Oxidative Stability and Nuclear Magnetic Resonance Analyses of Linoleic Acid Encapsulated in Cyclodextrins[1]

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ABSTRACT: The effects of α- and β-cyclodextrin (CD) on the oxidative stability of linoleic acid (LA) at 35°C were studied by measuring headspace oxygen depletion in airtight 35-mL serum bottles. LA was encapsulated in α-CD or β-CD in an aqueous solution during homogenization at 8000 rpm for 1 min and then dried under vacuum for 60 h at room temperature. Headspace oxygen was measured by thermal conductivity gas chromatography. The rate of oxygen depletion for the control, which contained LA only, was 93.8 μmole/L·h. The rates of oxygen depletion for LA, encapsulated at a 1:1 mole ratio (mole CD/moles LA) in α-CD and β-CD, were 13.8 and 111 μmoles/L·h, respectively. When LA was encapsulated in α-CD and β-CD at a 2:1 mole ratio (moles CD/moles LA), the rates of oxygen depletion were 0.573 and 53.9 μmoles/L·h, respectively. Although α-CD protected LA from reaction with oxygen at both ratios, the rate of oxygen depletion by LA encapsulated in β-CD at a 1:1 mole ratio was not statistically different from the control. β-CD protected LA from reaction with oxygen at a 2:1 mole ratio. ^{1}H nuclear magnetic resonance spectra of the complexes formed from 1:1 mole ratios of LA and CD indicated that LA was encapsulated in α-CD or β-CD. *JAOCS 74*, 1329–1333 (1997).

KEY WORDS: Cyclodextrin, encapsulation, kinetics, linoleic acid.

The oxidative deterioration of fats and vegetable oils in food is of paramount importance in the food industry because it results in the loss of essential fatty acids with the consequent loss of nutritional value and the development of flavors that are unacceptable to consumers. Oxidation of unsaturated fatty acids is due to the reaction of molecular oxygen with fatty acids. Hydroperoxides are the primary products of the reaction, but they decompose to give an array of secondary products, including esters, aldehydes, alcohols, ketones, lactones, and hydrocarbons. Some of these secondary products have off-flavors and are responsible for the rancid odor and flavor of fat. The usual approach to minimizing oxidation is by the use of antioxidants, which can be added directly to food or utilized in packaging materials.

Cyclodextrins (CD) are produced by the bacterial enzyme CD glycosyltransferase acting on starch. The enzyme hydrolyzes and then cyclizes starch into nonreducing cyclic oligosaccharides that contain six, seven or eight glucose units and are referred to as α-CD, β-CD, and γ-CD, respectively. The hydroxyl groups at carbon-6 of the glucoses are all on the rim of one end of the CD molecule, and the hydroxyl groups at carbons-2 and -3 are on the rim of the other end (1). The glucose molecules are held in a rigid chair conformation, due primarily to the formation of intramolecular hydrogen bonds between the hydroxyl at carbon-2 of one glucose molecule and the hydroxyl at carbon-3 of the adjacent glucose molecule (2). The distance between the oxygens involved in this hydrogen bonding is 3.01 Å for α-CD and 2.86 Å for β-CD, indicating that the hydrogen bonding in β-CD is stronger than in α-CD (1).

The inner diameter of these molecules is 5–6 Å, 6–8 Å, and 8–10 Å for α-, β-, and γ-CD, respectively (3). The inner cavity is hydrophobic because it is lined with carbon and hydrogen atoms and oxygen atoms involved in acetal or hemiacetal linkages. Consequently, when these molecules are dissolved in water, their nonpolar interior becomes a thermodynamically favorable environment for other hydrophobic molecules.

Many uses in the food, cosmetic, and pharmaceutical industries have been described for these molecules to take advantage of this ability to encapsulate nonpolar molecules. Recent studies have shown that encapsulation of unsaturated fatty acids in CD does indeed slow oxidation of the fatty acid (4–10).

Jyothirmayi *et al.* (8) demonstrated that, when a fatty acid was complexed in a CD molecule, signals of the carboxyl proton at $\delta = 12$ ppm noticeably decreased.

The objectives of this research were to study the effects of encapsulation in α- or β-CD on the oxidative stability of LA at 1:1 and 2:1 mole ratios (moles CD/moles LA) at 35°C and to relate these results to nuclear magnetic resonance (NMR) analyses of the complexes formed by LA and the CD.

EXPERIMENTAL PROCEDURES

Materials. α- and β-CD were supplied by American Maize Products Company (Hammond, IN). Linoleic acid was obtained from Aldrich Chemical Company (Milwaukee, WI). Deuterated (d_6)-dimethyl sulfoxide (99.96 atom% D) was obtained from Isotec, Inc. (Miamisburg, OH).

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Serum bottles, aluminum caps, and Teflon-coated septa were obtained from Supelco, Inc. (Bellefonte, PA).

Sample preparation. Samples were prepared from 104 μL LA and the appropriate amount of α- or β-CD to obtain the CD/fatty acid mole ratio of 1:1 or 2:1. The masses of CD used for the 1:1 mole ratio sample were 0.324 g α-CD and 0.378 g β-CD. The volume of distilled water added to bottles containing α-CD and β-CD was 5.0 and 10.0 mL, respectively, due to the differences in solubilities of the CD at room temperature: 14.5 and 1.85 g/100 mL, respectively. Duplicate sample bottles were prepared for each mole ratio. Controls were prepared that contained only LA. Immediately after addition of LA to an aqueous sample, each sample was homogenized at 8000 rpm for 1 min with a Brinkmann Polytron homogenizer (Kinematica, Lucerne, Switzerland). The sample bottles were wrapped in aluminum foil to prevent oxidation of the fatty acids by light. The sample bottles were transferred to a vacuum oven, and the samples were dried completely at room temperature and 28 in. Hg in a Mastergauge Model 3640 vacuum oven from National Appliance Company (Portland, OR). The bottles were sealed with Teflon-lined rubber septa and aluminum caps. The temperature of the samples was maintained at 35°C during each experiment in a Model 25 reciprocal shaking bath from Precision Scientific Company (Chicago, IL).

Headspace oxygen analysis of LA encapsulated in CD. Autoxidation of the unsaturated fatty acid was evaluated by measuring the headspace oxygen depletion while the samples were maintained at 35°C. Headspace oxygen in the sample bottles was analyzed by injecting a 100-μL headspace air sample from each serum bottle into a Hewlett-Packard 5890 gas chromatograph (Avondale, PA), equipped with a stainless-steel molecular sieve column (13×, 80:100; Alltech, Deerfield, IL) and a thermal conductivity detector. High-purity helium (99.99%) was used as a carrier gas. The flow rate was 40 mL/min. The gas chromatograph oven temperature was maintained at 40°C. The injector port and detector temperatures were maintained at 120 and 150°C, respectively. Duplicate injections were performed for each sample bottle. Headspace oxygen content at 0 h of storage time was measured as soon as each sample was prepared. Electronic counts were integrated on a Hewlett-Packard HP 3396A integrator.

The electronic counts for the chromatographic oxygen peak in 100 μL of headspace gas were converted to mmoles/L oxygen by using the gas law assumptions.

Air contains 20.946% oxygen (11). Therefore, 100 μL of air contains 20.946 μL oxygen. At room temperature (29°C) and one atmosphere pressure, 20.946 μL oxygen contains 8.45×10^{-7} moles oxygen according to the ideal gas law, $PV = nRT$ ($R = 0.080205$ L·atm/mole·K).

At room temperature, 100 μL air produced 21.297 electronic counts of oxygen (average of six trials, coefficient of variance = 0.14%). The quantity 8.45×10^{-7} moles O_2/100 μL headspace sample equals 8.45 mmoles O_2/L. When this number was divided by 21.297 counts O_2/sample air, the factor 0.3967 mmoles/L was obtained. The number of electronic counts for each measurement was multiplied by this factor to convert the counts to mmoles/L O_2.

Headspace oxygen of the samples was measured over a 48- or 54-h period at frequent intervals.

The rate of oxygen depletion by LA, unencapsulated or encapsulated in α- or β-CD, was determined by plotting mmoles/L oxygen against time for the given mole ratio. The slope of the regression line represents the rate of oxygen depletion in mmoles/L·h. The data used to determine the rate of the reaction do not include the plateau portion of the curve that occurred at the end of the reaction. The rates of oxygen depletion are converted to μmoles/L·h.

Statistical analysis. Two measurements for each of the duplicate samples were made for headspace oxygen. Mean values are reported. The data were analyzed by analysis of variance (ANOVA) (12). Fisher's least significant difference at a 5% level of significance was used as a scale resolver to compare the headspace oxygen values at each given time. Two-way ANOVA for oxygen was carried out to analyze variability in oxygen headspace.

NMR sample preparation and analysis. ^{1}H NMR spectra were determined at 35° for LA in d_6-dimethylsulfoxide (DMSO), α-CD in DMSO, β-CD in DMSO, α-CD with encapsulated LA in a 1:1 mole ratio in DMSO, α-CD with encapsulated LA in a 2:1 mole ratio in DMSO, and β-CD with encapsulated LA in 1:1 and 2:1 mole ratios in DMSO. The samples were sonicated in an Ultrasonic FS-28 sonicator (Fisher Scientific, Springfield, NJ) for 45 min to ensure the encapsulation of LA in CD.

High-resolution ^{1}H NMR spectra were recorded in d_6-DMSO on a Bruker AM-500 NMR spectrometer (Billerica, MA), operating at a base frequency of 500.13 MHz. Proton resonance assignments were based on chemical shifts in ppm relative to tetramethylsilane with DMSO as the internal reference.

RESULTS AND DISCUSSION

Headspace oxygen analyses of LA encapsulated in CD. Headspace oxygen in the sample bottles that contained LA only (control) decreased from 8.4 to 3.9 mmoles/L, whereas the headspace oxygen in the sample bottles that contained LA encapsulated in α-CD at a 1:1 mole ratio decreased from 8.4 to 7.7 mmoles/L during the same time interval.

Figure 1 illustrates the rate of headspace oxygen depletion in the sample bottle with LA encapsulated in α-CD at 35°C for 48 h. The rate of headspace oxygen depletion for the control sample with LA alone was 93.8 μmole/L·h as determined by linear regression analysis. However, the rate of headspace oxygen depletion of the LA encapsulated in α-CD at a 1:1 mole ratio was 13.8 μmole/L·h. Therefore, encapsulation in α-CD protected LA from reaction with oxygen. The rate of headspace oxygen depletion further decreased to 0.573 μmole/L·h when the mole ratio of α-CD/LA was increased to 2:1. There was a statistically significant difference between the control and the sample at the 1:1 mole ratio ($P < 0.05$). There was no statistically significant difference, however, be-

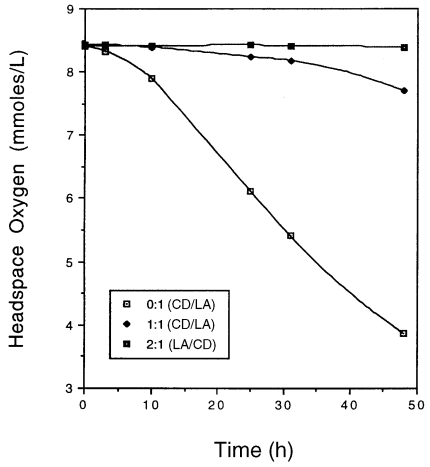

FIG. 1. Headspace oxygen depletion for linoleic acid (LA) encapsulated in α-cyclodextrin (CD) at 35°C.

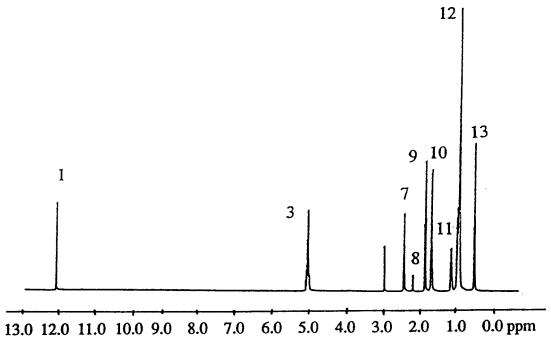

FIG. 3. ^{1}H nuclear magnetic resonance (NMR) spectrum of LA in d_6-dimethylsulfoxide (DMSO). Peaks are numbered and identified in Table 1. See Figure 1 for other abbreviations.

cause, in each spectrum, the proton signals of the carboxyl group tween the 1:1 mole ratio and the 2:1 mole ratio ($P > 0.05$). This suggests that, at a 1:1 mole ratio, α-CD encapsulation effectively lowers the oxidation of LA.

Figure 2 illustrates the rate of headspace oxygen depletion in the sample bottles that contained LA encapsulated in β-CD at 35°C for 54 h. The headspace oxygen content in the sample bottles with LA only (control) decreased from 8.3 to 3.8 mmoles/L, whereas the headspace oxygen content in the sample bottles with LA encapsulated in β-CD at a 1:1 mole ratio decreased from 8.3 to 2.5 mmoles/L over the same time period. The results, which suggest that LA encapsulated in β-CD at a 1:1 mole ratio actually reacted with oxygen faster than the control sample, which contained LA alone, were not statistically significant ($P > 0.05$). The rate of headspace oxygen depletion in the sample bottles that contained LA encapsulated in β-CD at a β-CD/LA mole ratio of 2:1 was 53.9 μmoles/L·h ($P < 0.05$). This result indicates that, at a 2:1 mole ratio, β-CD protects LA from reaction with oxygen but to a lesser extent than α-CD protects LA.

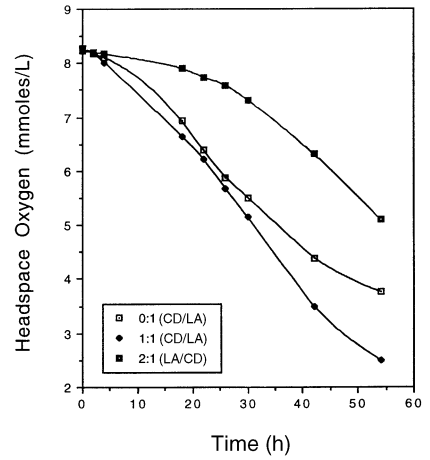

FIG. 2. Headspace oxygen depletion for LA encapsulated in β-CD at 35°C. See Figure 1 for abbreviations.

NMR analyses of LA encapsulated in CD. The proton NMR spectrum of LA in d_6-DMSO is shown in Figure 3. Peaks of all spectra are identified with numerical labels and are shown in Table 1. The proton of the carboxyl group is a sharp peak at δ = 12 ppm (8). The spectra for α- and β-CD are shown in Figure 4. The proton of the 6-hydroxyl group of the CD is at δ = 4.5 ppm (8).

Figure 5 shows NMR spectra of the complex formed by LA and α- or β-CD at a 1:1 mole ratio. Results indicate that LA is encapsulated in CD because the proton signals from the carboxyl group of LA at δ = 12 ppm decreased into the baseline, compared to the spectra of LA alone in Figure 3. Also, the signal from the glucose 6-hydroxyl group of the CD at δ = 4.5 ppm decreased into the baseline for the LA complex formed with α-CD, but not with β-CD. These observations agree with those by Jyothirmayi *et al.* (8).

Although not shown, NMR spectra of the 2:1 mole ratio complexes indicate that LA is complexed with both α- and β-CD be-

of LA at δ = 12 ppm disappeared almost completely.

TABLE 1
Identification of Nuclear Magnetic Resonance (NMR) Peaks[a]

Number of peak	Identification of peak	δ (ppm)
1	COOH of LA	12.0
2	2,3-OH of CD	5.4
3	CH=CH (C-9,-10,-12,-13) of LA	5.3
4	Anomeric C of CD	4.8
5	6-OH of CD	4.5
6	H-2,-3,-4,-5,-6 of CD	3.2–3.7
7	C-11 of LA	2.7
8	DMSO	2.5
9	C-2 of LA	2.2
10	C-8,-14 of LA	2.0
11	C-17 of LA	1.5
12	C-3 to C-7, C-15,-16 of LA	1.2–1.3
13	C-18 of LA	0.8

[a]^{1}H NMR resonance assignments were recorded in d_6 (DMSO) and based on chemical shifts in ppm relative to tetramethylsilane with DMSO as the internal reference; CD, cyclodextrin; LA, linoleic acid.

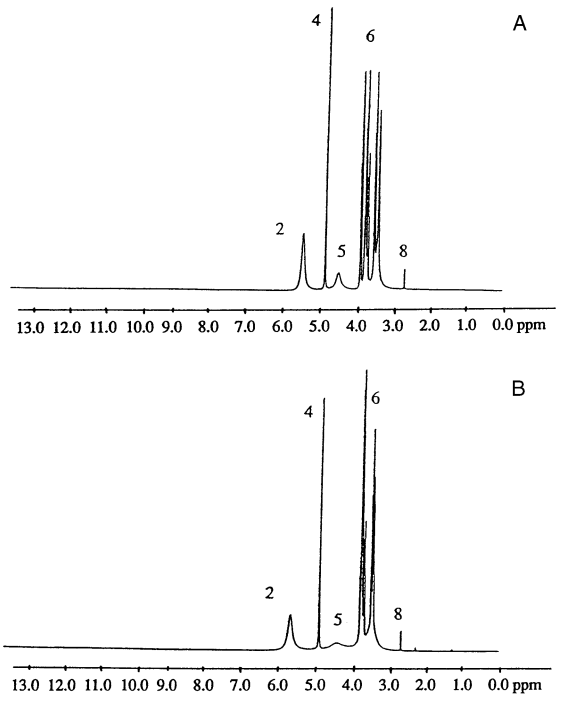

FIG. 4. ^{1}H NMR spectra of α-CD (A) and β-CD (B) in d_6-DMSO. Peaks are numbered and identified in Table 1. See Figures 1 and 3 for abbreviations.

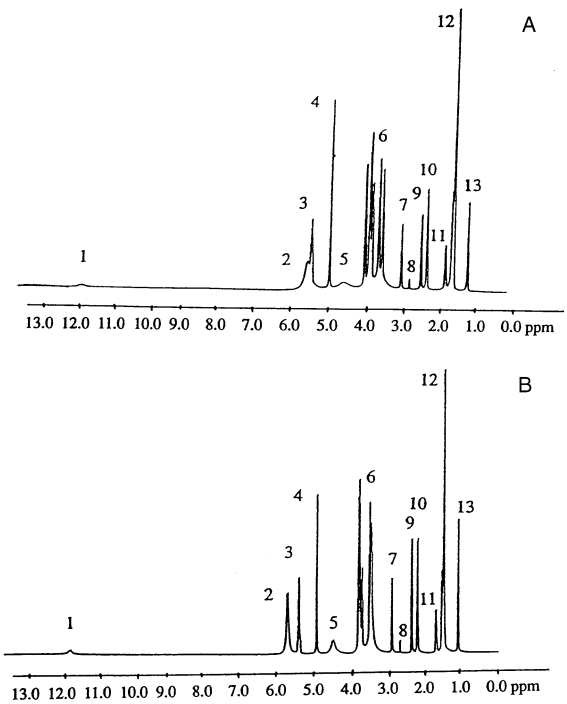

FIG. 5. ^{1}H NMR spectra of 1:1 mole ratio complexes of α-CD (A) and β–CD (B) with LA. Peaks are numbered and identified in Table 1. See Figures 1 and 3 for abbreviations.

Relationship between oxidative stability and NMR analyses of LA encapsulated in CD. α- and β-CD encapsulate LA at 1:1 or at 2:1 mole ratios as indicated by the disappearance of the proton signal of the carboxyl group of LA at δ = 12 ppm. The disappearance of this signal coincides with increased oxidative stability for LA encapsulated in α-CD at both 1:1 and 2:1 mole ratios (moles CD/moles LA). However, the disappearance of the carboxyl signal of LA at δ = 12 ppm coincides with apparent (although not statistically significant, $P > 0.05$) decreased oxidative stability over the time period studied for LA encapsulated in β-CD at a 1:1 mole ratio, but coincided with increased oxidative stability at a 2:1 mole ratio. It is our opinion that, even though LA encapsulates in β-CD at a 1:1 mole ratio, the cavity of β-CD, which was about 2 Å larger than the cavity of α-CD, enables some nonpolar oxygen molecules to enter the hydrophobic cavity of β-CD, thereby creating a miniature reaction chamber that enables some reaction between LA and oxygen.

When LA is encapsulated in β-CD, the rate of reaction of LA with oxygen decreases from 111 at a 1:1 mole ratio to 53.9 μmoles/L·h at a 2:1 mole ratio because excess CD molecules may trap nonpolar oxygen inside the hydrophobic cavity, resulting in decreased oxygen availability for reaction with the LA.

In summary, NMR studies confirmed the oxygen depletion studies and indicated that, at 35°C, α-CD forms a complex with LA which protects LA from reaction with oxygen at a 1:1 mole ratio. However, the complex formed by LA and β-CD at a 1:1 mole ratio does not protect the LA from reaction with oxygen because the cavity of the β-CD is possibly large enough to incorporate some nonpolar oxygen molecules to create a miniature reaction chamber and facilitate the reaction between LA and oxygen. β-CD has to be present in at least a 2:1 mole ratio (moles CD/moles LA) to protect LA from reaction with oxygen. Further study is required to explore why the proton signal from the glucose 6-hydroxyl group of the CD at δ = 4.5 ppm decreases into the baseline after complexation of LA with α-CD, but not with β-CD.

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