Antioxidative Properties and Enzymatic Synthesis of Ascorbyl FA Esters

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ABSTRACT: Efficient synthesis of unsaturated FA esters of ascorbic acid is possible with only a small excess of one of the reactants in *t*-amyl alcohol using *Candida antarctica* lipase as biocatalyst. Using free acids, we obtained yields that were comparable to yields reached using vinyl-activated acyl donors (71, 80, and 86% yields of esters with FA excesses of 1:1, 1:1.5, and 1:2, respectively). As very low water activity is needed to achieve sufficiently high yields of product, molecular sieves were used to improve the ascorbyl ester yields. Ascorbyl oleate is more amorphous and has a much lower m.p. and lower enthalpy of fusion than ascorbyl palmitate. This leads to a higher solubility of ascorbyl oleate in oil, resulting in an increased antioxidant effect compared to that of the palmitate. In an accelerated storage test using deodorized rapeseed oil, samples incubated with ascorbyl palmitate showed noticeable oxidation after 1 wk of storage, whereas samples incubated with ascorbyl oleate displayed negligible oxidation for 9 and 4 wk at 30 and 40°C, respectively.

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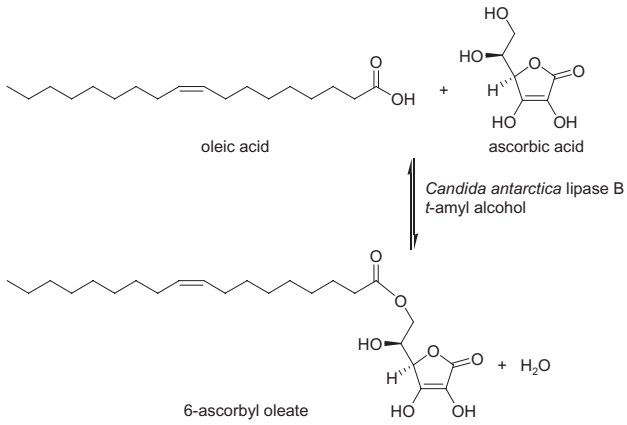
KEY WORDS: Antioxidant, ascorbyl oleate, ascorbyl palmitate, esterification, lipase.

Ascorbyl palmitate is a fat-soluble antioxidant commonly used in foods to prevent oxidation of sensitive fats and oils. Ascorbyl palmitate has a disadvantage, however, in that it has relatively low solubility in oils. The solubility can be improved by the addition of lecithin, although this causes the product to become hygroscopic. Another way to improve the solubility of an ascorbyl FA ester is to modify its structure to make it less crystalline. This can be done, for example, by introducing a double bond in the FA. Oleic acid is unsaturated, readily available, inexpensive, and approved for use in foods. This makes it an attractive candidate to replace the palmitic acid in an ascorbyl ester.

The chemical synthesis of saturated ascorbyl FA esters is straightforward (1). A similar procedure for the synthesis of ascorbyl oleate has been described in the literature (2) but has proven hard to repeat. The difficulties are due to the double bond of oleic acid, which is too sensitive for the reaction conditions needed for the chemically catalyzed esterification. Enzymatic catalysis, however, can be performed at lower reaction temperatures and under milder conditions, enabling the enzymatic synthesis of ascorbyl oleate (Scheme 1). Because



SCHEME 1

the reactants differ in polarity, the number of suitable solvents available is limited. We used *t*-amyl alcohol because it dissolves the reactants at high concentrations and does not deactivate the enzyme.

In 1971, Arakawa *et al.* (3) reported on the first enzymatic synthesis of ascorbyl laurate in aqueous solution. In 1990, Enomoto *et al.* (4) filed a patent on the esterification in organic solvents, and esterfications in *t*-amyl alcohol were reported by Humeau *et al.* (5) in 1995. Since then, not only saturated FA have been used, but also various unsaturated FA (6–8) as well as of phenyl butyric acid (9) and methyl lactate (10). These syntheses have typically reached yields below 50% unless activated acyl donors or a large excess of the FA was used.

In this study we show that enzymatic catalysis can be used for the synthesis of ascorbyl oleate. We also discuss some requirements for a synthetically favorable reaction and some potential pitfalls in this and similar syntheses when using tertiary alcohols as solvent. We further assess some physical properties of ascorbyl oleate and show that it has a favorable antioxidative effect on rapeseed oil compared with ascorbyl palmitate.

MATERIALS AND METHODS

Enzymatic synthesis. Ascorbic acid (99%), palmitic acid (99%), and oleic acid (99%) were purchased from Sigma-Aldrich (Stockholm, Sweden). Novozym 435, immobilized *Candida antarctica* lipase B, was a gift from Novozymes A/S

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(Bagsvaerd, Denmark). Syntheses of ascorbyl palmitate were performed enzymatically with 100 mg (0.57 mmol) ascorbic acid and palmitic acid in molar ratios of 1:1, 1:1.5, and 1:2 (ascorbic acid/palmitic acid) in 10.0 mL *t*-amyl alcohol at 60°C. The reactions were started by the addition of 50 mg of Novozym 435. Water activity of the reaction medium was kept constant with the addition of three portions of 3-Å molecular sieves (400 mg), which were exchanged during the reaction. Samples were taken throughout the reaction and did not exceed 1% of the total volume.

Ascorbyl oleate was synthesized by mixing 4.5 g oleic acid (16 mmol) and 5.53 g (32 mmol) ascorbic acid in 184 mL of *t*-amyl alcohol together with 2.5 g molecular sieves and 600 mg Novozym 435 at 65°C.

HPLC analysis. Analysis was done with RP-HPLC using a Supelco Discovery C18 column (15 cm × 4.6 mm, 5 μm; Sigma-Aldrich), 1 mL/min of a 96:4 mixture of methanol/water fortified with 0.5% acetic acid. Detection of ascorbic acid and ascorbyl esters was done spectrophotometrically at 254 nm. FA were determined with a Sedex 45 ELSD (Sedere, Alfortville, France) at 30°C and 2 bar air pressure using a calibration curve obtained separately. The product conversion was calculated as $C_{ester}/(C_{ascorbic\ acid} + C_{ester})$ for each sample. Retention times were approximately 1.9, 3.3, and 4.5 min for the ascorbic acid, ascorbyl ester, and FA, respectively.

Purification. The ascorbyl oleate product was purified by washing with hexane (160 + 30 + 30 mL) to remove unreacted oleic acid. Excess ascorbic acid was removed by dissolving the product in diethyl ether followed by filtration.

NMR. Ascorbyl oleate was analyzed with a NMR spectrometer (Bruker, Rheinstetten, Germany): ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.35 (*m*, 2H), 4.8 (*s*, 1H), 4.45 (*s*, 1H), 4.25 (*d*, 2H), 2.35 (*m*, 2H), 2.05 (*m*, 4H), 1.65 (*m*, 2H); 1.3 (*br*, 20H), 0.9 (*t*, 3H).

Antioxidant effect. The antioxidant effect of the synthesized ascorbyl oleate was compared with that of ascorbyl palmitate (Danisco Ingredients, Brabrand, Denmark) in accelerated storage tests at 30 and 40°C. The antioxidants were added to fresh, deodorized low-erucic acid rapeseed oil (Karlshamns AB, Sweden) at a concentration of 600 mg/kg. The FA composition of the oil was 58.5% oleic acid (18:1), 21.5% linoleic acid (18:2), 10.5% linolenic acid (18:3), 4.5% palmitic acid (16:0), and 1.5% stearic acid (18:0). The tocopherols, analyzed by HPLC, comprised 200 ppm α-, 320 ppm γ-, and 7 ppm δ-tocopherols. Duplicate samples of 50 g were placed in dark glass jars covered by a loose aluminum foil. The jars were kept in a dark heating cabinet held at the desired storage temperature. Jars were taken out at predetermined time intervals and analyzed for PV using a standard titrimetric method (11). The base oil with no added antioxidant was used as control.

Melting point and crystallinity. The melting properties of commercial ascorbyl palmitate and synthesized ascorbyl oleate were determined by DSC (Mettler TA8000). The ascorbyl esters (6–8 mg) were weighed into standard 40-μL aluminum pans and heated from 35 to 150°C at 5°C/min under nitrogen purging.

X-ray powder diffraction also was carried out to determine ascorbyl ester crystallinity. A Siemens D5000 operating with a Cu anode at 40 mA and 40 kV at ambient temperature was used for the studies. Diffractograms were taken in the long-spacing (0.7–10° 2-θ) and the short-spacing regions (17–27° 2-θ).

RESULTS AND DISCUSSION

Control of water activity. The approaches used in the literature for the enzymatic synthesis of ascorbyl esters can be divided into two groups. The first approach is to remove the water or alcohol formed from the reaction with either molecular sieves or reduced pressure to shift the equilibrium, or to use a vinyl ester as the acyl donor. The second approach uses none of these measures to remove the co-product formed from the reaction mixture. Despite the well-known fact that the yield of an equilibrium reaction benefits from having one of the products removed from the mixture, relatively few published experiments use water- or methanol-removal strategies such as molecular sieves or reduced pressure. The use of a vinyl ester as acyl donor, however, is commonly practiced.

The techniques used to control water activity that were evaluated in this work included vapor phase equilibration with a salt solution (12), water diffusion through silicon tubing with a circulating saturated salt solution (13), and molecular sieves. Vapor phase equilibration was considered unsuitable because the *t*-amyl alcohol vapor reacted with the salts tested (LiCl and $MgCl_2$), which resulted in irreversible transport of *t*-amyl alcohol from the reaction vessel to the salt solution reservoir. This resulted in a change in reaction volume and loss of water activity control. Immersing silicon tubing, through which saturated salt solutions circulated into the reaction mixture as a means of controlling the water activity, also was tried. The *t*-amyl alcohol, however, diffused through the tubing and reacted with the salt solution, forming complexes that precipitated and blocked the tubing and that also resulted in solvent loss from the reaction mixture and loss of water activity control. This problem is representative for a solvent that diffuses through silicon tubing and precipitates the salt. In this respect, silicon tubing-mediated equilibration is perhaps better suited for water-insoluble solvents.

Because the common technique for controlling water activity with saturated salt solutions was not suitable for this reaction system, molecular sieves were used to remove the water formed in the reaction. Molecular sieves have been shown to dry *t*-butyl alcohol to a water content of 13 ppm but only at a slow rate, thought to be dependent on its relatively high viscosity (14). To ensure a water activity as low and constant as possible throughout the reaction, the sieves were exchanged as the reaction proceeded and as water accumulated.

Reaction progress. We studied the esterification of ascorbic acid with a FA in *t*-amyl alcohol on a small scale using palmitic acid as the acyl donor. The syntheses were carried

out at three different FA concentrations at low water activity. Ascorbic acid and palmitic acid were mixed in *t*-amyl alcohol together with the molecular sieves. The reaction was started with the addition of Novozym 435 (immobilized lipase B from *C. antarctica*). After about 50 h, the reactions had reached a steady state. The product yield, monitored with time by HPLC, is shown in Figure 1.

Earlier reports of enzymatic synthesis of ascorbyl palmitate in *t*-amyl alcohol or *t*-butanol reported only 6 (6) or 14% yields (7) using a 1:1 ratio of ascorbic acid and acyl donor (Table 1). Bradoo *et al.* (15) reported a 50% yield in hexane when using equimolar amounts of ascorbic and palmitic acids catalyzed by *Bacillus stearothermophilus* lipase without removing co-product. By increasing the palmitic acid mole concentration to 2.5 times that of the ascorbic acid and adding molecular sieves, they obtained a 97% yield of ascorbyl ester.

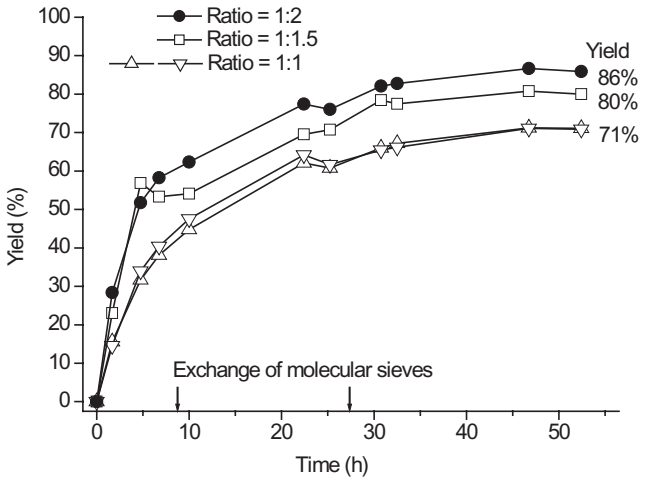

FIG. 1. Yield of ascorbyl palmitate in the lipase-catalyzed esterification of ascorbic acid and palmitic acid as a function of time at three molar ratios of ascorbic acid to palmitic acid in *t*-amyl alcohol and in the presence of molecular sieves. Duplicates are shown for the reaction at ratio 1:1.

In this study, we used ascorbic acid/palmitic acid molar ratios of 1:1, 1:1.5, and 1:2 and dried the reaction system with molecular sieves to obtain yields of 71, 80, and 86%, respectively. This can be compared to the results of Sakashita *et al.* (16), who reported an 88% yield of ascorbyl stearate with 2000 ppm water in *t*-butanol, and Yan *et al.* (17), who reported an 84% yield using a 1.5-fold molar excess of vinyl stearate in dried *t*-butanol (Table 1).

From this it can be concluded that our reaction system, which uses free acid and careful drying of the reaction medium, seems to be as efficient in driving the reaction toward completion as one using an activated acyl donor. Presumably, as the ascorbyl ester is formed it can be hydrolyzed in the presence of water.

When using activated acyl donors such as vinyl palmitate, ascorbyl esters will form at a high rate and reach high yields, but the ester product will hydrolyze if the medium is not carefully dried, which results in the same final composition for vinyl-activated acyl donors as for the free acids (Viklund, F., and K. Hult, unpublished results). Thus, it is very important to control the water activity of the reaction to reach high ester yields.

Because the ascorbic acid and ascorbyl esters are sensitive to oxidation, it also is important to check for oxidation of the products as the reaction progresses to obtain a high yield. Reactant or product oxidation would be seen as a decrease in yield with time, which is not the case (Fig. 1).

Ascorbyl oleate synthesis. Ascorbyl oleate was synthesized in a scaled-up reaction with careful removal of the water from the reaction with molecular sieves (Table 1). A twofold excess of ascorbic acid was used to maximize the utilization of the high-purity oleic acid. The reaction was stopped after 100 h. HPLC analysis indicated a yield of 87% based on oleic acid, which agrees very well with the 86% achieved for the palmitate ester when palmitic acid was used in excess. The product was purified by washing with hexane to remove the excess oleic acid, followed by dissolution in diethyl ether and filtration to remove unreacted ascorbic acid. The purity was

TABLE 1
Comparison of Lipase-Catalyzed Syntheses of Ascorbyl FA Esters

Acyl donor	Solvent	Catalyst	Temp. (°C)	Ratio[a]	Yield (%)	Drying	Reference
Methyl palmitate	*t*-Amyl alcohol	Novozym 435[b]	55	1:1	6	—	6
				1:5	47	—	
Methyl palmitate	*t*-Amyl alcohol	Novozym 435	50	1:1	14	—	7
				1:3	26	—	
				1:5	32	—	
Methyl palmitate	*t*-Amyl alcohol	Novozym 435	70	1:5	80[c]	Reflux distillation	18
Palmitic acid	Hexane	Lipase from *Bacillus stearothermophilus*	50	1:1	50	—	15
				1:2.5	97	Molecular sieves	
Vinyl stearate	*t*-Butanol	Amano PS	40	1.5	88	2000 ppm water	16
Vinyl palmitate	*t*-Butanol	Chirazyme L-2[b]	40	1.5	84	Molecular sieves	17
Palmitic acid	*t*-Amyl alcohol	Novozym 435	60	1:1	71	Molecular sieves	This study
				1:1.5	80	Molecular sieves	
				1:2	86	Molecular sieves	
Oleic acid	*t*-Amyl alcohol	Novozym 435	65	2:1	87	Molecular sieves	This study

[a]Ratio of ascorbic acid to acyl donor.
[b]Novozym 435 (Novozymes A/S, Bagsvaerd, Denmark) and Chirazyme L-2 are different preparations of immobilized lipase B from *Candida antarctica.*
[c]Isolated yield.

>97% (w/w), and the isolated yield of ascorbyl oleate was 29% (2.1 g) based on oleic acid. To improve the workup procedure, preparative silica gel chromatography was tested; however, silica gel chromatography was found to be unsuitable because the ascorbyl oleate oxidized and discolored on the column.

The purified ascorbyl oleate was a white, waxy solid. Structural verification of ascorbyl oleate was done with ^{1}H NMR. No oxidation of the oleoyl group was detected, and the product was exclusively the 6-*O*-monoester. The reaction rates (data not shown) and yields of ascorbyl palmitate and oleate are similar, but the chemical sensitivity and the physical properties of ascorbyl oleate demand other synthetic routes and workup procedures.

Melting properties and crystallinity. Ascorbyl palmitate melted cleanly in one single melting peak at 114–120°C, with an enthalpy of fusion of 129 J/g. It crystallized in a double-chain-packing crystal form (d = 45 Å) with short-spacing diffraction peaks at 4.2, 4.3, 4.0, 3.9, and 3.8 Å. In contrast, ascorbyl oleate exhibited only weak crystallinity, which was characterized by two weak peaks at 4.5 and 4.2 Å and double-chain packing (d = 35 Å). The amorphous structure of the ascorbyl oleate also was evident in the DSC thermograms, which had a broad melting peak at 83–84°C and an enthalpy of fusion of only 20–30 J/g. The low enthalpy of fusion and the observed diffraction pattern indicate that ascorbyl oleate solidifies primarily in a liquid crystalline structure with nonordered fatty acyl chains and a structured polar head group.

Antioxidant effect. The PV, as a function of storage time for the control and the two antioxidants at 30 and 40°C, are shown in Figures 2 and 3, respectively. Ascorbyl oleate performed significantly better than ascorbyl palmitate and the control at both temperatures. For ascorbyl palmitate there was a small but significant increase in the PV at short storage times at both temperatures, whereas ascorbyl oleate had a clear induction period of 4 wk at 40°C and 9 wk at 30°C before peroxide development accelerated.

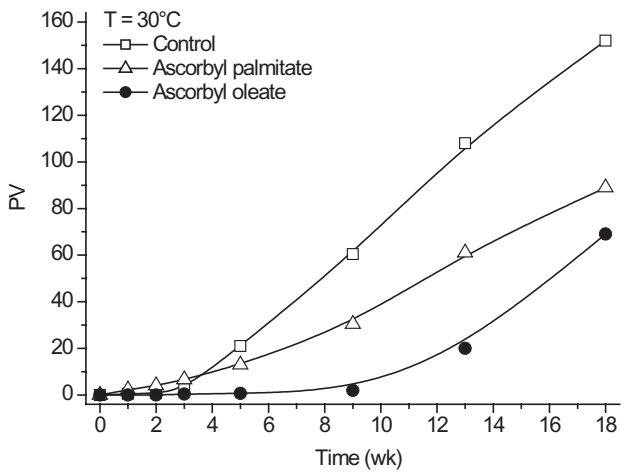

FIG. 2. Comparison of antioxidant effects for ascorbyl palmitate and ascorbyl oleate (600 mg/kg) in deodorized rapeseed oil at 30°C.

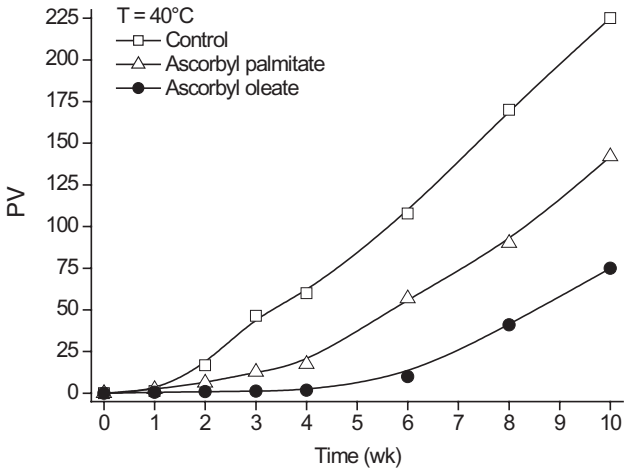

FIG. 3. Comparison of antioxidant effects for ascorbyl palmitate and ascorbyl oleate (600 mg/kg) in deodorized rapeseed oil at 40°C.

The remaining tocopherol content in the oil samples was analyzed after 10 wk at 40°C. The base oil at 40°C had no detectable tocopherol levels, whereas the sample with ascorbyl palmitate contained 9–10 ppm (3% remaining) γ-tocopherol. The oil protected by ascorbyl oleate had 184 ppm (57% remaining) γ-tocopherol and 6 ppm (86% remaining) δ-tocopherol left after 10 wk at 40°C.

The results show that when used as an antioxidant in oil, ascorbyl oleate is more effective than ascorbyl palmitate in inhibiting oil oxidation.

ACKNOWLEDGMENTS

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