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Production of Saturated Acyl L-Ascorbate by Immobilized Lipase Using a Continuous Stirred Tank Reactor

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6-*O*-Decanoyl, 6-*O*-dodecanoyl, or 6-*O*-tetradecanoyl L-ascorbate was continuously produced at 50 °C using a continuous stirred tank reactor (CSTR) with an immobilized lipase, Chirazyme L-2 C2, from *Candida antarctica*. Acetone was used as the reaction medium. For each saturated acyl L-ascorbate, the productivity of ca. 60 g/L reactor/day was achieved for at least 11 days. The solubility of the saturated acyl L-ascorbate in the soybean oil or water was measured at various temperatures. The solubilities in both the soybean oil and the water were higher for L-ascorbate with a shorter acyl chain. The acyl chain dependence of the solubility in water was stronger than that of the solubility in soybean oil. The temperature dependences of the solubility in both soybean oil and water could be expressed by the van't Hoff equation, and the dissolution enthalpy (ΔH) values for the soybean oil and water were about 20 and 90 kJ/mol, respectively, irrespective of the acyl chain length. The radical scavenging activities of L-ascorbic acid and the saturated acyl L-ascorbates against 1,1-diphenyl-2-picrylhydrazyl free radical were ca. 95% for all of the compounds, and the introduction of a saturated acyl group to the L-ascorbic acid did not affect the activity.

KEYWORDS: Acyl L-ascorbate; immobilized lipase; continuous stirred tank reactor; solubility; radical scavenging activity

INTRODUCTION

L-Ascorbic acid, which is known as vitamin C, is a water soluble vitamin with high reductivity. It is widely used as an additive in foods and cosmetics, whereas its lipophilic derivative acylated with a long-chain fatty acid such as hexadecanoic or octadecanoic acid is also used as an additive in foods rich in lipids. As compared to the chemical synthesis, the enzymatic synthesis using lipase has some advantages such as the direct use of unmodified substrates, moderate reaction conditions, and high regiospecificity of the enzyme. Because the lipase-catalyzed reaction in a conventional aqueous system thermodynamically favors the hydrolysis, a microaqueous organic solvent or solvent-free system is usually used to shift the reaction toward the synthesis. Recently, some papers have been published about the lipase-catalyzed synthesis of acyl L-ascorbates in an organic solvent (*1*−*11*). The syntheses have mainly been conducted in a batch reaction, although a continuous reaction would be preferred for the large-scale production. We reported the continuous syntheses of acyl erythritol or saccharides using a packed-bed reactor (*12*, *13*). The continuous production of 6-*O*-acyl L-ascorbates has not been reported.

Knowledge about the properties of the acyl L-ascorbates is important for their use as a food additive. We recently reported their surfactant properties (*14*). The solubility of acyl L-ascorbate in water or oil would be an important property for its application. We showed that 6-*O*-acyl L-ascorbates possessed an antioxidative ability for the autoxidation of unsaturated fatty acids (*11*). This would be due to the radical scavenging activity of the 6-*O*-acyl L-ascorbate.

In this study, we examined the continuous production of the 6-*O*-decanoyl, dodecanoyl, or tetradecanoyl L-ascorbate through the immobilized lipase-catalyzed condensation of L-ascorbic acid and decanoic, dodecanoic, or tetradecanoic acid in acetone using a continuous stirred tank reactor (CSTR). The solubility of acyl L-ascorbate in water or soybean oil was measured at various temperatures, and the dissolution enthalpy (ΔH) was evaluated from the van't Hoff plots. In addition, the radical scavenging activities of L-ascorbic acid and the acyl L-ascorbates against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical were measured.

MATERIALS AND METHODS

Materials. Immobilized lipases, Chirazyme L-1 from *Pseudomonas cepacia*; L-2, L-2 C2, and L-2 C3 from *Candida antarctica* type B; L-5 from *Candida antarctica* type A; and L-9 and L-9 C2 from

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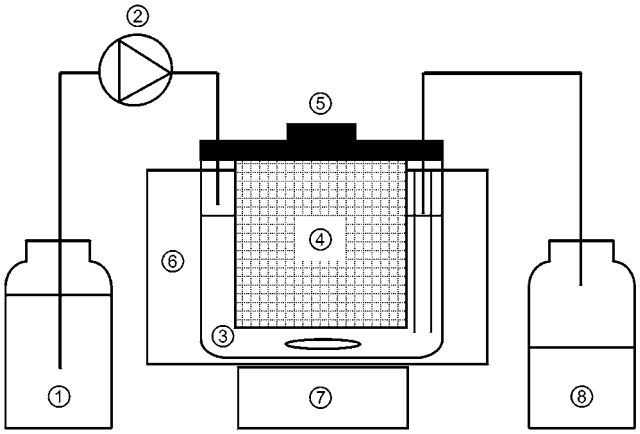

Figure 1. Scheme of CSTR for the continuous synthesis of saturated acyl L-ascorbates; (1) feed reservoir, (2) pump, (3) reactor, (4) basket packed with immobilized lipase, (5) lid, (6) water bath, (7) magnetic stirrer, and (8) effluent reservoir.

Rhizomucor miehei, were purchased from Roche Molecular Biochemicals, Mannheim, Germany. The octanoic, decanoic, dodecanoic, tetradecanoic, and hexadecanoic acids, acetone, soybean oil, and molecular sieves 4A 1/16 were obtained from Wako Pure Chemical Industries, Osaka, Japan. L-(+)-Ascorbic acid and hexadecanoyl L-ascorbate were obtained from Nacalai Tesque, Kyoto, Japan. The DPPH free radical and octadecanoyl L-ascorbate were obtained from Tokyo Kasei Kogyo, Tokyo, Japan. The chemicals were of analytical grade.

Synthesis of Saturated Acyl L-Ascorbate Using Various Immobilized Lipases in a Batch Reactor. L-Ascorbic acid (0.25 mmol) and a specific amount of decanoic, dodecanoic, or tetradecanoic acid were weighed into an amber glass vial with a screw-cap, and 100 mg of one of the immobilized lipases and 5 mL of acetone were then added to the vial. In the case where the effect of the addition of a desiccant on the conversion was examined, 250 mg of the molecular sieve 4A 1/16 was added. The vial was then immersed in a water bath at 50 °C to commence the condensation reaction with vigorous shaking. At appropriate intervals, 10 μL of the reaction mixture was sampled.

Continuous Production of Saturated Acyl L-Ascorbate Using a CSTR. A schematic diagram of the apparatus is shown in **Figure 1**. Chirazyme L-2 C2 particles (3.0 g by dry weight) were packed into a 78 mm × 62 mm i.d., 0.2 mm mesh size, stainless steel basket. The volume of the solvent in the reactor was 350 mL. The tube for withdrawing the reaction mixture was covered by concentric tubes to prevent the wash-out of any undissolved L-ascorbic acid powder. At the beginning of the operation, 40 g of L-ascorbic acid was added to the reactor after removing the upper lid. During the continuous operation, about 10 g of L-ascorbic acid was converted to the desired product and about 10 g of the acid, dissolved in the effluent, was left everyday from the reactor. Therefore, 20 g of L-ascorbic acid was added daily to the reactor. A fatty acid (decanoic, dodecanoic, or tetradecanoic acid) was dissolved with acetone at the concentration of 200 mmol/L, and the mixture was fed to the 90 mm × 85 mm i.d. reactor at a specified flow rate by an LC-3A pump (Shimadzu, Kyoto, Japan). The reactor was immersed in a water bath at 50 °C, and the reaction mixture was mixed by a magnetic stirrer. No disruption of the immobilized lipase by mixing was observed. To obtain the relationship between the conversion and the mean residence time, τ, the flow rate was varied in the range of 1.5−9.9 mL/min for each fatty acid. After a steady state was achieved (usually the substrate solution of three times the reactor volume was fed), the effluent was sampled. The product concentration was determined using a high-performance liquid chromatography (HPLC).

In long-term operations, the feed solution was fed at 2.0 mL/min, corresponding to the residence time of 175 min. At first, the dodecanoic acid solution was fed for 5 days, and then after changing the feed solution to decanoic acid solution, the production of decanoyl ascorbate was continued for 3 days. Furthermore, tetradecanoyl ascorbate was then continuously produced under similar conditions for 3 days. At appropriate intervals, the effluent was sampled and the dodecanoyl, decanoyl, or tetradecanoyl ascorbate concentration in it was determined.

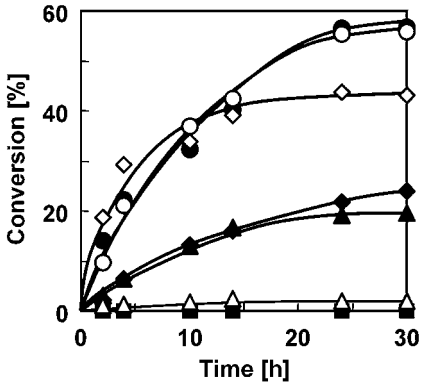

Figure 2. Changes with time in conversion of dodecanoyl L-ascorbate in acetone (5 mL) at 50 °C through condensation of L-ascorbic acid (0.25 mmol) and dodecanoic acid (1.875 mmol) using various immobilized lipases (100 mg). Chirazyme L-1, (◆), L-2 (◇), L-2 C2 (●), L-2 C3 (○), L-5 (■), L-9 (▲), and L-9 C2 (△). The curves were empirically drawn.

Solubility of Saturated Acyl L-Ascorbate in Water or Soybean Oil. The purified acyl ascorbate (10 mg to 1 g) was weighed in an amber vial, and 1−4 mL of distilled water or soybean oil was added. After the vial was immersed in a water bath at 50 °C for 1 h, the vial was transferred to a chamber or water bath kept at 5, 25, 37, or 50 °C for 24 h. The solution was filtered using a 0.2 μm pore diameter Dismic-13 JP membrane filter (Advantec Toyo, Tokyo, Japan) as quickly as possible, and the filtrate (10 μL) was analyzed using an HPLC.

Radical Scavenging Activity of Saturated Acyl L-Ascorbate. On the basis of a reported method (*15*, *16*), the radical scavenging activity of the saturated acyl ascorbate was quantified. Four milliliters of a 50% ethanol solution of each acyl ascorbate (0.125 mmol/L) and 1 mL of ethanol solution of DPPH free radical (0.500 mmol/L) were added to an amber vial. The headspace of the vial was filled with nitrogen gas, and it was tightly sealed. The vial was then vigorously shaken and incubated for 20 min at 25 °C. The radical scavenging activity of each acyl ascorbate was measured by the decolorization of DPPH free radical at 516 nm using a UV-1200 UV−vis spectrophotometer (Shimadzu), and the activity was expressed by the percent loss of the initial absorbance.

Analysis and Purification of Saturated Acyl L-Ascorbates. The analysis was carried out using an LC-10AT HPLC (Shimadzu) with a 300 mm × 4.6 mm i.d. Nucleosil 5C18 ODS column (Chemco Scientific, Osaka, Japan) and a SPD-10A UV detector (Shimadzu) operated at 245 nm. The eluent used was a mixture of methanol, water, and phosphoric acid (90/10/0.1 v:v:v). The sample was appropriately diluted by the eluent, and 20 μL was applied to the column. The flow rate was 1.0 mL/min for the decanoyl, dodecanoyl, and tetradecanoyl ascorbates and 1.2 mL/min for the hexadecanoyl and octadecanoyl ascorbates. The retention times of the decanoyl, dodecanoyl, tetradecanoyl, hexadecanoyl, and octadecanoyl ascorbates were 4.9, 6.0, 7.9, 9.2, and 13.9 min, respectively. The calibration curve was prepared using each isolated acyl ascorbate, which was dissolved in the eluent at known concentrations.

Each acyl ascorbate was isolated from the effluent according to the reported methods (*7*) with a slight modification. L-Ascorbic acid and acetone separated in the purification process were recycled back to the continuous production.

RESULTS AND DISCUSSION

Condensation of L-Ascorbic Acid and Dodecanoic Acid by Various Immobilized Lipases. Figure 2 shows the transient changes in the conversion to dodecanoyl L-ascorbate through the condensation of L-ascorbic acid and dodecanoic acid

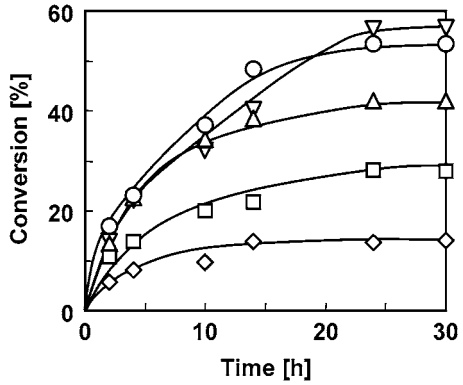

Figure 3. Effect of molar ratio of dodecanoic acid to L-ascorbic acid (0.25 mmol) on conversion to the product in acetone (5 mL) at 50 °C; the molar ratios of dodecanoic acid to L-ascorbic acid were 1 (◇), 2 (□), 5 (△), 7.5 (▽), and 10 (○). The amount of Chirazyme L-2 C2 was 100 mg. The curves were empirically drawn.

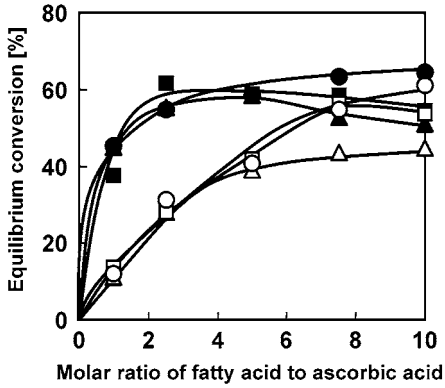

Figure 4. Effect of the addition of molecular sieves on the equilibrium conversion for the condensation of L-ascorbic acid and fatty acid; decanoic (○, ●), dodecanoic (□, ■), and tetradecanoic (△, ▲) acid. Closed and open symbols represent their presence and absence, respectively. The amount of molecular sieves was 250 mg in 5 mL of acetone. The other conditions were the same as in **Figure 3**. The curves were empirically drawn.

catalyzed by seven immobilized lipases (Chirazyme L-1, L-2, L-2 C2, L-2 C3, L-5, L-9, and L-9 C2) in acetone at 50 °C. The conversion was calculated based on the amount of L-ascorbic acid added, which was the limiting reactant. The immobilized lipases from *C. antarctica* type B (Chirazyme L-2 C2 and L-2 C3) gave high conversions (ca. 56%), as compared with the other lipases. The carrier of Chirazyme L-2 C2 is an acryl resin and that of L-2 C3 is undisclosed, but there was no difference in the conversion between the lipases. The immobilized lipases from *R. miehei* and *C. antarctica* type A (Chirazyme L-9 or L-9 C2 and L-5) hardly catalyzed the synthesis of dodecanoyl L-ascorbate. Chirazyme L-2 C2, which gave the highest conversion, was used as the catalyst throughout this study.

Effects of Molar Ratio of Fatty Acid to L-Ascorbic Acid and the Addition of Molecular Sieves on the Conversion to Product. Changes with time during the conversion of dodecanoyl L-ascorbate at various molar ratios of dodecanoic acid to L-ascorbic acid are shown in **Figure 3**. Because the solubility of L-ascorbic acid in acetone at 50 °C was ca. 26 mmol/L, undissolved L-ascorbic acid remained in the reaction mixture. The molar ratio was an overall one including the undissolved L-ascorbic acid. The conversion was higher at the higher ratios, and the conversions at the ratios of 7.5 and 10 were almost the same.

Because water is one of the products in the conversion, its removal is expected to derive the reaction toward the product formation. For the syntheses of the decanoyl, dodecanoyl, and tetradecanoyl L-ascorbates, the equilibrium conversions were measured in the presence of molecular sieves 4A 1/16 (50 mg/mL) at various molar ratios of fatty acid to L-ascorbic acid and were compared with the conversions in the absence of the molecular sieves. The reaction was completed within 30 h for each acyl L-ascorbate in both the presence and the absence of the molecular sieves. As shown in **Figure 4**, the addition of the molecular sieves significantly raised the equilibrium conversion at the low molecular ratios, but it had no significant effect at the high ratios. **Figure 4** also indicates that the acyl chain length scarcely affected the equilibrium conversion in both the presence and the absence of the molecular sieves.

Continuous Production of Saturated Acyl L-Ascorbate Using a CSTR. A fatty acid solution was fed to the stirred tank reactor with Chirazyme L-2 C2 at various flow rates, and the concentration of acyl L-ascorbate in the effluent was observed after a steady state was attained. The concentration of fatty acid in the feed solution was 200 mmol/L, since it gave the maximum concentration of the product (data not shown). Molecular sieves were not used in this continuous production based on the low solubility of L-ascorbic acid in acetone and the results shown in **Figure 4**. The relationship between the mean residence times, τ, and the concentrations of the decanoyl, dodecanoyl, or tetradecanoyl L-ascorbate in the effluent is shown in **Figure 5**. For each acyl L-ascorbate, the concentration of more than 18 mmol/L was attained at $\tau \geq 175$ min. Therefore, the reactor was operated at the mean residence time of 175 min for the long-term production of the acyl L-ascorbates.

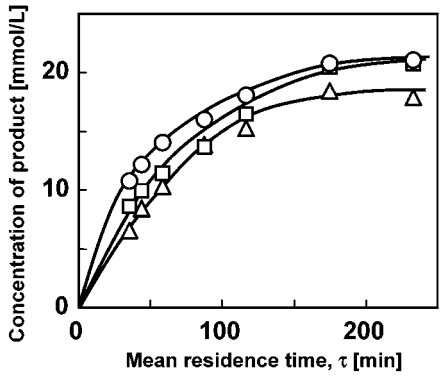

Figure 5. Relationship between the mean residence time, τ, and the concentration of decanoyl (○), dodecanoyl (□), or tetradecanoyl (△) L-ascorbate in the CSTR. The curves were empirically drawn.

The long-term operational stability of the immobilized enzyme was examined for the synthesis of the decanoyl, dodecanoyl, and tetradecanoyl L-ascorbates at $\tau = 175$ min. As shown in **Figure 6**, the productivity of dodecanoyl L-ascorbate for 5 days was constant and was determined to be ca. 60 g/L reactor/day. The decanoyl and tetradecanoyl L-ascorbates were also produced at similar conversions, the immobilized enzyme was stable, and the reactor was steadily operated for at least 11 days.

Solubilities of Saturated Acyl L-Ascorbates in Water and Soybean Oil. The solubilities of the saturated acyl L-ascorbates in water or soybean oil were measured at various temperatures. Although L-ascorbic acid is insoluble in soybean oil (*11*) and highly soluble in water, the acylation of L-ascorbic acid significantly improved its solubility in soybean oil but decreased

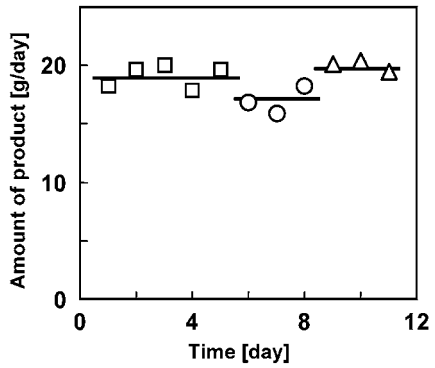

Figure 6. Continuous production of decanoyl (○), dodecanoyl (□), or tetradecanoyl (△) L-ascorbate using the CSTR with immobilized lipase, Chirazyme L-2 C2, at a flow rate of 2.0 mL/min and at 50 °C.

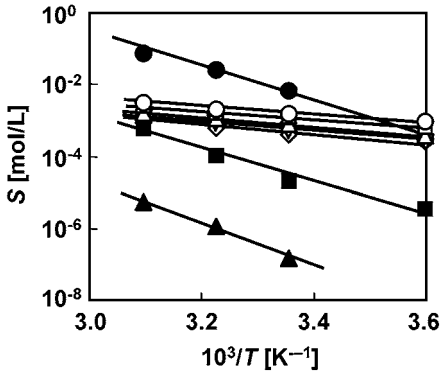

Figure 7. van't Hoff plots for the solubilities, *S*, of decanoyl (○, ●), dodecanoyl (□, ■), tetradecanoyl (△, ▲), hexadecanoyl (▽), and octadecanoyl (◇) L-ascorbates in soybean oil (open symbols) and water (closed symbols). The curves were empirically drawn.

the solubility in water. The temperature dependence of the solubilities, *S*, of the decanoyl, dodecanoyl, tetradecanoyl, hexadecanoyl, and octadecanoyl L-ascorbates in water or soybean oil (**Figure 7**) could be expressed by the following van't Hoff equation:

$$\mathrm{d}\ln S/\mathrm{d}\,(1/T) = -\Delta H/R \qquad (1)$$

where ΔH is the dissolution enthalpy, R is the gas constant, and T is the absolute temperature. The HPLC peaks of the hexadecanoyl and octadecanoyl L-ascorbates in aqueous solution were not detected at every temperature because of their very low solubilities in water. The solubilities of the acyl L-ascorbates in both soybean oil and water were higher for those with a shorter acyl chain. The dependence of the solubility in water on the acyl chain length of the acyl L-ascorbate was much stronger than that of the solubility in soybean oil.

The plots in **Figure 7** produced a straight line for each acyl L-ascorbate. The dissolution enthalpy, ΔH, was evaluated from the slope. The relationship between the carbon number of the acyl chain and the ΔH for the solubilization of the saturated acyl L-ascorbates in soybean oil or water is shown in **Figure 8**. The ΔH values were about 20 kJ/mol for soybean oil and about 90 kJ/mol for water.

Radical Scavenging Activity of Saturated Acyl L-Ascorbate. L-Ascorbic acid plays a role as a water soluble antioxidant by scavenging radicals. The radical scavenging activities of L-ascorbic acid and the decanoyl, dodecanoyl, tetradecanoyl, and hexadecanoyl L-ascorbates against DPPH free radical were measured. The scavenging activity of each tested compound was about 95%, and there was no negative effect of the acyl group and acyl chain length. It was found that the high activity of the L-ascorbic acid was not affected by the introduction of a saturated acyl group to the hydroxyl group at the C-6 position of the L-ascorbic acid.

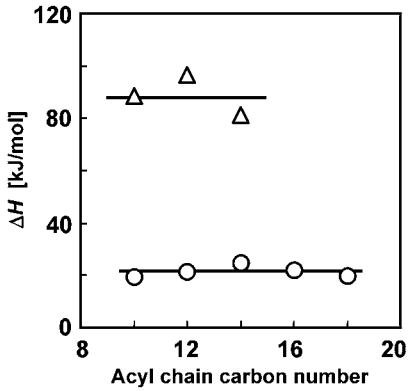

Figure 8. Relationship between the carbon number of acyl chain and the dissolution enthalpy, ΔH, for solubilization of saturated acyl L-ascorbates in soybean oil (○) or water (△).

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