Lipase-catalyzed Synthesis of C-6 Saturated and Unsaturated Fatty Acid Esters of L-Ascorbic Acid

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Abstract: L-Ascorbic acid (AsA) was reacted at 55°C for 5-7 h with 5 eq of palmitic, stearic, oleic, linoleic, linolenic and conjugated linoleic acids in 2-methyl-2-butanol (tertiary amyl alcohol, TAA) under catalysis by immobilized lipase (Candida antarctica) to give 37-55% of pure 6-esters of AsA. The esters were isolated in elemental-pure form without chromatography. The esterification reaction also proceeded in acetonitrile and dimethyl sulfoxide, but not in nitromethane or dimethylformamide. Adding drying salts and water-consuming reagents or adding a low level of water to reaction mixtures in TAA gave reduced yields of L-ascorbyl 6-palmitate. When the 6-oleate ester was stirred with lipase and oleic acid in TAA under the conditions used in esterification, about 44% of the ester was found in equilibrium with 56% of free AsA. t-1-Amyl palmitate was synthesized chemically; that ester was not detected in the lipase-catalyzed esterification. Those data show that primary hydroxyls were acylated selectively by the lipase, and that yields were optimum when the water in a reaction mixture was controlled.

Key words: L-ascorbic acid, unsaturated fatty acid esters, lipase

6-Esters of AsA with saturated fatty acids are readily prepared in >80% yield by reacting excess fatty acid with AsA near room temperature in concentrated sulfuric acid.13 The strong acid serves as reaction solvent and catalyst, and it also acts as desiccant to drive the equilibrium towards ester formation. AsA, unlike a sugar, is stable in concentrated (>98%) sulfuric acid probably because AsA forms a hydroxyclyl cation12 involving C-1, C-2, C-3, OH-1, OH-2 and OH-3. Unlike saturated fatty acids, unsaturated fatty acids do not react smoothly with AsA in concentrated sulfuric acid. Instead, they form complex mixtures of products from which we were unable to isolate the 6-esters. The patent literature describes the synthesis of L-ascorbyl 6-oleate5 using boron trifluoride plus concentrated sulfuric acid.1 The strong acid serves as reaction solvent and catalyst, and it also acts as desiccant to drive the equilibrium towards ester formation. AsA, unlike a sugar, is stable in concentrated (>98%) sulfuric acid probably because AsA forms a hydroxyclyl cation12 involving C-1, C-2, C-3, OH-1, OH-2 and OH-3. Unlike saturated fatty acids, unsaturated fatty acids do not react smoothly with AsA in concentrated sulfuric acid. Instead, they form complex mixtures of products from which we were unable to isolate the 6-esters. The patent literature describes the synthesis of L-ascorbyl 6-oleate5 using boron trifluoride plus concentrated sulfuric acid.1 The strong acid serves as reaction solvent and catalyst, and it also acts as desiccant to drive the equilibrium towards ester formation. AsA, unlike a sugar, is stable in concentrated (>98%) sulfuric acid probably because AsA forms a hydroxyclyl cation12 involving C-1, C-2, C-3, OH-1, OH-2 and OH-3. Unlike saturated fatty acids, unsaturated fatty acids do not react smoothly with AsA in concentrated sulfuric acid. Instead, they form complex mixtures of products from which we were unable to isolate the 6-esters. The patent literature describes the synthesis of L-ascorbyl 6-oleate5 using boron trifluoride plus concentrated sulfuric acid.1

In 1991 Japanese workers9 reported that a lipase suspended in an organic solvent produced 6-esters of AsA (or of erythorbic acid) by its reaction with an organic acid or its methyl ester, either saturated or unsaturated. Since then, other investigators have employed immobilized lipase in acetonitrile, t-butanol, t-amyl alcohol, and acetonitrile to synthesize a variety5-16 of 6-esters of AsA, including caproate, caprylate, caprate, laurate, myristate, palmitate, linoleate, α- and γ-linolenate (18:3n-3 and 3n-6), arachidonate (20:4n-6), eicosapentaenoate (20:5n-3), docosahexaenoate (22:6n-3), and phenylbutyrate. Most yields have been moderate (~50%), but a 91% yield of 6-palmitate was achieved when AsA was reacted 48 h at 40°C with two equivalents of the vinyl ester of palmitic acid in t-butanol.10 Moreover, the AsA 6-palmitate was isolated in >98% purity without chromatography.

We report here our work with an immobilized lipase to prepare L-ascorbyl 6-acylates of oleic, linoleic, conjugated linoleic and linolenic acids, as well as with palmitic and stearic. We also describe a simple isolation procedure that gives pure 6-esters.

It is of interest to covalently attach L-ascorbic acid to long-chain unsaturated fatty acids, as previously pointed out by others,6,14 in order to inhibit the autooxidation of PUFA and to increase dispersibility of PUFA in foods by imparting amphility. Consumption of n-3 PUFA is associated with reduced risks of developing cardiovascular disease, arthritis, inflammation, diabetes and cancer.17 Conjugated linoleic acid also exhibits anti-cancer effects, immune stimulation, and cholesterol reduction.18 Dr. James R. Millis of Cargill, Minnetonka, MN, USA first suggested in 1997 that we esterify AsA with conjugated linoleic acid. The website of Biotechnical Resources, Manitowoc, WI contains an extensive bibliography on conjugated linoleic acid.

MATERIALS AND METHODS

Materials. Novozym 435, was from NOVO Nordisk BioChem North America, Inc. (Franklinton, NC). The activity of Novozym 435 was reported by the supplier to be 7000 propyl laurate units (PLU)/g as measured by the disappearance of lauric acid (titration) during a esterification of n-propyl alcohol at 60°C for 15 min. Several supplies of enzyme were received at different times and were stored at 5°C. In our laboratory the activity of a batch was judged by the yield of the 6-palmitate ester of AsA when AsA (1.36 mmol) was reacted with palmitic acid (6.8...
mmol) in TAA (20 mL) containing Novozym 435 (200 mg) at 55°C for 7 h (see below). New shipments of enzyme gave 54–56% L-ascorbyl 6-palmitate, but one batch of enzyme exposed to the atmosphere approximately 10 times over a period of 7 months gave 27% AsA 6-palmitate. The moisture content of the commercial lipase was determined by drying to constant weight in vacuo at 25°C over anhydrous calcium sulfate; moisture contents of two batches were 3.1 and 5.2%. A similar immobilized lipase from *C. antarctica* is available from Roche Diagnostics, Penzberg, Germany, under the trade name Chirazyme L-2. AsA, oleic acid, silica gel (230–400 mesh) for column chromatography and ethyl ether stabilized with ethanol were from Fisher Scientific Company, Pittsburgh, PA. Stearic acid, palmitic acid, linoleic acid, linolenic acid, conjugated linoleic acid, bromine, triphenylphosphine and TAA, all of highest purity, were from Sigma Chemical Co. (Milwaukee, WI), and sodium methoxide and molecular sieves 4 A were from Aldrich Chemical Company, Inc. (Milwaukee, WI). The water content of TAA (minimum 99%) was reported by the supplier to average 0.15% by Karl Fisher assay. Conjugated linoleic acid was a mixture of 50% 9(cis or trans), 11(cis or trans)-diene, 40% 10(trans), 12(cis)-diene, and 10% 10(cis), 12(cis)-diene. Other chemicals were reagent-grade, and water was deionized to an ultrapure state.

**General methods.** Elemental analysis was done after drying a sample to constant weight at 25°C by Desert Analytics, Tucson, AZ, USA. Nuclear magnetic resonance spectra were obtained at 400 MHz for 1H and at 100 MHz for 13C in acetone-*d6* using the solvent’s residual 1H peak at δ = 2.05 ppm and the 13C peak at δ = 29.92 ppm as reference signals, respectively. Other spectra were measured in deuterated dimethyl sulfoxide using its residual 1H signal at δ = 2.50 ppm and 13C signal at δ = 39.51 ppm as references. Ultraviolet spectroscopy was done in absolute ethanol on a Varian Spectrophotometer (Model DMS 80, Varian Associates, Walnut Creek, CA). Thin-layer chromatography (TLC) was performed on microscope plates coated with silica gel-G (Kieselgel G, Type 60, Brinkmann Instruments Co., Westbury, NY). Plates were developed with solvent mix A, chloroform/methanol/acetic acid (8/1/0.1, v/v/v) or solvent mix B, hexane/ethyl ether 9/1 (v/v). Components were visualized by spraying with 50% sulfuric acid followed by heating at 105°C. Silica gel column chromatography was done on some esterification reaction mixtures at 2–3% loading on silica gel (100 g) using an elution rate of ~1 mL/min and a fraction size of 5 mL. Components were eluted sequentially with 100 mL each of hexane, followed by 5, 10 and 15 vol % methanol in chloroform. For esterification reactions catalyzed by lipase in TAA, the first material eluted from a column as determined by thin-layer chromatography was a mixture of unreacted fatty acid and an unknown, and the second eluate was the 6-ester of AsA. An unknown in the first fraction eluted from the column remained at the origin on thin-layer chromatograms developed in solvent mix B after a small amount of sodium methoxide was added, indicating the component was a fatty acid ester. In a control experiment L-ascorbyl 6-oleate (55 mg) was applied to a silica gel column, and 53 mg (96%) of vacuum-dried product was recovered, and the two materials showed the same mobility on a thin-layer chromatogram.

**Lipase-catalyzed esterification of l-ascorbic acid in t-amyln Alcohol (TAA).** All reactions and purifications of products were done at least twice, and the mean yields reported. Novozym 435 (200 mg) was added to a TAA (20 mL, 180 mmol) solution of AsA (240 mg, 1.36 mmol) and palmitic acid (1.74 g, 6.80 mmol), stearic acid (1.94 g, 6.80 mmol), oleic acid (2.15 mL, 6.80 mmol), linoleic acid (2.11 mL, 6.80 mmol), conjugated linoleic acid (2.11 mL, 6.80 mmol) or linolenic acid (2.12 mL, 6.80 mmol). The headspace in the flask was filled with nitrogen (ultrapure grade), and the flask wrapped in black plastic film. The reaction mixture was stirred with a magnetic stir bar in a glass-stoppered flask at 55°C, and TLC (solvent mix A) showed components with relative mobilities (Rf) of 0.3, 0.6 and 0.9 for AsA, L-ascorbyl fatty acid ester, and the fatty acid plus an unknown, respectively. TLC of the palmitate reaction mixture with solvent mix B showed all components remained at the origin except a minor unknown component having relative mobility of 0.3. Tertiary amyl palmitate, which was synthesized chemically (see below) showed at Rf 0.5 using solvent mix B, and it was never observed in a lipase-catalyzed esterification reaction done in TAA.

The level of AsA ester with Rf ~0.6 detected by TLC using solvent A appeared to reach a plateau in approximately 4 h. A reaction mixture was filtered after 4–7 h reaction time, and the solvent removed by evaporation below 30°C using a mechanical vacuum pump. The residue was mixed with hexane (250 mL), and ultra-pure nitrogen was bubbled through the milky-appearing mixture. The flask was stopped, covered with black plastic film, and held at 5°C for 30 min during which time a precipitate formed. The clear liquid phase was decanted from the precipitate, and the precipitate was washed with hexane (2–5 × 200 mL) until the liquid phase was free of fatty acid as determined by TLC using developing solvent A. Ethyl ether (300 mL) and water (20 mL) were added and nitrogen was bubbled through the mixture for 1 min. The organic phase was recovered and washed once more with water (20 mL). TLC showed AsA had been removed and the ether phase showed a single spot at Rf ~0.6. Anhydrous magnesium sulfate was added, and the head space filled with nitrogen gas. After standing overnight the mixture was filtered and ether removed by reduced pressure using a mechanical pump. Whenever a 6-ester was purified by silica gel chromatography, the hexane-washed residue was applied to the column.

**Identification of 6-esters of AsA.** The residual 6-stearate (48%) and 6-palmitate esters (55%) of AsA were recrystallized from a mixture of ethyl ether and hexane, and the products gave, respectively, m.p. 114–116 and 112–114°C; literature11 117.5–118 and 116–117°C. The H and 13C nmr spectra of the recrystallized 6-stearate ester was the same as for the crude 6-stearate ester, which was a white solid. Carbon-13 chemical shifts (δ, ppm) in the proton-decoupled spectrum of the 6-stearate ester measured in deuterated acetone solution were as follows: 175.2 (C=O of stearoyl), 173.3 (C-1), 154.4 (C-3), 120.2 (C-2), 77.3 (C-4), 68.2 (C-5), 65.9 (C-6), 35.2–23.8 (CH2 of
Fatty Acid Esters of L-Ascorbic Acid at C-6

Table 1. Carbon-13 chemical shifts (δ, ppm) of 6-fatty acid esters of L-ascorbic acid in acetone.

<table>
<thead>
<tr>
<th>Carbon on AsA</th>
<th>Unreacted</th>
<th>6-Palmitate</th>
<th>6-Stearate</th>
<th>6-Oleate</th>
<th>6-Linoleate*</th>
<th>6-Linolenate</th>
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<tr>
<td>C1</td>
<td>170.6</td>
<td>173.7</td>
<td>173.3</td>
<td>173.6</td>
<td>170.5</td>
<td>174.9</td>
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<td>C2</td>
<td>119.9</td>
<td>120.0</td>
<td>120.1</td>
<td>119.8</td>
<td>118.4</td>
<td>120.0</td>
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<tr>
<td>C3</td>
<td>151.7</td>
<td>151.3</td>
<td>154.4</td>
<td>151.6</td>
<td>152.3</td>
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<tr>
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<td>76.2</td>
<td>77.3</td>
<td>76.0</td>
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<td>77.1</td>
</tr>
<tr>
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<td>67.6</td>
<td>68.2</td>
<td>67.5</td>
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<td>68.0</td>
</tr>
<tr>
<td>C6</td>
<td>63.7</td>
<td>65.4</td>
<td>65.9</td>
<td>65.4</td>
<td>65.4</td>
<td>65.7</td>
</tr>
</tbody>
</table>

Carbon on Acyl Group

| C=O          | 170.6     | 173.3       | 170.8      | 172.8    | 177.5        |
| CH3          | 34.5–23.4 | 35.2–23.8   | 34.5–23.3  | 39.8–22.2| 35.0–21.5    |
| CH2          | 14.4      | 14.6        | 14.4       | 14.0     | 14.9         |
| C=C          | —         | —           | 130.5      | 129.8    | 127.8        |

* Determined in deuterated dimethyl sulfoxide using carbon peak at δ=39.51 ppm as reference signal.

stearyl) and 14.6 (CH₂ of stearoyl). Proton chemical shifts (δ, ppm) of the 6-stearate ester were as follows: 4.64 (d, 1 H, H-4) and 4.21–4.07 (m, 3 H, H-5, H-6 and H-6'). The methylene protons (32 H) and the methyl protons (3 H) of the stearoyl group were at δ 2.4–1.2 and 0.9, respectively. The ¹³C-NMR data of all the 6-esters of AsA is given in Table 1.

The unsaturated fatty acid esters of AsA all showed a single spot on TLC. Physically all were solids at 25°C, but mechanically the solids increased in softness as the degree of unsaturation increased. After drying in a vacuum desiccator over Drierite, they gave the following yields and melting points: 6-oleate, 254 mg (42%), m.p. 68–72°C; 6-linoleate, 214 mg (36%), m.p. 58–66°C; 6-conjugated linoleate, 210 mg (35%), m.p. 55–65°C; and 6-linolenate 206 mg (35%), m.p. 54–64°C.


Other esterification reactions. The esterification reaction with oleic acid was performed as described above, except at reaction times of 3, 6, 7 and 8 h, and the yields of the 6-oleate ester of ascorbic acid were 40, 42, 40 and 37%, respectively.

When acetonitrile was substituted for TAA as the reaction solvent, the yield of the crude 6-oleate AsA was 211 mg (40%) instead of 48%. Nitromethane also was tried as the reaction solvent, but the unsaturated fatty acids were only partially soluble therein, and only low levels of the desired products were obtained after column chromatography. When dimethyl sulfoxide was employed as the reaction solvent, the desired product was observed, and the yield was judged by TLC to be the same as that observed in TAA; but the isolation of AsA 6-oleate from dimethyl sulfoxide was not attempted. No AsA ester was observed by TLC when dimethylformamide was used as the reaction media.

A series of experiments were done to change the water concentration in the esterification reaction in TAA with palmitic acid. When anhydrous sodium sulfate (1 g) was added initially to the reaction mixture, 77 mg of the 6-palmitate ester (13% yield) was obtained; molecular sieves (4 Å) (1 g), 207 mg of product (35% yield); triethyl orthoformate (1 mL, 6.0 mmol), 86 mg of product (14% yield); and triisopropyl orthoformate (1 mL, 4.5 mmol), 92 mg of product (15% yield). The yield from the blank reaction mixture with no water-adsorbing or water-reacting additive was 55% of AsA 6-palmitate. Adding water (2.0 and 10.0 μL, 1 and 5% based on Novozym 435, or 0.11 and 0.55 mmol) reduced the yield of L-ascorbic acid 6-palmitate to 44 and 21%, respectively.

Lipase-catalyzed esterification of L-ascorbic acid in hexane. Another approach to esterify AsA with palmitic acid using lipase was attempted. To create a film of AsA on an inert carrier, silica gel (60-200 mesh, 480 mg) was added to a methanolic solution of AsA (240 mg), and the methanol was evaporated under reduced pressure. The homogeneous powder was dried over anhydrous calcium sulfate for 72 h. When stirred in methanol, the powdery product was found to have retained 96% of added AsA as determined by iodometric titration.

An open erlenmeyer flask (100 mL) containing hexane (20 mL), Novozym 435 (0.2 g), palmitic acid (1.744 g) and AsA (240 mg) absorbed on silica gel (480 mg) was placed in a desiccator containing a saturated aqueous solution of sodium nitrate (equilibrium relative humidity 74% at 25°C). The desiccator was held at 40°C for 48 h. Isolation of the palmitate ester gave 7% of the desired product. A similar yield was found in a reaction done in an open beaker containing hexane (50 mL), Novozym 435 (0.2 g), oleic acid (2.15 mL), and AsA (240 mg) absorbed on silica gel (480 mg). The beaker was placed in the desiccator over a saturated aqueous solution of sodium nitrate at 40°C for 48 h, during which time ~20 mL of hexane evaporated from the beaker.

Loss of L-ascorbyl 6-oleate upon lipase-catalyzed reaction with excess fatty acid in t-amyl alcohol. L-Ascorbyl 6-oleate (602 mg, 1.36 mmol), oleic acid (1.72 mL, 5.44 mmol), Novozym 435 (200 mg) and TAA (20 mL) were combined and stirred at 55°C for 4 h. Those concentrations matched the concentrations of AsA, fatty acid (total), and lipase in the lipase-catalyzed esteri-
fication of AsA with oleic acid. The reaction mixture was filtered, solvent removed by vacuum below 60°C, and the residue subjected to column chromatography on silica gel. The recovery of the 6-oleate was 265 mg (46%). We also used L-ascorbyl 6-stearate (605 mg) to determine equilibration concentrations under the same conditions, and 275 mg of L-ascorbyl 6-stearate was recovered (44%). When the enzyme was omitted in the above reactions, the L-ascorbyl 6-oleate and 6-stearate were recovered quantitatively.

Esterification of t-amyl alcohol with palmitic acid.

Esters of fatty acids with tertiary alcohols have been prepared chemically using the following procedure. To a solution of triphenylphosphine (0.262 g, 0.001 mol) solution in dichloromethane (20 mL) bromine was added dropwise with stirring until a faint yellow color persisted. A dichloromethane solution (20 mL) of palmitic acid (0.256 g, 0.001 mol) at room temperature was added, and the mixture shaken for 3-4 min. Then t-amyl alcohol (2.7 mL) was added, and the mixture was allowed to stand at room temperature for another 10 min with occasional shaking. Water was added, and the dichloromethane layer was isolated, dried over anhydrous sodium sulfate, and the solvent removed. Thin-layer chromatography of the reaction mixture using solvent mix B showed developed with ethyl ether, and the two faster moving components collected. TLC using solvent mix B showed the solvent-extraction method, which generally boosts yields by at least 5%.

RESULTS AND DISCUSSION

Immobilized C. antarctica lipase.

Novozym 435 is a commercial lipase from the fungus Candida antarctica, but produced in transgenic Aspergillus oryzae; it is immobilized on a macroporous acrylic resin. The enzyme is reported to be lipase B with a molecular weight of 33 kDa, and to have properties of both an esterase that hydrolyzes water-soluble substrates and a lipase that hydrolyzes insoluble esters at the organic-water interface. C. antarctica lipase is one of a few lipases capable of esterifying sugars with fatty acids, whereas most others catalyze transesterification reactions starting with vinyl or isopropenyl esters of fatty acids. In addition, C. antarctica lipase has been used to produce high-molecular weight polyesters in solvent-free polymerizations of lactones.

Esterification of L-ascorbic acid with fatty acids.

Esterification of AsA with palmitic and stearic acids catalyzed by Novozym 435 in TAA gave 48-55% of esters that were isolated in pure form without chromatographic fractionation. The palmitate and stearate esters, as well as the other esters prepared in this work (Table 1), were all 6-esters of AsA. Proton nmr gave integrated intensities of signals consistent with a monooester product, as did elemental analysis. Carbon-13 nmr spectra showed the signal of C-6 in an ester had been deshielded by 3-4 ppm compared to that of AsA (Table 1). The esters showed an absorption maximum (λmax) of 245 nm in ultraviolet spectra measured in ethanol, which is consistent with no esterification at C-2.

The L-ascorbyl 6-esters of unsaturated fatty acids were solids with broad melting points. Esterification of AsA with oleic, linoleic, conjugated linoleic and linolenic acids using the immobilized lipase in TAA gave 35-42% purified monooester after silica gel column chromatography. Because the reverse reaction, lipase-treatment of the 6-ester of AsA with excess fatty acid in TAA gave 44-46% recovery of ester (see below), the yields of the 6-esters of AsA in the esterification reaction may be near the maximum. The order of decreasing yields for the 6-esters of AsA was palmitate > stearate > oleate, in agreement with others. Those results have been explained by the difficulty of fitting a long-chain fatty acid into the deep elliptical-funnel active site of the C. antarctica lipase.

Isolation and purification of the 6-fatty acid esters of AsA by silica gel column chromatography sometimes gave poor recovery of the desired ester. It appears that when water contaminated a reaction mixture that was applied to a column, either (i) O2-oxidation and then degradation occurred or (ii) unreacted AsA became ionized and the acidity catalyzed the degradation of the product. It is recommended that the 6-esters of AsA be isolated by the solvent-extraction method, which generally boosts yields by at least 5%.

Humeau and coworkers reported that when the molar ratio of AsA to methyl palmitate was varied from 1:1, 1:3, 1:5, and 1:7, respectively, the yields of ascorbyl palmitate were 6, 48, 68 and 50%. In our laboratory when a molar ratio of 1:5 of AsA to oleic acid was used in TAA, the yield of AsA oleate was 48%, and when the ratio was changed to 1:2.5, the yield decreased to 33%. We also used oleic acid methyl ester as acyl donor at a 1:5 molar ratio of AsA : oleic, but the yield of L-ascorbyl 6-oleate was the same as with oleic acid.

Lipase-catalyzed loss of L-ascorbil 6-oleate in TAA.

L-Ascorbyl 6-oleate and 6-stearate were treated with the same level of immobilized lipase in TAA under reaction conditions where the molar concentrations of fatty acid and AsA in TAA were identical to those in the esterification reaction. The recoveries of L-ascorbil 6-oleate and 6-stearate after column chromatography were 44-46%.
Those results indicate that approximately an equal amount of ester and unreacted AsA are in equilibrium under the conditions of the reaction when a 5 molar excess of fatty acid to AsA is used, which agrees with Watanabe and co-workers. The solvent TAA is present in about a 130 molar excess to AsA, yet TAA is not esterified. TAA was found (see below) to contain an unknown alcohol that might compete with AsA for the fatty acid in the esterification reaction. In addition, the solvent may contain some water compared to the 1.4 mmol of AsA in a reaction (see below). However, when the solvent volume was reduced by 50% in the esterification reaction, no increase in L-ascorbyl 6-oleate yield was realized. It appears that the water required for activity of the lipase (see below) may limit the yield of the ester. It is known that some water (0.5–3.0% based on lipase) is required for lipase-catalyzed esterifications in a non-aqueous environment. Apparently, the 3–5% moisture that is bound to the immobilized C. antarctica lipase is sufficient for efficient catalysis, since the yields of AsA 6-esters are at a maximum starting with dry solvents.

Preparation of t-amyl palmitate containing a contaminating ester.

t-Amyl palmitate was synthesized chemically and identified by proton NMR. A literature search revealed t-amyl palmitate has not been reported previously. Thin-layer chromatography in solvent mix B showed the chemically synthesized t-amyl palmitate contained a low-level contaminant with slower mobility than the predominant ester. Presumably the minor component is the palmitate ester of the AsA was retained in the adsorption process. Reaction of t-amyl alcohol with palmitic acid also was performed. Interestingly, the ester formed by enzyme catalysis showed only one product on thin-layer chromatography and it had the same mobility as the contaminating product in the chemically synthesized t-amyl palmitate. Thin-layer chromatography showed the lipase-produced ester formed rapidly and appeared to reach a plateau level after 0.5–1.0 h. The contaminating ester was highly hydrophobic, so in the preparation of an AsA 6-ester it was removed along with the ether-soluble unreacted fatty acid.

Lipase-catalyzed esterification of l-ascorbic acid and attempts to improve the yield of ester.

The water formed in the esterification reaction plus that introduced with the lipase and solvent (TAA) could account for the approximately 50% equilibrium yield of ester. Perhaps removing the water could drive the equilibrium towards the ester. However, adding reagents that either adsorbed water, such as molecular sieves and anhydrous sodium sulfate, or adding reagents that reacted with water, such as triethyl orthoformate, did not improve the yield of ester.

Adding water at 1 and 5 wt % (based on lipase) to the reaction of AsA (1.4 mmol) with palmitic acid (6.8 mmol) reduced the yield of AsA 6-palmitate from 55 to 44 and 21%, respectively. The water added was only 0.1 and 0.5 mmol, which amounted to 4–17% of new water compared to the total water (2.9 mmol) already present. Water in a typical esterification reaction originates from three sources; (1) 200 mg of commercial lipase (~0.5 mmol), (2) that formed in the esterification reaction (~0.8 mmol), and (3) that estimated to be in 20 mL of TAA (~1.6 mmol). Previously, a maximum yield of 55% l-ascorbyl 6-lauroate was formed in acetonitrile when AsA (1 mmol) was reacted with 5 eq of lauric acid in the presence of 2.8 mmol of water per mmol of AsA or per 400 mg of Novozym 435. In our work, reactions typically contained ~2.1 mmol water per mmol of AsA or per 200 mg of Novozym 435.

A second approach to attempt to increase ester yields was to use a non-protic solvent for the esterification reaction. However, when acetonitrile was used here as solvent and palmitic acid as acyl donor, the yield of the L-ascorbyl 6-palmitate was the same (55%) as when TAA was used, but the yield of the 6-oleate ester was only 35% in acetonitrile versus 48% in TAA. When dimethyl sulfoxide was used as solvent, TLC indicated that esterification of AsA had occurred, but because of the low volatility of dimethyl sulfoxide no attempt was made to isolate the desired product. Thin-layer chromatography indicated no esterification of AsA occurred in dimethylformamide.

6-Palmitate ester of AsA prepared in hexane.

Sugar esters have been prepared in 22–86% by a lipase-catalyzed reaction in hexane in an open container placed in a humid atmosphere (equilibrium relative humidity 74% at 25°C). In our work AsA was adsorbed onto the surface of silica gel, and iodometric titration showed the AsA was retained in the adsorption process. Reaction of the adsorbed AsA with palmitic acid in hexane in a humid atmosphere for 48 h, however, gave only a 7% yield of isolated L-ascorbic 6-palmitate. An immobilized lipase from Bacillus stearothermophilus SB-1 has been reported to form 97% AsA 6-palmitate after 5-cycles of reacting AsA (50 mM) with 5 eq of palmitic acid in hexane at 60°C for 2 h. The conversion was calculated by titration of unreacted fatty acid, and isolation and purification of the 6-ester were not reported.

CONCLUSIONS

6-Acyl esters of AsA are produced by immobilized lipase (Candida antarctica) catalysis under mild conditions (25–60°C, 7–48 h) starting with AsA in dry acetone, n-butanol and t-amyl alcohol. Yields are moderate in reactions containing almost equal weights of AsA and immobilized lipase and with 3–9 eq of fatty acid based on AsA. However, yields exceed 90% starting with vinyl esters of fatty acids, where available. Long-chain 6-fatty esters of AsA are readily isolated and purified from reaction mixtures by solvent-extraction to remove unreacted reagents plus a trace of a highly hydrophobic unknown ester. The L-ascorbyl 6-esters of polyunsaturated fatty acids have been shown to resist autoxidation. Immobilized, thermostable C. antarctica lipase is available from two commercial sources; Roche Molecular Biochemicals (Chirazyme L-2) and Novo Nordisk (Novozym 435).
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REFERENCES


