Lipase-Catalyzed Synthesis of Unsaturated Acyl L-Ascorbates and Their Ability to Suppress the Autoxidation of Polyunsaturated Fatty Acids

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ABSTRACT: L-Ascorbic acid and various polyunsaturated fatty acids (PUFA) were condensed at 55°C by the immobilized lipase Chirazyme L-2 in dry acetone to produce the unsaturated acyl ascorbates. The PUFA moieties of the products were much more resistant to autoxidation at 65°C and nearly 0% relative humidity than the corresponding unmodified PUFA. The effects of the molar ratio of ascorbic acid or linoleoyl ascorbate to linoleic acid on the autoxidation of linoleic acid were examined. The autoxidation of linoleic acid was effectively suppressed at molar ratios greater than or equal to 0.2 when either ascorbic acid or linoleoyl ascorbate was mixed with linoleic acid. The addition of lauroyl ascorbate, synthesized through the enzyme-catalyzed condensation of ascorbic acid and lauric acid in acetone, to docosahexaenoic acid also significantly suppressed the autoxidation of docosahexaenoic acid at molar ratios of ≥0.2.

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KEY WORDS: Acyl ascorbate, L-ascorbic acid, autoxidation, condensation, immobilized lipase, polyunsaturated fatty acid.

Much attention has been paid to the use of polyunsaturated fatty acids (PUFA) as components in foods (1). However, PUFA are susceptible to autoxidation (2,3), and the autoxidation causes deterioration of the foods. L-Ascorbic acid is a hydrophilic antioxidant with a strong reducing ability. The lipase-catalyzed synthesis of acyl ascorbate in a solid-phase system (4) or in an organic solvent (5–9) has been reported. However, its ability to suppress lipid autoxidation has not been reported. In a previous paper (10), we reported the synthesis of 6-*O*-eicosapentaenoyl L-ascorbate by the lipase-catalyzed condensation of eicosapentaenoic acid and L-ascorbic acid in acetone and compared its autoxidation process to that of the unmodified eicosapentaenoic acid. In the work described in this paper, some PUFA L-ascorbates were synthesized using an immobilized lipase from *Candida antarctica*, Chirazyme® L-2, and their autoxidation processes were then observed. The PUFA used were linoleic, α-linolenic, γ-linolenic, arachidonic, and docosahexaenoic acids. The effect of the molar ratio of unmodified L-ascorbic acid or linoleoyl ascorbate to linoleic acid on the suppression of the autoxidation of linoleic acid was examined. We previously reported the lipase-catalyzed condensation of ascorbic acid with various medium-chain fatty acids having carbon numbers of 6, 8, 10, and 12 in acetonitrile (11). Therefore, the ability of lauroyl ascorbate to suppress the autoxidation of docosahexaenoic acid was also evaluated in the present work.

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EXPERIMENTAL PROCEDURES

Materials. γ-Linolenic and docosahexaenoic acids were supplied by the Maruha Corporation (Tokyo, Japan), and their purities were both greater than 95% based on gas chromatographic (GC) analysis. L-Ascorbic acid, linoleic acid, acetone, and hexane were purchased from Nacalai Tesque (Kyoto, Japan). α-Linolenic, arachidonic, and lauric acids were purchased from Sigma Chemical (St. Louis, MO). Immobilized lipase from *C. antarctica*, Chirazyme® L-2 c.-f. C2, was obtained from Roche Molecular Biochemicals (Mannheim, Germany). The enzyme is the same as Novozym®435 according to the manufacturer. Soybean oil was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Condensation reaction. Acetone was first dehydrated by adding 5 Å molecular sieves. The water content of the acetone was about 0.01% (vol/vol), and was determined for each experiment by a Karl-Fischer titration. L-Ascorbic acid (0.125 mmol) and a PUFA [linoleic acid (0.577 mmol)], γ-linolenic acid (0.600 mmol), arachidonic acid, (0.638 mmol), α-linolenic acid (0.611 mmol), and docosahexaenoic acid (0.648 mmol)] were weighed into an amber glass vial with a screw cap, and 200 mg of Chirazyme L-2 and 2.5 mL of dehydrated acetone were added to the vial. The headspace of the vial was filled with nitrogen, and the vial was tightly sealed. The vial was then immersed in a waterbath at 55°C with vigorous shaking to commence the condensation reaction. At appropriate intervals, 10 μL of the reaction mixture was taken and mixed with 50 μL of a 50 mM solution of toluene in high-performance liquid chromatography (HPLC) eluent [acetonitrile/tetrahydrofuran/0.1% (vol/vol) phosphoric acid (50:22:28 by vol) as the internal standard for the HPLC analysis and then with 40 μL of HPLC eluent. The analysis was carried out by HPLC with a YMC-Pack C8 column (4.6 mm × 250 mm; YMC Inc., Kyoto, Japan) and an ultraviolet (UV) detector (245 nm). The mixture (20 μL) was applied to the column and eluted with the eluent at 1.5 mL/min. The retention times of

linoleoyl, γ-linolenoyl, arachidonoyl, α-linolenoyl, and docosahexaenoyl ascorbates, which were synthesized in this study, were 7.0, 5.8, 6.6, 5.6, and 6.2 min, respectively. After the sampling, the headspace was again filled with nitrogen to prevent the oxidation of the substrates and product.

Purification of product. The product was purified by preparative HPLC with a YMC-Pack ODS-AQ column (20 mm i.d. × 250 mm; YMC Inc.) using a mixture of methanol and water [85:15 (vol/vol) for linoleoyl ascorbate, 87:13 for γ- and α-linolenoyl ascorbates, and 90:10 for arachidonoyl and docosahexaenoyl ascorbates] as the eluent. The volume of sample applied was 500 μL, and the flow rate of each eluent was 9.9 mL/min. The retention times of linoleoyl, γ- and α-linolenoyl, arachidonoyl, and docosahexaenoyl ascorbates were 43.4, 22.3, 21.8, 18.8, and 18.2 min, respectively. The purification was repeated until an amount of each product sufficient for autoxidation experiments was obtained.

Autoxidation. About 11–20 mg of PUFA ascorbate was dissolved in 1 mL of methanol. Aliquots of the solution (20 μL each) were placed in flat-bottomed glass cups (1.5 cm i.d. and 3.0 cm height), and the methanol was then evaporated under reduced pressure. The cups were placed in a desiccator with a petri dish containing phosphorus pentoxide to regulate the relative humidity at nearly 0%. The desiccator was stored in the dark at 65°C. At appropriate intervals, a cup was removed, and 10 μL of a hexane solution of methyl palmitate (0.025 mL/mL) was added to the cup. Methyl palmitate was used as the internal standard for the GC analysis. After the evaporation of hexane, an aliquot (200 μL) of sodium methoxide in methanol (1.0 g/L) was added. The cup was stored at 70°C for 30 min to transesterify the PUFA ascorbate to the PUFA methyl ester. The transesterification then was stopped by adding 10 mL of acetic acid to the cup. The methanol was removed under reduced pressure. The residue was then dissolved in 500 μL of hexane for GC analysis with flame-ionization detection (FID) (12).

The autoxidation of unmodified PUFA was conducted as follows. The cups described above were filled with 2.5 μL of a solution containing 15–20 μL of PUFA dissolved in 1 mL of hexane. After evaporation of the hexane, the autoxidation was carried out under the conditions described above. At appropriate intervals, a cup was taken out, and the unoxidized PUFA was quantified by GC analysis.

The autoxidation of linoleic acid mixed with an appropriate amount of ascorbic acid or linoleoyl ascorbate was also evaluated. Thirty microliters of linoleic acid and the specified amount of L-ascorbic acid were dissolved in 2 mL of methanol, or 7.5 μL of linoleic acid and the specified amount of linoleoyl ascorbate were dissolved in 500 μL of methanol. Aliquots of this solution (25 μL each) were placed in the cups, and the methanol was removed under reduced pressure. The autoxidation of the linoleic acid was then observed under the conditions described above. In these experiments, the autoxidation of the linoleoyl moiety of linoleoyl ascorbate was not observed because the moiety was not transesterified to methyl linoleate.

The autoxidation of docosahexaenoic acid mixed with lauroyl ascorbate at various molar ratios was measured as follows. Forty microliters of docosahexaenoic acid was dissolved in 1.0 mL of methanol, and 9.82 mg (0.0274 mmol) of lauroyl ascorbate was dissolved in 0.5 mL of methanol. Aliquots of the docosahexaenoic acid solution (250 μL each, corresponding to 0.0274 mmol of docosahexaenoic acid) were placed in the cups, and 500, 250, 100, or 25 mL of lauroyl ascorbate solution was added to give ascorbate/fatty acid molar ratios of 1.0, 0.5, 0.2 or 0.05. The subsequent procedures were the same as in the experiments with linoleic acid. The conditions for the GC analysis of the unoxidized docosahexaenoic acid were the same as those for the analysis of the eicosapentaenoyl ascorbate (10).

Solubility of linoleoyl ascorbate in hexane or soybean oil. Twenty milligrams of linoleoyl ascorbate or ascorbic acid were weighed in an amber vial, and 2 mL of hexane or soybean oil was added. The vial was immersed in a water bath at 50°C for 1 h, and the vial was then transferred to another water bath and vigorously shaken at 25°C for 24 h. The solution was filtered using a Dismic-13 JP membrane filter (Advantec Toyo, Tokyo, Japan; pore diameter 0.2 μm). When hexane was used as the solvent, the filtrate (20 μL) was directly analyzed by high-performance liquid chromatography (HPLC) with a Cosmosil® NH_2-MS column (4.6 mm i.d. × 250 mm; Nacalai Tesque) and a UV detector (245 nm) to determine the concentration of linoleoyl ascorbate or ascorbic acid. The eluent was a 50:50 (vol/vol) mixture of methanol and 0.25% (wt/vol) potassium dihydrogenphosphate, and its flow rate was 0.8 mL/min (13). The retention times of linoleoyl ascorbate and ascorbic acid were 8.7 and 10.3 min, respectively. For soybean oil, the filtrate was diluted twice with hexane and then analyzed by HPLC under the same conditions. The calibration curves were prepared using linoleoyl ascorbate and ascorbic acid dissolved in the eluent at known concentrations.

RESULTS AND DISCUSSION

Condensation of PUFA and L-ascorbic acid. Figure 1 shows the changes with time in the concentration of the polyunsatu-

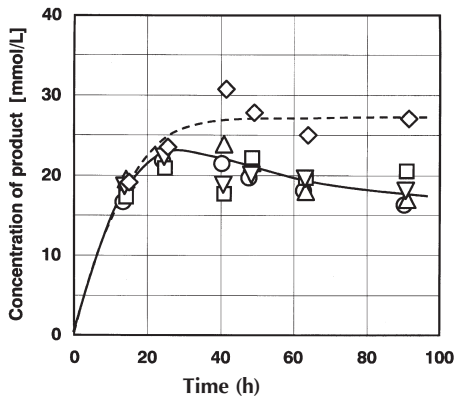

FIG. 1. Changes with time in concentration of polyunsaturated acyl L-ascorbates during immobilized-lipase-catalyzed condensation of polyunsatutated fatty acids and L-ascorbic acid in dehydrated acetone at 55°C. The polyunsaturated fatty acids used were (○) linoleic, (△) γ-linolenic, (□) arachidonic, (▽) α-linolenic, and (◇) docosahexaenoic acid.

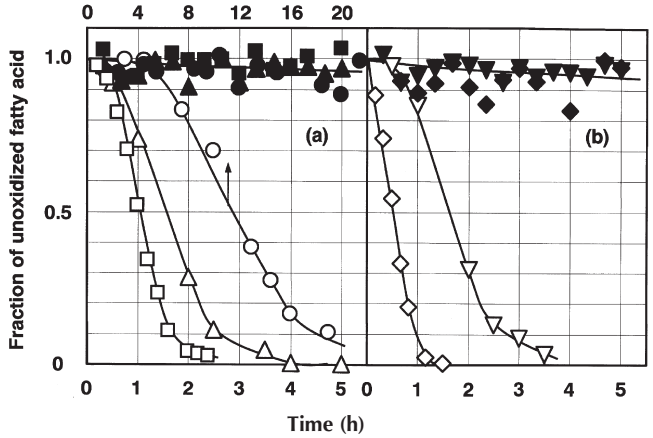

FIG. 2. Autoxidation of polyunsaturated fatty acids and their ascorbates at 65°C and nearly 0% relative humidity. (a) n-6 series: (○) linoleic acid, (●) linoleoyl ascorbate, (△) γ-linolenic acid, (▲) γ-linolenoyl ascorbate, (□) arachidonic acid, and (■) arachidonoyl ascorbate; (b) n-3 series: (▽) α-linolenic acid, (▼) α-linolenoyl ascorbate, (◇) docosahexaenoic acid, and (◆) docosahexaenoyl ascorbate. The time scale shown on the upper axis of (a) is for linoleic acid and linoleoyl ascorbate.

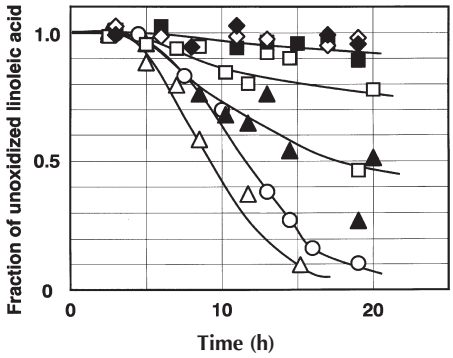

FIG. 3. Autoxidation of linoleic acid mixed with L-ascorbic acid at various molar ratios. The molar ratios of ascorbic acid to linoleic acid were (○) 0, (△) 0.01, (▲) 0.05, (□) 0.1, (■) 0.2, (◇) 0.5, and (◆) 1.0. The autoxidation was observed at 65°C and nearly 0% relative humidity.

rated acyl L-ascorbates produced through the lipase-catalyzed condensation of the corresponding PUFA and L-ascorbic acid in dehydrated acetone. Although the structures of the products were not identified in this study, the products can be reasonably presumed to be 6-*O*-acyl ascorbates because the ascorbates esterified with eicosapentaenoic acid (10) and lauric acid (11) by the enzyme were identified by ^{1}H nuclear magnetic resonance (NMR) to be the corresponding 6-*O*-acyl ascorbates. In every case, the molar ratio of fatty acid to ascorbic acid was *ca.* 5, and no desiccant was added to the reaction system to remove the water produced. The concentration of each product reached a maximal yield of about 23 mmol/L (except for docosahexaenoyl ascorbate, the maximum concentration of which was *ca.* 27 mmol/L) within 2 d. The concentration gradually decreased during the latter half of the experiment. The decrease could be ascribed to the oxidative degradation of PUFA and/or the ascorbate moiety of the product owing to their instability, although the headspace was filled with nitrogen after each sampling.

Comparison of autoxidation between PUFA and corresponding ascorbate. Figure 2 shows the autoxidation processes of the unmodified PUFA and the PUFA moiety of the PUFA ascorbates at 65°C and nearly 0% relative humidity. The unmodified PUFA were almost completely autoxidized within 5 h, except for linoleic acid, which was autoxidized within 1 d, whereas all the PUFA ascorbates were significantly resistant to autoxidation. Ninety percent or more of the PUFA moieties remained in the unoxidized state during the test period for each of the ascorbates.

Effect of addition of ascorbic acid or linoleoyl ascorbate on the autoxidation of linoleic acid. Figure 3 shows the autoxidation at 65°C and *ca.* 0% relative humidity of linoleic acid mixed with ascorbic acid in various molar ratios. At molar ratios of ascorbic acid to linoleic acid greater than or equal to 0.2, the autoxidation of linoleic acid was suppressed nearly completely for at least 20 h even under the severe conditions employed.

Similar results were obtained when linoleoyl ascorbate was mixed with linoleic acid in various molar ratios and the autoxidation of linoleic acid was measured at 65°C and *ca.* 0% relative humidity (Fig. 4). As with addition of the unmodified ascorbic acid, the autoxidation of linoleic acid was almost completely suppressed at molar ratios of greater than or equal to linoleoyl ascorbate to linoleic acid 0.2.

Suppression of autoxidation of docosahexaenoic acid by addition of lauroyl ascorbate. From the results shown in Figure 4, it was expected that the addition of saturated acyl ascorbate to a PUFA would suppress the autoxidation of the PUFA. Therefore, we measured the autoxidation of docosahexaenoic acid, which is very susceptible to autoxidation, mixed with lauroyl ascorbate in various molar ratios. As shown in Figure 5, the autoxidation of docosahexaenoic acid was significantly suppressed at molar ratios of lauroyl ascorbate to docosahexaenoic acid greater than or equal to 0.2.

Solubility of linoleoyl ascorbate in hexane and soybean oil. As shown in Figures 3–5, both the unmodified and acyl-

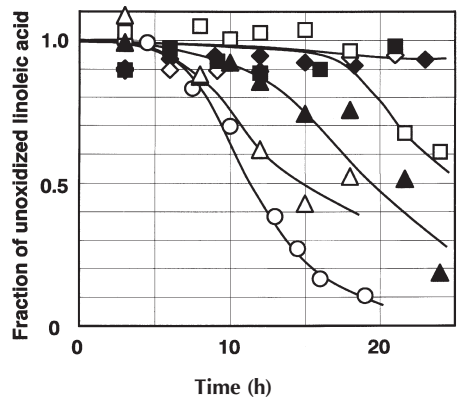

FIG. 4. Effect of the addition of linoleoyl L-ascorbate to linoleic acid on autoxidation of linoleic acid at 65°C and nearly 0% relative humidity. The molar ratios of linoleoyl ascorbate to linoleic acid were (○) 0, (△) 0.01, (▲) 0.05, (□) 0.1, (■) 0.2, (◇) 0.5, and (◆) 1.0.

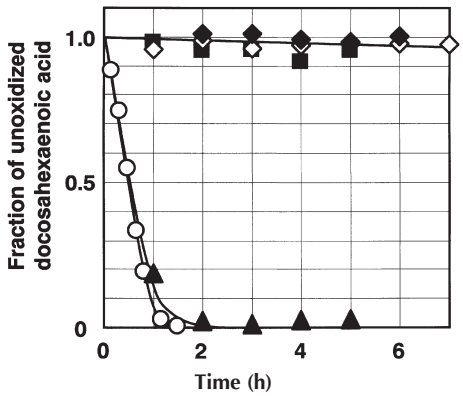

FIG. 5. Suppression of autoxidation of docosahexaenoic acid by the addition of lauroyl L-ascorbate. The molar ratios of lauroyl ascorbate to docosahexaenoic acid were (○) 0, (▲) 0.05, (■) 0.2, (◇) 0.5 and (◆) 1.0. The autoxidation was observed at 65°C and nearly 0% relative humidity.

ated ascorbic acids were effective in suppressing oxidation of PUFA. Advantages of the acylated ascorbic acids would be their greater hydrophobicity and consequently better solubility in hydrophobic environments. Therefore, the solubilities of linoleoyl ascorbate in hexane and soybean oil, measured at 25°C, were 1.14 and 1.82 mmol/L, respectively. The solubilities of unmodified ascorbic acid in hexane and soybean oil were too low to be evaluated under our analytical conditions. It is notable that despite these substantial differences in solubility in hydrophobic solvents, under the experimental conditions employed here there was no difference between the abilities of ascorbic acid and its fatty acid esters to prevent PUFA oxidation.

The polyunsaturated acyl ascorbates could be synthesized through the lipase-catalyzed condensation of the corresponding fatty acids and L-ascorbic acid, and the ascorbates were highly resistant to autoxidation. The addition of saturated or unsaturated acyl ascorbate to a PUFA in molar ratios of ≥0.2 was very effective in suppressing autoxidation of the PUFA. The reason why the molar ratio of 0.2 was critical for the suppression of autoxidation remains unclear. Whereas ascorbic acid is a hydrophilic antioxidant with low solubility in hydrophobic materials, the solubility of acyl ascorbates in hexane and soybean oil is on the order of millimoles per liter. Thus, acyl ascorbates may be promising food additives with the ability to suppress the autoxidation of unsaturated fatty acids or their acylglycerols.

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