Improvement of Oxidative Stability of Conjugated Linoleic Acid (CLA) by Microencapsulation in Cyclodextrins

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Oxidative stability of conjugated linoleic acid (CLA) encapsulated in α-, *β*-, and *γ*-cyclodextrins (designated CLA/CDs microencapsules) was studied by measuring the headspace-oxygen depletion in airtight serum bottles and by measuring the peroxide values (POV). The rate of oxygen depletion was reduced from 41.0 (control) to 21.5, 2.1, 1.2, and 1.1 μmol/L·h^{-1} by CLA/α-CD microencapsules at 1:1, 1:2, 1:4, and 1:6 mole ratios, respectively, indicating that CLA oxidation was completely protected by a 1:4 mole ratio of CLA/α-CD. Such a protective effect by CLA/*β*-CD or CLA/*γ*-CD microencapsules was achieved at a 1:6 mole ratio, but the effect by CLA/*β*-CD was slightly greater than that by CLA/*γ*-CD. The protective effect of α-, *β*-, and *γ*-CDs for CLA oxidation was confirmed by their POV-reducing abilities in CLA/CDs. These results suggest that α-CD was the most effective for the protection of CLA oxidation by microencapsulation, followed by *β*-CD and *γ*-CD.

Keywords: *Conjugated linoleic acid (CLA); cyclodextrins (CDs); microencapsulation; oxidation*

INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term for a group of positional (C8,C10; C9,C11; C10,C12; and C11,C13) and geometric (*cis*,*cis; cis*,*trans; trans*,*cis*; and *trans*,*trans*) isomers of octadecadienoic acid (linoleic acid) with a conjugated double-bond system (Ha et al., 1987; Sehat et al., 1999). It was first identified as comprising an anticarcinogenic principal present in grilled ground beef (Ha et al., 1987). Later, it was found in many other food sources, especially dairy products (Chin et al., 1992; Ha et al., 1989; Parodi, 1997; Shantha et al., 1992).

Chemically synthesized CLA, composed of approximately 48% each of *cis*,9-*trans*,11 CLA and *trans*,10-*cis*,12 CLA, inhibited carcinogen-induced neoplasia in several animal models (Ha et al., 1987, 1990; Ip et al., 1991, 1996, 1997; Ip and Scimeca, 1997; Liew et al., 1995). The CLA inhibited the proliferation of human malignant melanoma, colorectal, and breast cancer cells (Cunningham et al., 1997; Durgam and Fernandes, 1997; Schonberg and Krokan, 1995; Shultz et al., 1992a, 1992b; Visonneau et al., 1997; Wong et al., 1997). In addition to the anticarcinogenic activity, the CLA exhibited several other biological activities in animal models: reduction of atherosclerosis (Lee et al., 1994), modulation of immunity (Cook et al., 1993; Miller et al., 1994), stimulation of growth (Chin et al., 1994), reduction of body fats (Park et al., 1997), and reduction of lipid oxidation (Ip et al., 1991).

Antioxidant activity of CLA was first recognized in 1990 from in vitro experimental results (Ha et al., 1990). Several studies to date have examined the antioxidant property of CLA; however, the activity still remains controversial. Some of the studies have shown that CLA acted as an antioxidant, whereas some other studies have demonstrated that CLA might be a prooxidant. Antioxidant activity of CLA was observed in mammary gland tissues from rats fed CLA, when evaluated by the thiobarbituric acid reactive substances (TBARS) values (Ip et al., 1991). TBARS values of meats from pigs and chickens, and eggs from hens fed various levels of CLA, were significantly reduced during storage at 4 °C, compared with that of the control (Lee et al., 1999a, 1999b; Park et al., 1998). However, CLA did not reduce TBARS values in pork patties mixed with CLA (Joo et al., 2000). In in vitro studies, CLA was oxidized as rapidly as linoleic acid (Chen et al., 1997; van der Berg et al., 1995). These studies suggest that CLA must be protected from oxidation when it is to be used in food systems as fortifiers or additives.

α-Cyclodextrin (α-CD), *β*-cyclodextrin (*β*-CD), and *γ*-cyclodextrin (*γ*-CD), consisting of 6, 7, and 8 D(+)-glucopyranose units connected by α(1→4) glycosidic bond, respectively, are well-known cyclodextrins (CDs) for food industry uses (Hedges et al., 1995; Saenger, 1980). CDs are known to form inclusion complexes with various compounds, ranging from polar to highly nonpolar agents (Divakar, 1990; Saenger, 1984). Many nonpolar compounds have been encapsulated in CDs to use in the food, cosmetic, and pharmaceutical industries (Hedges et al., 1995). Free fatty acids and their derivatives are known to sequester in the hydrophobic inner cavity of CDs (Szejtli and Banky-Elod, 1975; Szente et al., 1998). Polyunsaturated fatty acids encapsulated in

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α- and β-CDs have been shown to be protected completely against oxidation even in pure oxygen (Jyothirmayi et al., 1991; Reichenbach and Min, 1997). Thus, we expect that CLA oxidation could be protected by microencapsulation in CDs.

The objective of this study is to investigate the oxidative stability of CLA microencapsulated in α-, β-, or γ-CD at various mole ratios, when reacted at 35 °C.

MATERIALS AND METHODS

Materials. CLA, composed of approximately 48% each of *cis*,9-*trans*,11 CLA and *trans*,10-*cis*,12 CLA, was synthesized from linoleic acid by alkaline isomerization at 180 °C (Ha et al., 1990). Linoleic acid (99.9%) was obtained from PREP, Inc. Nu Chek (Elysian, MN). The α-, β-, and γ-CDs were purchased from Sigma Chemical Co. (St. Louis, MO). Deuterated d_6-dimethyl sulfoxide (d_6-DMSO; 99.96 atom % D) was 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 reagent grade.

Preparation of CLA Microencapsules in CDs. Microencapsulation of CLA in CDs (designated CLA/CDs microencapsules) was prepared from the appropriate amount of α-, β-, or γ-CD and 0.36 mmol CLA (100 mg CLA) to obtain a 1:1, 1:2, 1:4, or 1:6 mole ratio of CLA/CDs. Distilled water (10 mL) and an appropriate amount of CDs were added to the sample bottle, containing 0.36 mmol CLA dissolved in 0.5 mL ethanol, and then vortexed for 30 s. The control sample was prepared with CLA alone, and a sample containing linoleic acid or CD alone was also prepared. The sample bottle was maintained in a water bath (70 °C) for 5 min, homogenized at 8000 rpm for 1 min with a homogenizer (Ika Works, Kuala Lumpur, Malaysia) under nitrogen, and cooled to room temperature. A magnetic bar was put in the sample bottle to facilitate the reaction of the sample with oxygen during incubation, and then the bottle was completely freeze-dried with a freeze drier (Bondiro, Ilshin Lab. Co. Ltd, Seoul, Korea). Each sample bottle was sealed with an open-top aluminum seal and silicone septum.

Headspace-Oxygen Analysis. The serum sample bottle, containing the CLA/CD microencapsules and a magnetic stirrer bar, was incubated in a shaking incubator (35 °C, 250 rpm; Scientific Instrument Company, Seoul, Korea) for 80 h. Headspace-oxygen content was measured over a period of time at frequent intervals (0, 10, 20, 30, 50, and 80 h). Headspace oxygen was analyzed in the temperature-controlled analytical room (25 °C) by injecting a 100-μL portion of headspace air from each serum bottle into a Hewlett-Packard 5890 series II plus GC (Little Fall, TX), equipped with a thermal conductivity detector (TCD) and a Carboxen-1000 (60∼80 mesh, 15 ft × 1/8 in stainless steel, 2.1-mm i.d., Supelco, Bellefonte, PA). Helium (99.99%) was used as a carrier gas with a flow rate of 40 mL/min. The oven temperature was maintained at 40 °C. The temperature of the injector and detector was maintained at 120 °C. The electronic response of oxygen in 100 μL of headspace air was recorded with a Hewlett-Packard 3365 integrator and converted to mmol O_2/L headspace air, according to the method described by Reichenbach and Min (1997). The headspace oxygen (mmol O_2/L headspace air) was plotted against incubation time (*h*) for given mole ratios of CLA/CDs. The slope of the regression line representing the rate of oxygen depletion in mmol $O_2/L \cdot h^{-1}$ was calculated from the headspace-oxygen depletion curve using the Sigma Plot program (SPSS Inc., 1998).

Peroxide Value (POV) Analysis. POV of samples was determined by the AOCS method, modified slightly (AOCS, 1992). POV was determined from the samples that measured headspace-oxygen content. Briefly, 10 mL of acetic acid/chloroform mixture (3:2, v/v) was added to the serum sample bottle and stirred for 2 min with a magnetic stirrer to exclude CLA from the CLA/CDs microencapsules. One mL of saturated KI solution was added to the bottle and stirred for another min, followed by the addition of 10 mL distilled water. The sample was titrated with 0.005 N sodium thiosulfate standardized, using a starch solution as an indicator. The blank test was also conducted. POV was calculated, following the formula: a POV (mequiv/kg) = $(S - B) \times N \times 1{,}000$/g sample weight, where *S* and *B* are mL of sodium thiosulfate solution consumed by sample and blank tests, respectively, and *N* is a standardized normality of sodium thiosulfate solution.

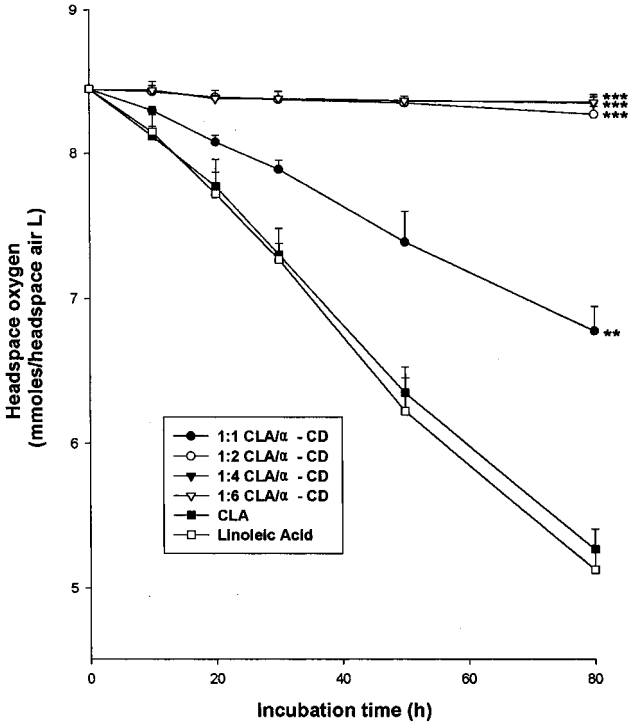

Figure 1. Headspace-oxygen depletion by CLA/α-CD microencapsulations. The CLA/α-CD microencapsules were incubated in a shaking incubator (35 °C, 250 rpm) for a period of 80 h. Two and three asterisks represent that the headspace-oxygen depletion is significantly different from that of the control, incubated for 80 h, at $p < 0.01$ and $p < 0.001$ levels, respectively, by Tukey's w significant test (Ott, 1984).

NMR Analysis. The samples, dissolved in d_6-DMSO, were sonicated in an Ultrasonic FS-28 sonicator (Fisher Scientific, Springfield, NJ) for 40 min to ensure the microencapsulation of CLA in CDs. High-resolution ^{1}H NMR spectra were determined at 35 °C and recorded in d_6-DMSO on a Bruker AW-500 NMR spectrometer (Bruken, Germany), operating at a base frequency of 500 MHz. Proton resonance assignments were based on chemical shifts in ppm, relative to tetramethylsilane in DMSO as the internal reference.

Statistical Analysis. Data were analyzed by two-way analysis of variance (SAS Inc., 1990). Tukey's w significant test at 5, 1, and 0.1% was used to compare the mean values (Ott, 1984).

RESULTS

Headspace-Oxygen Analysis of CLA/CDs Microencapsules. CLA/CDs microencapsules, prepared in airtight serum bottles (50 mL) with various mole ratios of CLA/CDs, were reacted with oxygen in a shaking incubator (35 °C, 250 rpm) for 80 h. The unreacted headspace-oxygen content was determined by GC. The protective effect of CDs for CLA oxidation was in the order of α-CD>β-CD>γ-CD at given mole ratios of CLA/CDs.

Figure 1 shows headspace-oxygen depletion by CLA/α-CD microencapsules. The headspace oxygen of the control sample (CLA alone) decreased from 8.5 to 5.3 mmol/L, whereas the headspace oxygen decreased from 8.5 to 6.8, 8.2, 8.3, and 8.4 mmol/L by CLA/α-CD at 1:1,

Table 1. Headspace-Oxygen Depletion Rate of CLA/CDs during Incubation at 35 °C

treatment (CLA/CDs mole ratio)	microencapsulation with α-CD	β-CD	γ-CD
1:0 (control)	41.0[a] (0.9982)**[b]	41.0 (0.9982)**	41.0 (0.9982)**
1:1	21.5(0.9689)**	37.7 (0.9962)**	38.3 (0.9986)**
1:2	2.1 (0.9901)**	28.8 (0.9984)**	30.8 (0.9980)**
1:4	1.2 (0.8951)*	14.3 (0.9964)**	19.5 (0.9991)**
1:6	1.1 (0.8422)*	2.5 (0.9909)**	5.8 (0.9942)**

[a] The headspace-oxygen depletion rate of CLA/CDs microencapsules (μmol/L·h^{-1}) was calculated by linear regression analysis (SPSS Inc., 1998). The headspace-oxygen depletion rate of linoleic acid was a 43.1 μmol/L·h^{-1}. [b] The number in parentheses indicates the r value obtained from the linear regression analysis (SPSS Inc, 1998), and one and two asterisks represent significance at $p < 0.05$ and $p < 0.01$ levels, respectively (SAS Institute Inc., 1990).

1:2, 1:4, and 1:6 mole ratios, respectively. The headspace oxygen of linoleic acid alone decreased from 8.5 to 5.1 mmol/L, which is not statistically different from that of the control. The rate of headspace-oxygen depletion by CLA/α-CD microencapsules, calculated from Figure 1 by the linear regression analysis, is represented in Table 1. The rate of headspace-oxygen depletion of the control was 41.0 μmol/L·h^{-1}, and it was reduced to 21.5 μmole/L·h^{-1} by CLA/α-CD microencapsules at a 1:1 mole ratio. It further decreased to 2.1, 1.2, and 1.1 μmol/L·h^{-1}, by increasing the mole ratio of CLA/α-CD to 1:2, 1:4, and 1:6, respectively. These results indicate that the oxidation of CLA was significantly protected by encapsulation in α-CD at a 1:1 mole ratio ($p < 0.05$); moreover, it was completely protected at a 1:4 mole ratio.

β-CD microencapsulation also protected CLA from oxidation, but it was less effective than α-CD. CLA/β-CD microencapsules at a 1:1 mole ratio did not reduce the CLA oxidation, as shown by comparing the headspace-oxygen depletion rate of the CLA/β-CD microencapsules at a 1:1 mole ratio (37.7 μmol/L·h^{-1}) to that of the control sample (41.0 μmol/L·h^{-1}) (Table 1). A significant reduction of the headspace-oxygen depletion was observed from the CLA/β-CD microencapsules at a 1:2 mole ratio as compared to that of the control ($p < 0.05$), and further significant reduction was observed by CLA/β-CD microencapsules at a 1:4 mole ratio ($p < 0.01$). CLA oxidation was almost completely protected by CLA/β-CD microencapsules at a 1:6 mole ratio, which is similar to the effect of CLA/α-CD microencapsules at a 1:4 mole ratio. These results indicate that the CLA/β-CD microencapsules at a 1:1 mole ratio actually reacted with oxygen similar to the control, and at a 1:2 mole ratio significantly reduced the CLA oxidation ($p < 0.05$). Unlike α-CD encapsulations, a 1:6 mole ratio of CLA/β-CD is required to give a similar protective effect exhibited by CLA/α-CD microencapsules at a 1:4 mole ratio.

The protective effect of γ-CD for CLA oxidation was similar to that of β-CD. Headspace-oxygen depletion by CLA/γ-CD at a 1:1 mole ratio was not reduced, as compared to that of control; however, it was significantly reduced by increasing the mole ratios of CLA/γ-CD to 1:2, 1:4, and 1:6 ($p < 0.05$). Like β-CD, γ-CD microencapsulation almost completely protected CLA from oxidation by a 1:6 mole ratio of CLA/γ-CD, but to a lesser extent than β-CD. These protective effects of γ-CD were also proven by the data of the headspace-oxygen depletion rate shown in Table 1.

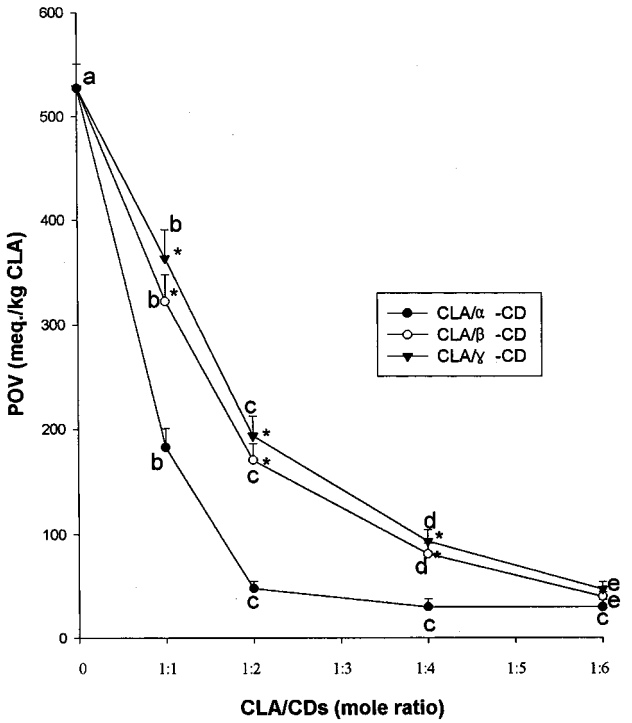

Figure 2. POV of CLA/CDs microencapsules at various mole ratios. The CLA/CDs microencapsules were incubated in a shaking water bath (35 °C, 250 rpm) for 80 h. The zero mole ratio of CLA/CDs means CLA alone. Different letters on the same line represent significantly different at $p < 0.05$ level by Tukey's w significant test. One asterisk represents significantly different from the POV of CLA/α-CD microencapsules at a given mole ratio ($p < 0.05$) by Tukey's w significant test (Ott, 1984).

POV of CLA/CDs Microencapsules. POV was determined from the samples that measured headspace-oxygen content. Figure 2 shows POV of CLA/CDs microencapsules at 1:1, 1:2, 1:4, and 1:6 mole ratios. POV reduction effect was in the order of α-CD > β-CD > γ-CD at given mole ratios of CLA/CDs.

The POV of the control sample was 535 mequiv/kg. It was significantly reduced to 182.5 mequiv/kg by CLA/α-CD microencapsules at a 1:1 mole ratio ($p < 0.05$), and it was further reduced to 48.3, 30.3, and 30.2 mequiv/kg by CLA/α-CD microencapsules at 1:2, 1:4, and 1:6 mole ratios, respectively. These results indicate that no further significant reduction of POV was seen by increasing the mole ratio of CLA/α-CD to more than 1:2.

CLA/β-CD microencapsules at a 1:1 mole ratio also significantly reduced POV to 322.5 mequiv/kg ($p < 0.05$). When the mole ratio of CLA/β-CD was increased to 1:2, 1:4, and 1:6, POV was further reduced to 170.7, 81.6, and 40.3 mequiv/kg, respectively. The POV obtained from the CLA/β-CD microencapsules was significantly ($p < 0.05$) higher than that from the CLA/α-CD microencapsules at the same mole ratio, indicating that β-CD is less protective for CLA oxidation than α-CD. The protective effect of CLA/γ-CD microencapsules from CLA oxidation was similar to that of CLA/β-CD microencapsules, but the effect of γ-CD is lower than that of β-CD. These results indicate that CLA/α-CD at a 1:4 mole ratio completely protected CLA from oxidation, but β- and γ-CD did not, even at a 1:6 mole ratio. These results are consistent with the results from headspace-oxygen depletion experiments (Figure 1 and Table 1).

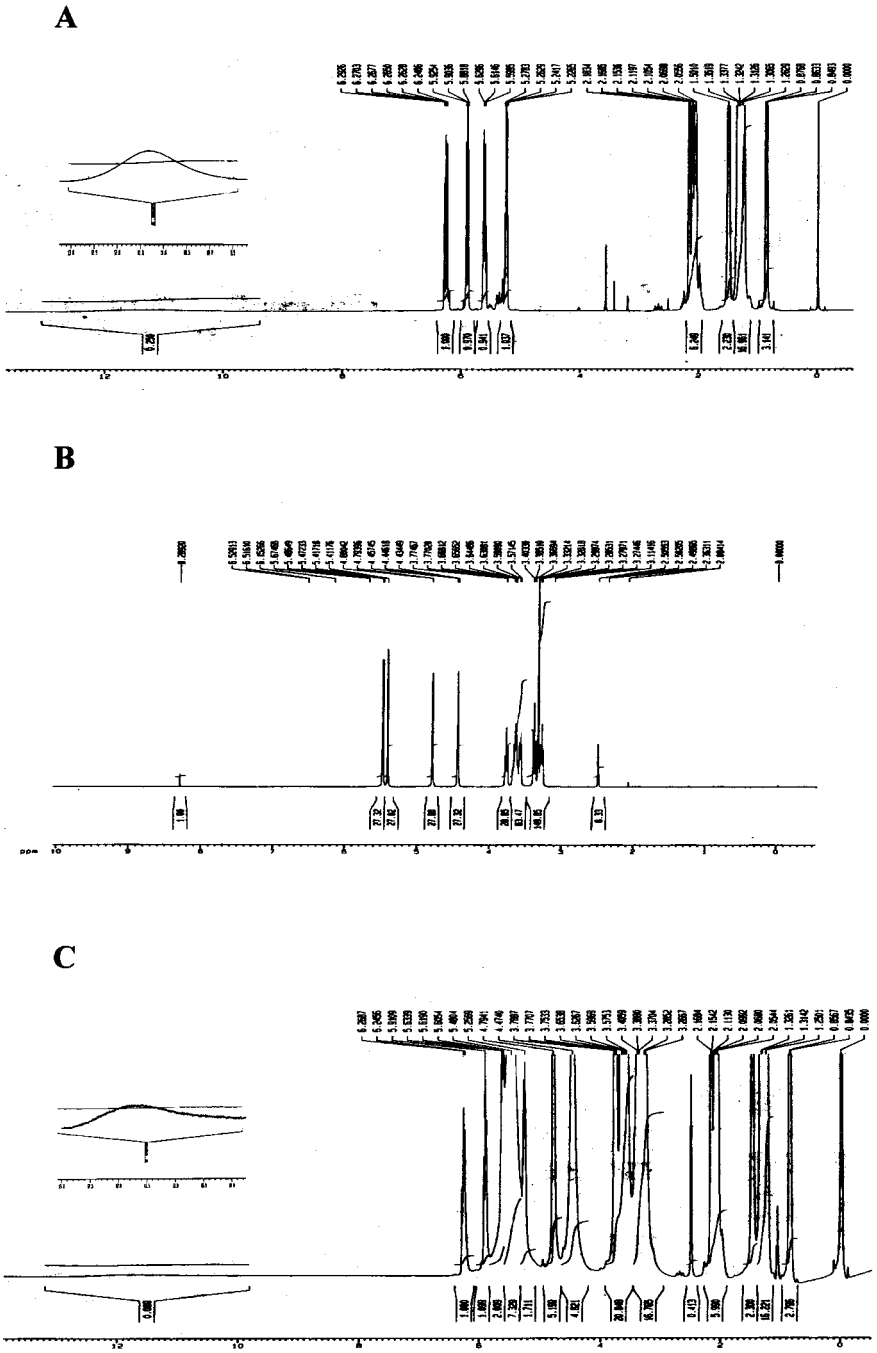

Figure 3. ^{1}H NMR spectra of CLA (A), α-CD (B), and CLA/α-CD microencapsules at a 1:1 mole ratio (C) in d_6-DMSO determined at 500 MHz. The assignments and chemical shifts (ppm) of peaks are shown in Tables 2 and 3.

NMR Analysis of CLA/CDs Microencapsules. The ^{1}H NMR spectra of CLA and α-CD, dissolved in d_6-DMSO, are shown in Figure 3A and B, respectively, and assignments of the proton signals are presented in Table 2. The proton signals of the methyl, conjugated diene, and carboxyl groups of CLA appeared at 0.84−0.87, 5.24−6.30, and 11.32 ppm (broad), respectively, whereas proton signals of α-CD appeared at 3.27−5.47 ppm. A typical ^{1}H NMR spectra of the CLA/α-CD microencapsules at a 1:1 mole ratio is shown in Figure 3C. Most of the proton signals of CLA and CDs were not affected by microencapsulation; however, the chemical shift of carboxyl proton of CLA moved to downfield or upfield, depending on the kinds of CDs and the mole ratios of CLA/CDs.

Table 3 shows the chemical shift of the carboxyl proton of CLA/CDs, and its relative area to the conjugated diene proton (6.28 ppm) at 1:1 and 1:4 mole ratios of CLA/CDs. Most significantly, the chemical shift of the carboxyl proton of CLA/α-CD microencapsules moved to downfield (11.74−11.88 ppm) from 11.32 ppm (broad) of free CLA. In contrast, the chemical shift of the carboxyl proton of CLA/*β*-CD or CLA/*γ*-CD microencapsules at a 1:1 mole ratio moved to upfield (10.20−10.48 ppm) and at a 1:4 mole ratio moved to downfield (11.81−11.82 ppm). The relative area of the carboxyl proton (0.250) to conjugated diene proton (6.28 ppm) of CLA was decreased to 0.069 and 0.115, respectively, by CLA/α-CD microencapsules at 1:1 and 1:4 mole ratios. It was further decreased to near zero by CLA/*β*-CD or CLA/*γ*-

Table 2. Assignment and Chemical Shift of ^{1}H NMR Signals of CLA/CDs Microencapsules at a 1:4 Mole Ratio

assignment	chemical shift (ppm)[a]			
	free	microencapsule		
		CLA/α-CD	CLA/β-CD	CLA/γ-CD
		CLA		
-COOH	11.32 (broad)	11.88 (broad)	11.82 (broad)	11.81 (broad)
-CH=CH−CH=CH−	5.24−6.30 (5.77)[b]	5.26−6.28 (7.77)	5.26−6.28 (5.77)	5.24−6.28 (5.76)
-C2−H	2.16−2.19 (2.18)	2.16−2.19 (2.18)	2.15−2.18 (2.17)	2.16−2.19 (2.18)
-C8, −C14−H	2.06−2.12 (2.09)	2.10−2.12 (2.11)	2.05−2.11 (2.08)	2.06−2.12 (2.09)
-C17−H	1.47−1.50 (1.49)	1.47−1.49 (1.48)	1.48−1.50 (1.49)	1.47−1.49 (1.48)
-C3−7, −C15, −C16−H	1.25−1.36 (1.31)	1.25−1.34 (1.30)	1.25−1.34 (1.30)	1.25−1.35 (1.31)
-C18−H	0.84−0.87 (0.86)	0.84−0.87 (0.86)	0.84−0.87 (0.86)	0.84−0.87 (0.86)
		α-CD		
-C2, −C3−OH	5.41−5.47 (5.44)	5.41−5.49 (5.45)		
-C1−H	4.79−4.80 (4.80)	4.79−4.80 (4.80)		
-C6−OH	4.43−4.46 (4.45)	4.43−4.45 (4.44)		
-C4, −C5, −C6−H	3.57−3.77 (3.67)	3.58−3.79 (3.69)		
-C2, −C3−H	3.27−3.40 (3.34)	3.27−3.41 (3.34)		
			β-CD	
-C2, −C3−OH	5.64−5.69 (5.67)		5.61−5.66 (5.64)	
-C1−H	4.82−4.83 (4.83)		4.82−4.83 (4.83)	
-C6−OH	4.40−4.43 (4.42)		4.40−4.41 (4.41)	
-C4, −C5, −C6−H	3.55−3.68 (3.62)		3.55−3.67 (3.61)	
-C2, −C3−H	3.28−3.37 (3.32)		3.30−3.37 (3.34)	
				γ-CD
-C2, −C3−OH	5.70−5.74 (5.72)			5.71−5.73 (5.72)
-C1−H	4.88−4.89 (4.89)			4.88−4.89 (4.89)
-C6−OH	4.48−4.50 (4.49)			4.47−4.49 (4.48)
-C4, −C5, −C6−H	3.53−3.63 (3.58)			3.53−3.64 (3.59)
-C2, −C3−H	3.30−3.37 (3.34)			3.33−3.37 (3.35)

[a] ^{1}H NMR spectra were recorded in d_6-DMSO and the assignments were based on chemical shift in ppm relative to tetramethylsilane in d_6-DMSO as internal reference. [b] The number in parentheses represents the median value of the high and the low chemical shifts of the proton assigned.

Table 3. Chemical Shift and Relative Area of the Carboxyl Proton of CLA/CDs Microencapsules

treatment	chemical shift (ppm)[a]	relative area[b]
free CLA	11.32 (broad)	0.250
CLA/α-CD		
1:1	11.74	0.069
1:4	11.88	0.115
CLA/β-CD		
1:1	10.48	0.001
1:4	11.82	0.003
CLA/γ-CD		
1:1	10.20	0.008
1:4	11.81	0.011

[a] ^{1}H NMR spectra were recorded in d_6-DMSO and assignments were based on chemical shift in ppm relative to tetramethylsilane in d_6-DMSO as internal reference. [b] The relative area of the carboxyl proton to the proton (6.28 ppm) of the conjugated diene of CLA.

CD microencapsules at both 1:1 and 1:4 mole ratios. These results indicate that when CLA is encapsulated in CDs, the carboxyl proton is in an electronic environment different from that of the carboxyl proton of free CLA (Abraham and Loftus, 1990).

DISCUSSION

CLA is quite susceptible to oxygen, resulting in oxidation; however, the present study shows that the oxidation of CLA is protected by microencapsulation in CDs. In the present study, CLA isomers (*cis*,9-*trans*,11 CLA and *trans*,10-*cis*,12 CLA) were not distinguished from each other for microencapsulation in CDs. The protective effects of CDs for CLA oxidation were in the order of α-CD > β-CD > γ-CD at given mole ratios of CLA/CDs. The CLA oxidation was completely protected by CLA/α-CD microencapsules at a 1:4 mole ratio, whereas a similar protective effect by both CLA/β-CD and CLA/γ-CD microencapsules was observed at a 1:6 mole ratio. The exact protective mechanism of CDs for the CLA oxidation is not completely understood; however, the observed suppression of CLA oxidation by microencapsulation with CDs might be attributed to the physical interference by CDs and to the insertion of CLA into CD molecules. Based on the literature related to CDs encapsulations and on the NMR data obtained from this study, several possible protective mechanisms of CDs for CLA oxidation, associated with the hydrophobicity and cavity size of CDs, could be postulated.

Evidence for the Microencapsulation of CLA in CDs. First of all, the microencapsulation of CLA in CDs can be confirmed by ^{1}H NMR spectral analysis of CLA/CDs microencapsules. No critical chemical shift of protons of CLA and CDs in CLA/CDs microencapsules was observed from one-dimensional ^{1}H NMR spectra, except for the minor changes in the chemical shift of the carboxyl proton of the CLA molecule (Tables 2 and 3).

Many investigators have observed the changes in the chemical shift and/or the decrease in the proton signal of the carboxyl group when polyunsaturated fatty acids were encapsulated in CDs, and they have suggested the interaction of the carboxyl protons with CD molecules through hydrogen bonding (Bru et al., 1995; Jyothirmayi et al., 1991; Reichenbach and Min, 1997). In the present study, the chemical shift of the carboxyl proton of CLA/α-CD microencapsules at both 1:1 and 1:4 mole ratios moved to downfield (11.74−11.88 ppm) from 11.32 ppm of the carboxyl proton of free CLA, whereas the chemical shift of carboxyl protons of CLA/β-CD and CLA/γ-CD moved to upfield at a 1:1 mole ratio (10.20−10.48 ppm),

and moved to downfield at a 1:4 mole ratio (11.81−11.82 ppm) (Table 3). These results suggest that the carboxyl proton of CLA formed the hydrogen bond with CD molecules by microencapsulation (Abraham and Loftus, 1990) and, thus, indicating that CLA was encapsulated in CDs. However, the exact molecular features of CLA/CDs microencapsules could not be characterized at this time.

Interestingly, no critical evidence for the association of the conjugated diene portion of CLA with CDs in CLA/CDs microencapsules was derived from the NOESY ^{1}H NMR spectral analysis of CLA/CDs microencapsules (data not shown); however, evidence for microencapsulation of the conjugated diene portion of CLA with CDs can be derived from the ^{1}H NMR spectral analysis of CLA/CDs microencapsules (Table 3). The relative area of the carboxyl proton to the conjugated diene proton of CLA was decreased from 0.250 to 0.069 and 0.115, respectively, by 1:1 and 1:4 mole ratios of CLA/α-CD. The ratio was further decreased to a zero level by CLA/β-CD and CLA/γ-CD microencapsules. These results suggest that the conjugated diene of CLA is microencapsulated with CDs, resulting in the different electronic environment of the carboxyl proton or the conjugated diene proton of CLA/CDs microencapsules from that of the carboxyl proton or the conjugated diene proton of free CLA (Abraham and Loftus, 1990; Bru et al., 1995; Jyothirmayi et al., 1991; Reichenbach and Min, 1997).

Reichenbach and Min (1997) observed the disappearance of the proton signal of the carboxyl group of linoleic acid by α-CD and β-CD microencapsulations. The disappearance of the proton signal of the carboxyl group was in accordance with increased oxidative stability for linoleic acid encapsulated in α-CD at both 1:1 and 1:2 mole ratios, but it was not consistently observed in β-CD. The disappearance of the carboxyl proton signal of linoleic acid by microencapsulation in α-CD or β-CD agrees with our results that the relative area ratio of the carboxyl proton to the conjugated diene proton in CLA/CDs microencapsules decreased (Table 3). However, the direct relationship of the proton signal change and the oxidative stability of CLA cannot be defined from this study or other studies (Reichenbach and Min, 1997).

Effect of Hydrophobicity of CDs on the Oxidative Stability of CLA. In terms of the hydrophobicity of CDs, when CDs are dissolved in water their outer surfaces have a hydrophilic nature induced by the hydroxyl group of 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 (Saenger, 1984). Thus, hydrophobicity of the inner cavity of CDs facilitates the insertion of the conjugated diene portion of CLA into the cavity of CDs.

The strength of hydrogen bonding in the cavity of CDs may influence the insertion of CLA into the cavity. Hydrogen bonding distance between the hydroxyl group at C2 of one D(+)-glucopyranose molecule and the hydroxyl group at C3 of the adjacent D(+)-glucopyranose molecule of CDs is 3.01 Å for α-CD and 2.86 Å for β-CD, suggesting that the hydrogen bonding in β-CD is stronger than α-CD (Saenger, 1984). Thus, the hydrophobic nature of the cavity of α-CD is greater than β-CD, and this may influence the liable insertion of the hydrocarbon chain of CLA into the cavity of CDs. This may result in more efficient protection of α-CD than β-CD or γ-CD for CLA oxidation.

Effect of Cavity Size of CDs on the Oxidative Stability of CLA. It is possible to presume that the conjugated diene portion of CLA is inserted into the cavity of CDs by microencapsulations. The cavity size and hydrophobicity of CDs may affect the insertion of the conjugated diene portion of CLA. The cavity sizes of α-, β-, and γ-CDs are found to be 5−6 Å, 6−8 Å, and 8−10 Å, respectively (Hedges et al., 1995). The exact size and shape of CLA are not known, but the structure of CLA (*cis*,9-*trans*,11 CLA and *trans*,10-*cis*,12 CLA) might rather closely resemble that of *trans*,9-*cis*,12 linoleic acid (7.9Å, width) than that of *cis*,9-*cis*,12 linoleic acid (11.3 Å, width) (Mishkel and Spritz, 1969), because the orientation of a *trans*-double bond is associated with straightening of the hydrocarbon chain. Therefore, the cavity sizes of α-, β-, and γ-CDs are large enough for the insertion of the conjugated diene portion of a CLA molecule. Once a CLA molecule is inserted into a CD molecule, the release feasibility of the CLA from CLA/CDs complexes might be in the order of CLA/γ-CD, CLA/β-CD, and CLA/α-CD complexes, which is in decreasing order of cavity size of CDs. This might be related to the observed results of α-CD > β-CD > γ-CD in the protection of CLA from oxidation. However, the present study did not provide any evidence that CLA is inserted into CD molecules.

The cavity size of the CDs is possibly large enough to incorporate some oxygen molecules to create a miniature reaction chamber and facilitate the reaction between CLA and oxygen. A substantial amount of oxygen could be captured in the CDs during experimental procedures (Reichenbach and Min, 1997). It is postulated that the oxygen amount captured in CDs could be in the order of γ-CD > β-CD > α-CD, due to the cavity size of CDs. The oxygen captured in the cavity of CDs will be used for initiation of the oxidation of CLA microencapsulated in CDs. Thus, the initiation of CLA oxidation will be fastest in CLA/γ-CD microencapsules and slowest in CLA/α-CD microencapsules. The protective effect of CDs for CLA oxidation (α-CD > β-CD > γ-CD) observed in the present study is in agreement with the decreasing cavity size of CDs.

Effect of the Mole Ratio of CLA/CDs on the Oxidative Stability of CLA. Another important factor for the protection of CLA from oxidation is the mole ratio of CLA/CDs. Regardless of the structural features of CDs, the lower the mole ratio of CLA/CDs was, the higher the protective effect for CLA oxidation exhibited (Figure 1 and Table 1). This might be correlated to the fact that the higher concentration of CDs exhibits greater physical interferences for the reaction of CLA with oxygen than the lower concentration of CDs.

Conclusions. In conclusion, CLA/α-CD microencapsules at a 1:4 mole ratio completely protected CLA from oxidation, when oxidized at 35 °C. The CLA protective effect of β-CD from oxidation is much lower than that of α-CD. A 1:6 mole ratio of CLA/β-CD is required to give a protective effect similar to that exhibited by CLA/α-CD microencapsules at a 1:4 mole ratio. The protective effect of γ-CD was similar to that of β-CD, but to a lesser extent than β-CD. POV-reducing efficiency of CDs was consistent with the protective effect of CDs for CLA oxidation. The protective efficiency of CDs for CLA oxidation may be, in part, attributed to the hydrophobicity of the inner cavity of CDs, which facilitates

insertion of the conjugated diene portion into the CD cavity, and the cavity size of the CDs, which is possibly large enough to incorporate some oxygen molecules and create a miniature reaction chamber to facilitate the reaction between CLA and oxygen. In addition, physical interference by CDs is not a negligible factor for the oxidative stability of CLA. Further study is required to explore the structural features of CLA/CDs microencapsules. It is of significance to note that β-CD is an appropriate material with which to microencapsulate CLA for industry use, because of its adequate protective effects for CLA oxidation and because it is much lower in cost than other CDs.

ABBREVIATIONS USED

CLA, conjugated linoleic acid; CD(s), cyclodextrin(s); CLA/CDs microencapsules, CLA microencapsulated in CDs; TBARS, thiobarbituric acid reactive substances; POV, peroxide value.

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