.

The stoichiometry of the mole ratio of CLA/CDs was further studied by GC analysis. **Figure 3** shows the composition of the CLA complexed with β-CD in the CLA/β-CD inclusion complex prepared with various mole ratios of CLA to β-CD. The composition of CLA was 25.3, 49.9, 79.6, 96.4, and 96.5% at the CLA/β-CD mole ratios of 1:1, 1:2, 1:3, 1:4, and 1:6, respectively. The composition of adsorbed CLA on the surface of the complexed and uncomplexed CLA remaining in solution decreased to nearly zero at a mole ratio greater than 1:4 of CLA/β-CD. These results indicate that 1 mol of CLA was complexed with 4 mol of β-CD. **Table 6** shows the percentage of the complexed CLA in CLA/α-CD and CLA/γ-CD inclusion complexes prepared at various mole ratios of CLA to α-CD and γ-CD, respectively. The percentage of CLA complexed with α-CD was increased to the 1:4 mole ratio of CLA/α-CD in a dose-dependent manner, but not to the 1:6 mole ratio, indicating that 1 mole of CLA complexed with 4–6 mol of α-CD. Likewise, the percentage of CLA complexed with γ-CD was not increased by a more ratio greater than 1:2 of CLA/γ-CD, indicating that 1 mole of CLA complexed with 2 mol of γ-CD.

Oxidative Stability of CLA in CLA/CDs Inclusion Complexes. Figure 4 shows the headspace oxygen depletion by CLA/CDs inclusion complexes prepared at a 1:6 mole ratio of CLA/CDs. The inclusion complexes in an airtight serum bottle were reacted in a shaking incubator (35 °C, 250 rpm) for 80 h, and then the unreacted headspace oxygen content was measured by GC. The headspace oxygen of control sample was 8.1 mmol/L. The headspace oxygen of the sample bottle containing CLA alone decreased from 8.1 to 3.7 mmol/L, whereas the headspace oxygen of CLA/α-CD, CLA/β-CD, and CLA/γ-CD inclusion complexes was not decreased as compared to control. No difference in the headspace oxygen depletion was seen between the inclusion complexes. CDs were not consumed by headspace oxygen at all. These results indicate that the oxidation of CLA was completely protected by complexation with either α-, β-, or γ-CD.

DISCUSSION

CLA insertion into the cavity of CDs is affected by the cavity size and hydrophobicity of CDs and the hydrogen bonding strength in the cavity of CDs (*19*, *27*). The cavity sizes of α-, β-, and γ-CDs are found to be 5–6, 6–8, and 8–10 Å, respectively (*28*). These sizes are enough for the insertion of CLA, the structure of which closely resembles that of *t*9,*c*12-linoleic acid (7.9 Å width) (*29*).

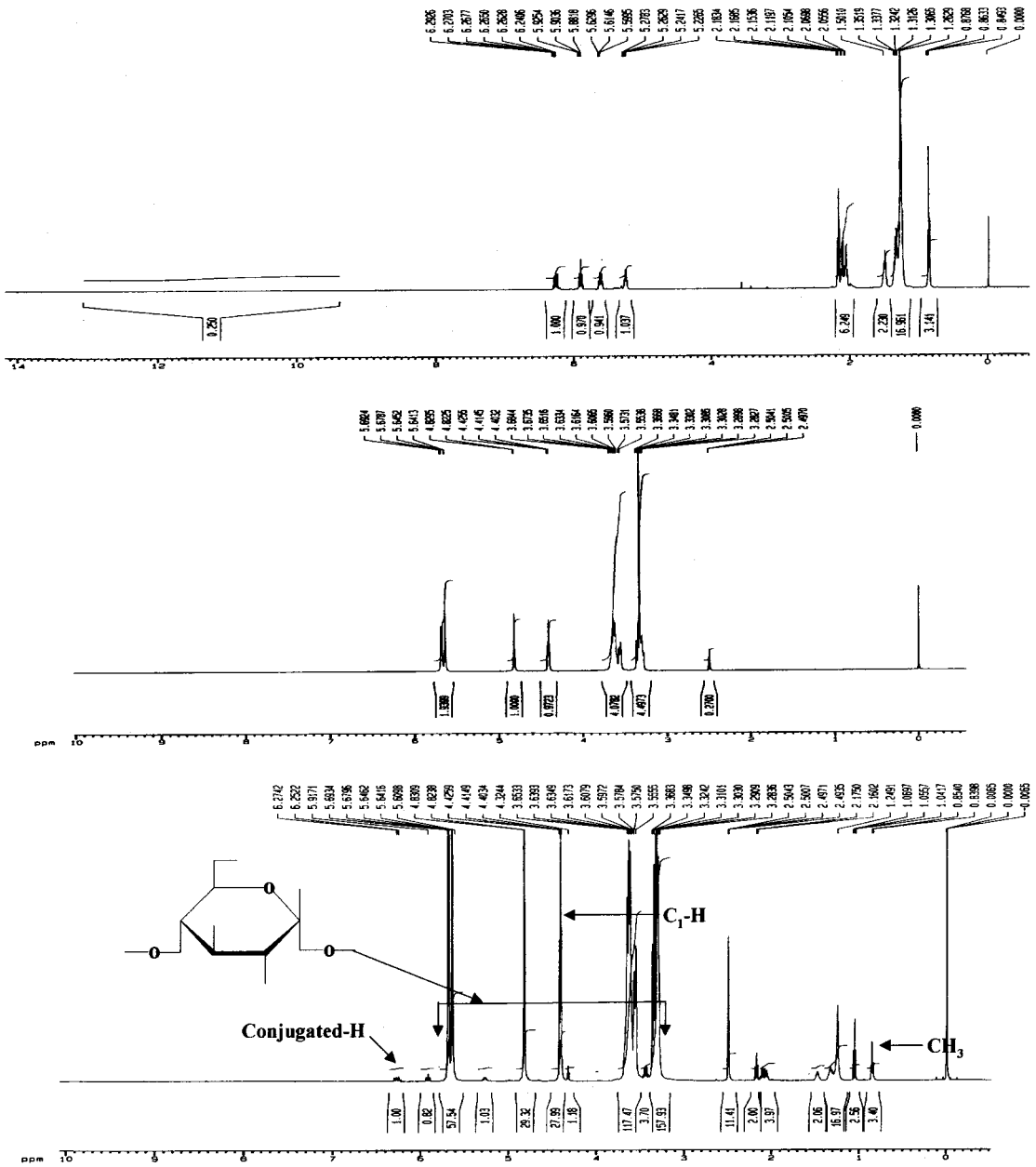

Figure 2. ^{1}H NMR spectra of CLA (top), β-CD (middle), and CLA/β-CD inclusion complex (bottom) in DMSO-d_6 determined at 500 MHz.

Table 5. Mole Ratio of CDs to CLA in CLA/CDs Inclusion Complexes Measured by ^{1}H NMR

CD	area of proton		mole ratio[c] (CD/CLA)
	C1–H (glucose)[a]	conjugated diene-H[b]	
α-CD	29.1 (6)[d]	1.0	4.9
β-CD	28.0 (7)	1.0	4.0
γ-CD	16.6 (8)	1.0	2.1

[a] Area of the α-D-glucopyranose C1–H (4.40–4.43 ppm) complexed with CLA (**Figure 2**). [b] Area of the conjugated diene-H (C9, 6.25–6.27 ppm) of the CLA complexed with CDs (**Figure 2**). [c] Ratio of the area of C1–H allocated to one α-D-glucopyranose unit to the area of conjugated diene-H of CLA. [d] The value in parentheses represents the number of α-D-glucopyranose units in a given CD.

When CDs are dissolved in water, the outer surfaces of the CDs have a hydrophilic nature induced by the hydroxyl group of the glucose moiety on CDs, and the internal cavity has a hydrophobic nature, which is strongly affected by the polarity of the CD group coating the inner channel (*30*). Thus, the hydrophobicity of the inner cavity of CDs facilitates the insertion of the CLA molecule(s) into the cavity of the CDs. Hydrogen-bonding distances between the hydroxyl group at C2 of one α-D-glucopyranose molecule and the hydroxyl group at C3 of the adjacent α-D-glucopyranose molecule of CDs are 3.01 Å for α-CD and 2.86 Å for β-CD, suggesting that the hydrogen bonding in β-CD is stronger than that in α-CD (*30*). Thus, the hydrophobic nature of the cavity of α-CD is greater than that of β-CD, and this may influence the insertion of the hydrocarbon chain of CLA into the cavity of CDs. γ-CD has a cavity size twice as large as that of α-CD and weaker hydrophobicity and hydrogen-bonding strength than α-CD. These properties of CDs suggest that CLA complexed more easily and stronger with α-CD than with β-CD, followed by γ-CD. This is in agreement with the fact that 1 mole of CLA complexed with 5 mol of α-CD, 4 mol of β-CD, and 2 mol of γ-CD as determined by GC and ^{1}H NMR analysis (**Figures 2** and **3**; **Tables 5** and **6**).

However, when analyzed by GC, the percentage of the complexed CLA in CLA/γ-CD was 88.9 at the 1:2 mole ratio of CLA to γ-CD, after which the percentage did not significantly

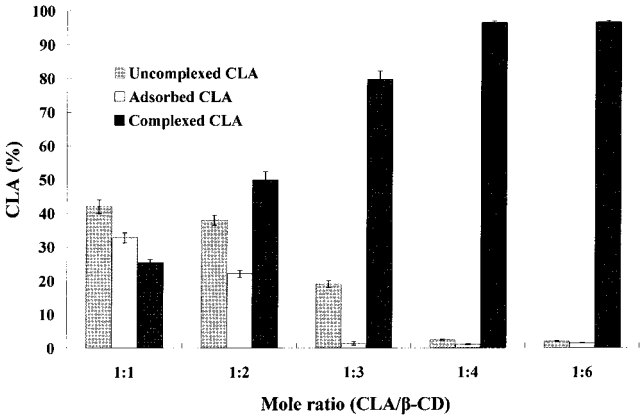

Figure 3. Distribution of CLA to the aqueous ethanol (uncomplexed CLA) and the surface (adsorbed CLA) and inside of the cavity of β-CD (included CLA) in CLA/β-CD complex. The CLA/β-CD complex was prepared at various mole ratios of CLA/β-CD, using 0.4 g of CLA and appropriate amounts of β-CD.

Table 6. Percentage of the Complexed CLA in CLA/α-CD and CLA/γ-CD Inclusion Complexes Prepared at Various Mole Ratios of CLA to α-CD and γ-CD[a]

	mole ratio of CLA/CDs[b]				
CD	1:1	1:2	1:3	1:4	1:6
α-CD	20.5 ± 1.5[c]	67.5 ± 2.1	81.8 ± 1.8	96.9 ± 0.5	98.8 ± 0.4
γ-CD	21.4 ± 2.4	88.9 ± 1.5	87.9 ± 1.2	88.8 ± 0.3	95.4 ± 0.6

[a] Amount of CLA was measured by GC. [b] Amount of CLA used for the preparation of CLA/CDs inclusion complexes was 0.4 g. [c] Mean ± SD of three experimental data.

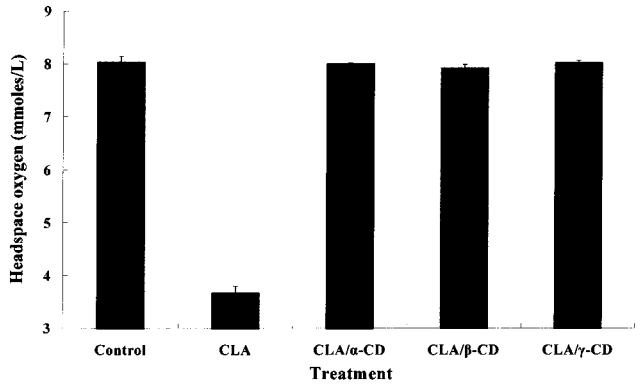

Figure 4. Headspace oxygen depletion by CLA/CDs inclusion complexes prepared at a 1:6 mole ratio of CLA/CDs. Oxidation of the CLA/CDs inclusion complexes was induced at 35 °C for 80 h. Oxygen content was determined at 40 °C by GC equipped with TCD and Carboxene-1000 stainless steel column and calculated according to the method described by Kim et al. (*19*).

increase over the 1:6 mole ratio (**Table 6**). This might suggest that 2 mol of CLA was inserted into the cavity of γ-CD, actually resulting in 2 mol of CLA complexed with 4 mol of γ-CD. It is uncertain why the percentage of CLA complexed with γ-CD did not exceed 90%, but it might be, in part, due to the larger cavity size, weaker hydrophobicity, and weaker hydrogen bonding of γ-CD, resulting in extraction of the inserted CLA from the cavity.

The CLA sample used was composed of about 47.2% *c*9,*t*11 CLA and 50.7% *t*10,*c*12 CLA isomers. These two isomers are similar in their chemical structures but slightly different in their bending angles around the conjugated diene. The *c*9,*t*11 CLA is more bent than the *t*10,*c*12 CLA isomer (*31*). The present study did not distinguish these two isomers for the complexation with CDs. We believe that the complexation of the CLA isomers with CDs is not so different from one another. Further research on the complexation of the CLA isomers with CDs is ongoing.

The complexation of CLA with CDs completely protected CLA from oxidation by inserting the conjugated diene group of CLA into the cavity of CDs (**Table 4** and **Figure 4**). Previously, we observed that the protective effect of CLA by microencapsulation with CDs was in the order α-CD > β-CD > γ-CD. When CLA was microencapsulated with CDs, unlike inclusion complexes, some of the CLA was adsorbed on the surface of CLA/CDs complexes and resulted in oxidation. The extent of adsorbed CLA amount on the surface was proportional to the molecular size of the CDs (γ- > β- > α-CD), which was inversely related to the protective effect of CLA from oxidation.

In conclusion, the ^{1}H NMR and GC analyses revealed that 1 mol of CLA complexed with 5 mol of α-CD, 4 mol of β-CD, and 2 mol of γ-CD and, possibly, 2 mol of CLA was complexed with 4 mol of γ-CD. The complex forms through carboxyl and methyl ends, centered on the conjugated diene portion of CLA. CLA in CLA/CDs inclusion complexes was stable at 37 °C for 80 h against oxidation.

ABBREVIATIONS USED

α-CD, α-cyclodextrin; β-CD, β-cyclodextrin; γ-CD, γ-cyclodextrin; CDs, α-, β-, and γ-cyclodextrins; CLA/CDs inclusion complexes, CLA inclusion complex with α-CD, β-CD, and γ-CD; GC, gas chromatography; IS, internal standard; RF, response factor.

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