Continuous Production of Acyl L-Ascorbates Using a Packed-Bed Reactor with Immobilized Lipase

Ko Kuwabara[a], Yoshiyuki Watanabe[a], Shuji Adachi[a,*], Kazuhiro Nakanishi[b], and Ryuichi Matsuno[a]

[a]Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan, and [b]Department of Bioscience and Biotechnology, Faculty of Engineering, Okayama University, Okayama 700-8530, Japan

ABSTRACT: Saturated acyl (6-*O*-caproyl, lauroyl, and myristoyl) and unsaturated acyl (6-*O*-oleoyl, linoleoyl, and arachidonoyl) L-ascorbates were continuously synthesized at 50°C using a system where a column packed with ascorbic acid powder and a packed-bed reactor with an immobilized lipase from *Candida antarctica* were connected in series. A productivity of 1.6–1.9 kg/L reactor·d was achieved for at least 11 d. The surface tension of the caproyl or lauroyl L-ascorbate in aqueous solution was measured at various temperatures and pH to estimate the critical micelle concentration (CMC) of the acyl L-ascorbate. The CMC values were independent of temperature but dependent on the pH. The value of the caproyl ascorbate increased with an increase in pH.

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KEY WORDS: Acyl L-ascorbate, critical micelle concentration, immobilized lipase, packed-bed reactor, surface tension.

L-Ascorbic acid (vitamin C) is a water-soluble vitamin with a high antioxidative activity that is used as an additive in foods and cosmetics. Its lipophilic derivative, chemically acylated with a long-chain FA such as palmitic or stearic acid, is also used as an additive in foods rich in lipids. The enzymatic synthesis using a lipase has some advantages such as high regiospecificity of the enzyme, the direct use of unmodified substrates, and moderate reaction conditions. A microaqueous organic solvent or solvent-free system is generally used to shift the reaction toward ester synthesis, because lipase-catalyzed condensation in a conventional aqueous system thermodynamically favors hydrolysis. Some studies have reported the lipase-catalyzed synthesis of acyl L-ascorbates in an organic solvent using a batch reaction (1–7); however, a continuous reaction would be preferred for large-scale production. We reported the continuous synthesis of acyl mannose or erythritol using a packed-bed reactor (8,9). We also reported the continuous production of saturated acyl L-ascorbates using a continuous stirred tank reactor (10). However, the productivities for the acyl erythritol, mannose, and L-ascorbate were *ca*. 29, 350, and 57 g/L reactor·d, respectively.

*To whom correspondence should be addressed at Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan. E-mail: adachi@kais.kyoto-u.ac.jp

Acyl L-ascorbates are amphiphilic compounds that have a surface activity. Their surfactant properties have been reported previously (11–14). The pH dependence of the critical micelle concentration (CMC) of acyl ascorbate has not been examined, in spite of the presence of an ionizing group in the ascorbate.

In this study, a reactor system, in which a column packed with L-ascorbic acid powder and another column packed with an immobilized lipase were connected in series, was proposed to continuously synthesize an acyl L-ascorbate, with a productivity much higher than those in our previous reports (8–10). 6-*O*-caproyl, lauroyl, myristoyl, oleoyl, linoleoyl, and arachidonoyl L-ascorbates were synthesized through the immobilized lipase-catalyzed condensation of L-ascorbic acid with capric, lauric, myristic, oleic, linoleic, and arachidonic acids, respectively, in acetone using this system. The surface tension of the caproyl or lauroyl L-ascorbate was also measured at various temperatures and pH to estimate the CMC value.

EXPERIMENTAL PROCEDURES

Materials. The immobilized lipase, Chirazyme® L-2 C2, from *Candida antarctica* type B was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Capric, lauric, and myristic acids and acetone were obtained from Wako Pure Chemical Industries (Osaka, Japan). Linoleic acid was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Oleic acid was purchased from Sigma Chemical (St. Louis, MO). Arachidonic acid was supplied by Suntory, Ltd. (Osaka, Japan). L(+)-Ascorbic acid was obtained from Nacalai Tesque (Kyoto, Japan). The purities of the unsaturated FA used were greater than 90%, and all the other chemicals were of analytical grade.

System for continuous production of acyl L-ascorbate. A schematic diagram of the reactor system is shown in Figure 1. The L-ascorbic acid powder (*ca.* 40 g) was packed into a cylindrical glass column (10 mm i.d. × 150 mm) and Chirazyme L-2 C2 particles (*ca.* 1.5 g by dry weight) were packed into a stainless steel column (4.6 mm i.d. × 150 mm). The columns were connected in series with a stainless steel tube having an i.d. of 0.8 mm. A FA (capric, lauric, myristic, oleic, linoleic, or arachidonic acid) was dissolved in acetone at a concentration of 25–250 mmol/L. The FA solution was fed into the column packed with L-ascorbic acid through a preheating coil (1.0 mm i.d. × *ca.* 1.0 m) and then pumped to the immobilized-enzyme

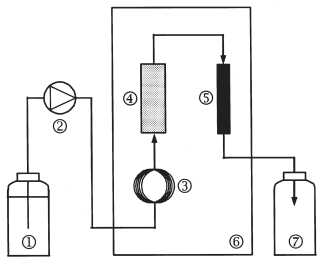

FIG. 1. Schematic of a packed-bed reactor for the continuous synthesis of acyl ascorbates. 1: feed reservoir; 2: pump; 3: preheating coil; 4: column packed with L-ascorbic acid; 5: column packed with immobilized lipase (Chirazyme® L-2 C2; Roche Molecular Biochemicals, Mannheim, Germany); 6: thermoregulated chamber; 7: effluent reservoir.

column at a specified flow rate. The preheating coil and columns were installed in a thermoregulated chamber at 50°C. For unsaturated FA, the headspace of the reservoir was filled with N_2 gas and the reservoir was placed in the dark. In the system, the operation was stopped to pack L-ascorbic acid powder in the column when the powder was exhausted. In this sense, the system was, to be exact, semicontinuous. However, the term "continuous" was used because interruption of the operation for packing was seldom necessary, owing to the low solubility of L-ascorbic acid in acetone.

After a steady state was achieved, the effluent was sampled and the product concentration was determined using HPLC. These operations were carried out at various flow rates for every FA to obtain the relationship between the conversion and the superficial residence time in the immobilized-enzyme column, τ.

An FA solution (200 mmol/L) was continuously fed into the system for 11 d at a flow rate of 0.5 mL/min, which corresponded to $\tau = 5$ min. The acid solution was changed in the order of arachidonic, oleic, linoleic, capric, lauric, and myristic acids. The arachidonic acid solution was fed for 1 d, and the other acids for 2 d. At appropriate intervals, the effluent was sampled and the concentration of the product in it was quantified.

Surface tension of saturated acyl L-ascorbates at various temperatures and pH. The caproyl and lauroyl L-ascorbates were dissolved in distilled water at various concentrations, and their surface tensions were measured at various temperatures by the Wilhelmy method using a CBVP-A3 surface tensiometer (Kyowa Kaimenkagaku). The surface tensions of the caproyl L-ascorbate dissolved in 0.01 mol/L sodium citrate buffers of pH 3 to 6 were also measured.

HPLC analysis and purification of acyl L-ascorbates. The quantitative analysis of an acyl L-ascorbate was carried out by HPLC with a Cosmosil 5C18-AR-II packed column (4.6 mm i.d. × 150 mm; Nacalai Tesque) and a UV (245 nm) detector. The eluent was a methanol/water/phosphoric acid mixture (90:10:0.1 by vol). The sample was appropriately diluted by the eluent and applied to the column (applied volume: 10 μL). The flow rate was 1.0 mL/min. The retention times of the caproyl, lauroyl, myristoyl, oleoyl, linoleoyl, and arachidonoyl L-ascorbates were 2.0, 2.4, 2.9, 3.9, 3.2, and 3.0 min, respectively. The calibration curve was prepared using each isolated acyl ascorbate, which was dissolved in the eluent at a known concentration.

The saturated acyl L-ascorbates were isolated from the effluent according to the reported methods with a slight modification (4). The unsaturated acyl L-ascorbates were recovered from the reaction mixture using a glass column (30 mm i.d. × 500 mm) packed with an ODS resin (ODS-AM, YMC Inc., Kyoto, Japan) after concentrating the mixture by rotary evaporation. A methanol/water/trifluoroacetic acid mixture (85:15:0.3 by vol) was used as the eluent, and its flow rate was *ca.* 10 mL/min. The desired product was fractionated by monitoring its elution using a YRD-833 refractometer (Shimamuratech). The acetone separated in the purification process was reused in the continuous production. The products synthesized through the condensation of ascorbic acid with oleic and arachidonic acids were identified by ^{1}H NMR as the 6-*O*-oleoyl and 6-*O*-arachidonoyl L-ascorbates, respectively: 6-*O*-oleoyl L-ascorbate (300 MHz, MeOH-d_4, TMS): δ 0.89 (*t*, *J* = 6.7 Hz, 3H, CH_3–), δ 1.29, 1.32 (2 *br s*, 20H, –CH_2–), δ 1.59 (*t*, *J* = 7.0, 2H, –C$\underline{H}_2$CH_2COO–), δ 2.01, 2.15 (2 *br s*, 4H, –C$\underline{H}_2$CH=CHC$\underline{H}_2$–), δ 2.36 (*t*, *J* = 7.5, 2H, –CH_2COO–), δ 4.08 (*m*, 1H, H-5), δ 4.21 (*m*, 2H, H-6), δ 4.72 (*d*, *J* = 2.1, 1H, H-4), δ 5.33 (*m*, 2H, –CH=CH–); 6-*O*-arachidonoyl L-ascorbate (300 MHz, MeOH-d_4, TMS): δ 0.97 (*t*, *J* = 7.6 Hz, 3H, CH_3–), δ 1.33 (*m*, 6H, –CH_2–), δ 1.66 (*m*, 2H, –C$\underline{H}_2$CH_2COO–), δ 2.08 (*m*, 4H, –C$\underline{H}_2$CH=CH–), δ 2.29, 2.37 (2*t*, *J* = 7.4, 2H, –CH_2COO–), δ 2.84 (*m*, 6H, –CH=CHC$\underline{H}_2$CH=CH–), δ 4.11 (*m*, 1H, H-5), δ 4.23 (*m*, 2H, H-6), δ 4.68 (*d*, *J* = 2.1, 1H, H-4), δ 5.35 (*m*, 8H, –CH=CH–).

RESULTS AND DISCUSSION

Effects of FA concentration and residence time in the reactor on product concentration. A capric, lauric, myristic, oleic, linoleic, or arachidonic acid solution in acetone was fed to the reactor system at the flow rate of 0.5 mL/min, which corresponded to a superficial residence time in the immobilized-enzyme column, τ, of 5.0 min. After a steady state was achieved, the concentration of the corresponding acyl L-ascorbate in the effluent was determined. Figure 2 shows the effect of the FA concentration on the product concentration. For every FA, the product concentration increased as the FA concentration in the feed increased, generally reaching the maximum value at FA concentrations greater than 200 mmol/L. Because the solubility of L-ascorbic acid in acetone at 50°C was 25.8 mmol/L, the molar ratio of a FA to L-ascorbic acid at the inlet of the immobilized-enzyme column would be *ca.* 8 at the point of maximum esterification. The concentrations of the oleoyl and linoleoyl L-ascorbates were lower than those of the other ascorbates at any FA concentration. This would be due to the lower purity of the oleic and linoleic acids used (*ca.* 90%). In subsequent experiments, the FA concentration in the feed was fixed at 200 mmol/L.

An FA solution was fed through the L-ascorbic acid column to the column packed with the immobilized lipase at various flow rates, and the corresponding acyl L-ascorbate concentration

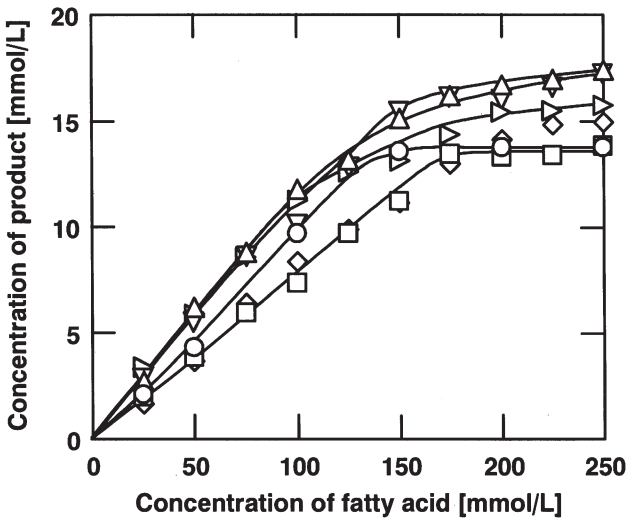

FIG. 2. Effect of the concentration of (○) arachidonic, (□) oleic, (◇) linoleic, (△) capric, (▽) lauric, or (▷) myristic acid on that of the product using the packed-bed reactor with immobilized lipase, Chirazyme L-2 C2, at the flow rate of 0.5 mL/min. For supplier of Chirazyme L-2 C2 see Figure 1.

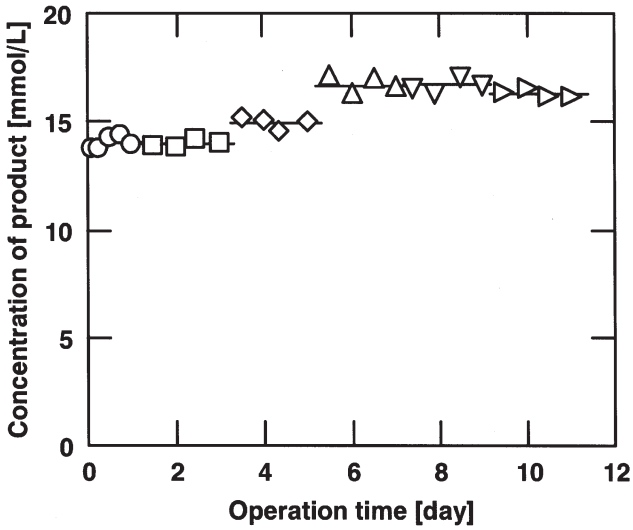

FIG. 4. Continuous production of (○) arachidonoyl, (□) oleoyl, (◇) linoleoyl, (△) caproyl, (▽) lauroyl, and (▷) myristoyl L-ascorbates using the packed-bed reactor at the flow rate of 0.5 mL/min and 50°C. The FA solution was 200 mmol/L.

in the effluent at a steady state was measured. Figure 3 shows the relationship between the τ value and the acyl L-ascorbate concentration in the effluent. For every acyl L-ascorbate, the maximum product concentration was given at τ = 5 min or longer.

Continuous production of various acyl L-ascorbates. Arachidonoyl L-ascorbate was synthesized for 1 d using the reactor system. The FA to be fed was changed to oleic acid, and the synthesis of oleoyl L-ascorbate was continued for 2 d. The linoleic, capric, lauric, and myristic acids were then fed to the system in this order to produce the corresponding ascorbates every 2 d (Fig. 4). For the synthesis of each acyl L-ascorbate, the τ value was fixed at 5 min. The system could be stably operated for 11 d, and no loss in the enzyme activity was observed. The product concentrations in the effluent were in the range of 14 to 17 mmol/L. The lower concentration of unsaturated acyl L-ascorbates compared to those of the saturated acyl L-ascorbates would be ascribed to the lower purity of the unsaturated FA. These product concentrations corresponded to the productivity of 1.6 to 1.9 kg/L reactor·d, depending on the molecular mass of the product. The productivity was much higher than that previously reported for the synthesis of acyl erythritol and mannose using a packed-bed reactor (8,9).

Surfactant properties of caproyl and lauroyl L-ascorbates. We previously reported the CMC of the saturated acyl L-ascorbates (acyl-chain lengths: 6 to 12) in distilled water at 25°C (13). In the present study, the surface tensions of the caproyl

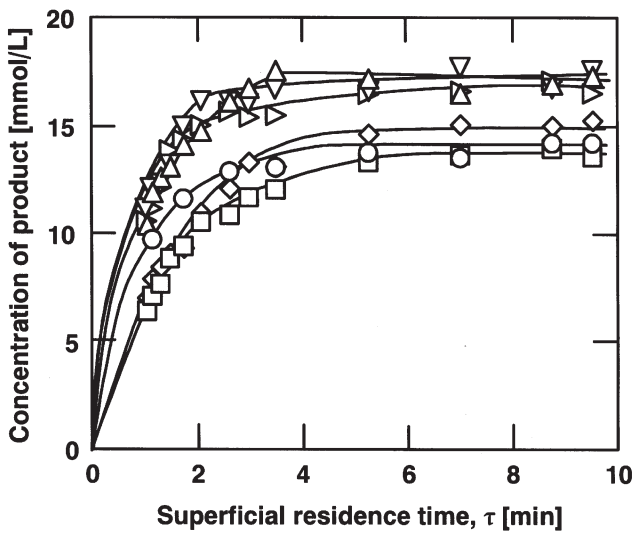

FIG. 3. Relationship between the superficial residence time, τ, and the concentration of (○) arachidonoyl, (□) oleoyl, (◇) linoleoyl, (△) caproyl, (▽) lauroyl, or (▷) myristoyl L-ascorbate using the packed-bed reactor at 50°C. The FA solution was 200 mmol/L.

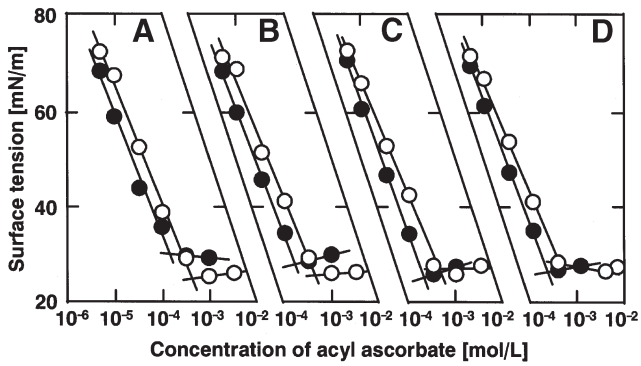

FIG. 5. Surface tensions of caproyl (○) and lauroyl (●) L-ascorbates in distilled water at (A) 30, (B) 35, (C) 40, and (D) 45°C.

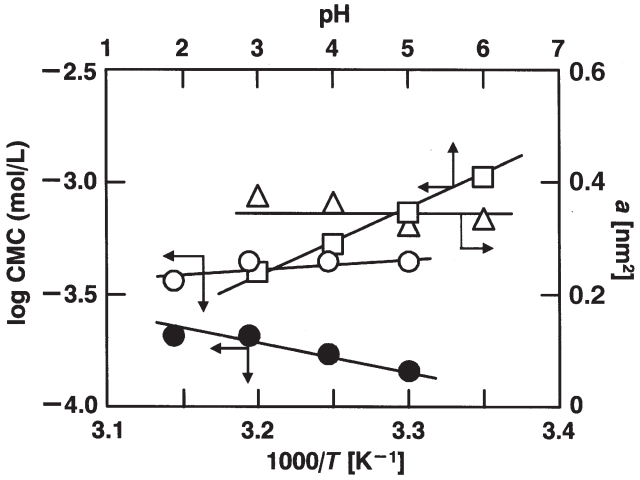

FIG. 6. Effect of temperature or pH on the critical micelle concentration (CMC) and the residual area per molecule (*a*) of the caproyl (open symbols) and lauroyl (●) L-ascorbates.

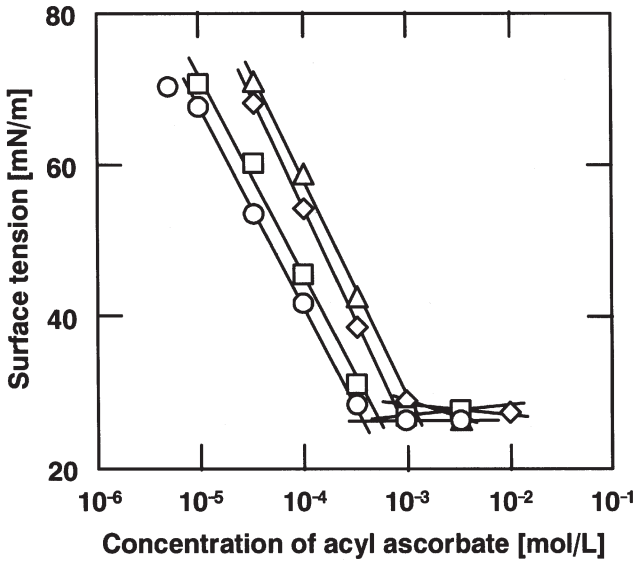

FIG. 7. Surface tension of caproyl L-ascorbate in 0.01 mol/L sodium citrate buffer solution at 30°C and pH (○) 3, (□) 4, (◇) 5, and (△) 6.

and lauroyl L-ascorbates dissolved in distilled water were measured at various temperatures by the Wilhelmy method to determine the temperature dependence of the CMC values (Fig. 5). The CMC value was estimated from the intersection of two lines for an ascorbate at each temperature. The surface excess, Γ, was evaluated from the slope of the line for the lower concentrations according to the following equation:

$$-\frac{d\gamma}{d\log C}=\frac{RT}{0.434}\Gamma \qquad [1]$$

where γ is the surface tension, C is the concentration of acyl L-ascorbate, R is the gas constant, and T is the absolute temperature. The reciprocal of the Γ value gives the residual area per molecule, a. The CMC and a values of each ascorbate are plotted vs. the 1000/T values in Figure 6. The temperature dependence of the CMC was very weak for both ascorbates. The a value was also independent of the temperature, and was about 0.35 nm^2 for both the caproyl and lauroyl ascorbates.

Figure 7 shows the effect of pH on the surface tension of the caproyl ascorbate solution at 30°C. The CMC value was higher at the higher pH as shown in Figure 6. The pK_a value of L-ascorbic acid is 3.77 (15). If we assume that the pK_a value of the ascorbyl moiety is the same as that of L-ascorbic acid, the degree of dissociation of the ascorbyl moiety increases at the higher pH. The exponential increase in the CMC with an increase in pH could be explained by the increase in the degree of dissociation (16). The a value was almost constant at any pH, and the value was the same as those obtained at different temperatures for the caproyl and lauroyl L-ascorbates. This indicated that the acyl L-ascorbates would be oriented so as to stick their acyl residues into the air, and that the a values were exclusively determined by the ascorbyl moiety.

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