

Synthesis and Properties of Ascorbyl Esters Catalyzed by Lipozyme TL IM using Triglycerides as Acyl Donors

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Received: 17 March 2010/Revised: 5 May 2010/Accepted: 7 July 2010/Published online: 23 July 2010
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Abstract Esters of L-ascorbic acid with long-chain fatty acids (E-304) are employed as antioxidants in foods rich in lipids. Although their enzymatic synthesis offers some advantages compared with the current chemical processes, most of the reported methods employ the immobilized lipase from *Candida antarctica* as biocatalyst and free fatty acids or activated esters as acyl donors. In order to diminish the cost of the process, we have investigated the synthesis of ascorbyl oleate and ascorbyl palmitate esters with the immobilized *Thermomyces lanuginosus* lipase Lipozyme TL IM—which is significantly less expensive than Novozym 435—and triglycerides as source of fatty acids. Lipozyme TL IM gave rise to a lower yield of 6-*O*-ascorbyl oleate than Novozym 435 when using triolein (64 vs. 84%) and olive oil (27 vs. 33%) as acyl donors. Both 6-*O*-ascorbyl oleate and 6-*O*-ascorbyl palmitate displayed excellent surfactant and antioxidant properties. The Trolox Equivalent Antioxidant Capability values for the oleate and palmitate were 71 and 84%, respectively, of those obtained

with L-ascorbic acid; however, both derivatives were able to stabilize soybean oil towards peroxide formation.

Keywords Vitamin C · Ascorbyl esters · L-Ascorbyl oleate · Lipase · *Thermomyces lanuginosus* · Enzymatic transesterification · Alkyl esters · Triglycerides · Surface tension · CMC · Antioxidant activity

Introduction

The modification of natural antioxidants to improve their chemical, oxidative and/or thermal stability, or to alter their hydrophile–lipophile balance (HLB), yields a series of “semisynthetic” antioxidants with a great impact in industry [1]. These derivatives are generally prepared under harsh conditions using strongly corrosive acids such as sulfuric acid or hydrogen fluoride [2, 3]. To overcome these shortcomings, new approaches based on the use of biological catalysts are being evaluated, which are characterized by mild reaction conditions, low energy requirements and a minimization of the isomerization and rearrangement side reactions [1, 4]. In addition, biocatalysts are biodegradable and display chemo-, regio- and/or stereospecificity resulting in decreased by-product formation thus avoiding the need for functional group protection and activation [5, 6].

L-Ascorbic acid (vitamin C) is the major water-soluble natural antioxidant. Acting as a free radical scavenger, L-ascorbic acid and its derivatives react with oxygen, thus removing it in a closed system. The combination of L-ascorbic acid and primary antioxidants like α -tocopherol renders a synergic effect that results in the “vitamin E recycling system” [7]. This mixture of vitamins is usually added to fatty products to retard the autooxidation of unsaturated fatty acids. However, due to the low miscibility

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of L-ascorbic acid with α -tocopherol or with any oil-based formula, it is well established the use of ascorbyl fatty acid esters instead of vitamin C [8].

Ascorbyl palmitate and stearate, classified by European Union with the code E-304 (fatty acid esters of ascorbic acid) are produced by reacting ascorbic acid with sulfuric acid followed by re-esterification with the corresponding fatty acid, and subsequently purified by recrystallization [9]. This chemical process has some disadvantages such as the use of strong acids, the low yields due to non-regio-selective reactions, the formation of by-products due to instability of vitamin C and the need of tedious product isolation [10, 11]. As an alternative, lipases, especially that from *Candida antarctica*, have been successfully used to catalyze the synthesis of ascorbyl esters in tertiary alcohols, acetone and even in ionic liquids [12], employing as acyl donors saturated and unsaturated free fatty acids, alkyl and vinyl esters [13, 14]. The enzymatic process is specific for the primary alcohol, yielding the corresponding 6-*O*-ascorbyl ester. Although ascorbyl palmitate is more soluble in fats than ascorbic acid itself, its low solubility and miscibility in edible fats and oils limits its use [9]. Ascorbyl esters of unsaturated fatty acids, e.g. oleate or linoleate, exhibit improved miscibility with α -tocopherol, oils or fatty products, and can be also employed as food additives (E-304).

In the present work, we have investigated the enzymatic synthesis of L-ascorbic acid fatty acid esters, in particular ascorbyl oleate and palmitate. In order to reduce the cost of the process, we have analyzed: (1) the use of less expensive biocatalysts than the immobilized lipase from *C. antarctica*, in particular the silica-granulated lipase from *Thermomyces lanuginosus*—Lipozyme TL IM [15]; (2) the use of triglycerides and oils as acyl donors as an alternative to fatty acids or activated esters.

Experimental Procedures

Chemicals and Enzymes

Immobilized lipases from *Thermomyces lanuginosus* (Lipozyme TL IM) and *Candida antarctica* (Novozym 435) were a kind gift from Novozymes A/S. Triolein, methyl oleate, oleic acid, tripalmitin, ethyl palmitate, palmitic acid, 2-methyl-2-butanol (2M2B, *tert*-amyl alcohol), (*R*)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid [(*R*)-Trolox] and 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) were from Sigma-Aldrich. Vinyl palmitate was from TCI (Tokyo, Japan). L-ascorbic acid (vitamin C) was supplied by Fluka. Extra-virgin olive oil was from La Española (Aceites del Sur S.A., Spain). All other reagents were of the highest available purity. The

solvent 2-methyl-2-butanol was dried before use over 3-Å molecular sieves (Sigma) at least for 24 h.

Determination of Lipase Activity

The hydrolytic activity was measured titrimetrically at pH 8.0 and 30 °C using a pH-stat (Mettler, Model DL 50). The reaction mixture contained tripropionin (0.3 ml, final concentration 80 mM), acetonitrile (0.7 ml) and Tris-HCl buffer (19 ml, 1 mM, pH 8.0) containing NaCl (0.1 M). The immobilized biocatalyst was then added and the pH automatically maintained at 8.0 using 0.1 N NaOH as titrant. Experiments were done in triplicate. One enzyme unit (U) was defined as that catalyzing the formation of 1 μ mol of fatty acid per min.

Enzymatic Synthesis of 6-*O*-Ascorbyl Esters

All reactions were performed in 30-ml amber glass sealed vessels at 40 °C, with orbital shaking (250 rpm). Ascorbic acid (0.5 mmol) and the corresponding acyl donor (fatty acid, alkyl or vinyl ester, 1.5 mmol) were dissolved in 5 ml of dried 2-methyl-2-butanol. The mixture was equilibrated for 10 min, and the biocatalyst (125 mg) was added. Aliquots were removed at intervals, filtered using a 0.45 μ m Durapore[®] membrane coupled to an eppendorf tube and analyzed by HPLC. For the reactions involving the use of triglycerides (tripalmitin, triolein) as acyl donors, the procedure was as described above but the amount of ascorbic acid and triglyceride was varied in the range 0.5–1.5 mmol (0.1–0.3 M). In the case of olive oil, the weight of oil added was the same (88–265 mg/ml) as in the experiments with triolein. Experiments were performed in duplicate. Standard deviations were lower than 5%.

HPLC Analysis

Reactions aliquots were analyzed by HPLC, using a 9012 pump (Varian) and a Nucleosil 100-C18 column (4.6 \times 250 mm, Análisis Vinicos, Tomelloso, Spain), maintained at 45 °C. Integration was carried out using the Varian Star 4.0 software. Detection was performed using an evaporative light-scattering detector DDL-31 (Eurosep) equilibrated at 60 °C. Methanol:water 95:5 (v/v) containing 0.1% (v/v) acetic acid was used as mobile phase (flow rate 1.2 ml/min) for 6 min. Then, a gradient from this eluent to pure methanol was performed in 1 min (min 6 to 7), after which the flow rate was increased to 1.7 ml/min in 1 min (min 7 to 8) and held for another minute (min 8 to 9). A new gradient from methanol to methanol:acetone (50:50 v/v) was performed in 2 min (min 9 to 11) and held for 6 min (min 11 to 17) at 1.7 ml/min. After this, the gradient returned to the initial conditions (min 17 to 25).

Purification and Characterization of 6-*O*-Ascorbyl Oleate

6-*O*-Ascorbyl oleate was synthesized as described in the general procedure and purified by semipreparative HPLC. The column was a Nucleosil-C18 (10 × 250 mm, 5 μm, Analisis Vinicos, Spain), and the mobile phase was 95:5 (v/v) methanol/H₂O at 4.8 ml/min. The fractions containing the product were pooled and the solvent evaporated. NMR spectra were recorded on a Varian Unity spectrometer (¹H NMR, 500 MHz; ¹³C NMR, 125 MHz), with a reverse probe and a gradient unit. The spectra were obtained at 30 °C, for samples of around 5 mg in solutions of 0.6 ml of deuterated methanol (CD₃OD). Proton chemical shifts refer to the methanol multiplet (3.30 ppm). Carbon chemical shifts refer also to the methanol multiplet (49.0 ppm). Mass spectrometry was determined with a MALDI-TOF system Reflex III (Bruker-Franzen). For the experiments the matrix employed was a saturated solution of 2,5-hydroxybenzoic acid.

Surfactant Properties

Determination of surface tension in water of ascorbyl esters was performed at 20 °C according to the Wilhelmy plate method, with a Krüss tensiometer (Processor tensiometer K-12, Hamburg, Germany), which determines directly the real tension values at the equilibrium, using a series of aqueous solutions of ascorbyl esters at various concentrations. The equilibrium time of the surface before the surface tension measurements was at least 1 h. The critical micelle concentration (CMC) was determined graphically from the abrupt change in the slope of the plot representing the surface tension versus logarithm of surfactant concentration (expressed in mM).

TEAC Assay

To measure the antioxidant activity of the new compounds we used the Trolox Equivalent Antioxidant Capability (TEAC) assay with some modifications to adapt to 96-well plates. This assay is based on the ability of antioxidants in reducing ABTS radical. Briefly, ABTS (7 mM final concentration) was added to an aqueous solution of 2.45 mM potassium persulfate and kept in the dark at room temperature for 15 h to obtain the ABTS radical, which was stable for 2 days. The ABTS^{•+} solution was diluted with ethanol to get an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at room temperature. In each well, 20 μl of a solution of Trolox (standard) or of the antioxidants (0.5–25 μM) in ethanol were added to 230 μl of adjusted ABTS^{•+} solution. The decrease of absorbance of ABTS^{•+} solution was monitored at 734 nm during 6 min using a

microplate reader (model Versamax, Molecular Devices) and the decrease of absorbance (ΔA_{734}) for each concentration was calculated determining the area under the curve. Experiments were performed at 30 °C in quadruplicate. The $\Delta A_{734\text{nm}}$ versus concentration curve was plotted for the different compounds and used to calculate the equivalent Trolox concentration. The TEAC value was determined as the ratio between the slopes of concentration- ΔA_{734} curves for the corresponding antioxidant and Trolox.

Evaluation of Oxidative Stability Under Accelerated Conditions

Oil stability was determined at 110 °C and 20 ml air/h using a Rancimat apparatus (Metrohm). The ascorbyl derivatives (50 ppm) were added to soybean oil. The range of conductivity was 200 μS. The soybean oil with no added antioxidant was used as control.

Results and Discussion

The enzymatic synthesis of ascorbyl esters has been widely investigated using saturated and unsaturated acyl donors. It is noteworthy that most of the studies have been carried out with the lipase B from *Candida antarctica* immobilized in different supports (lipases Novozym 435, Chirazyme L-2, etc.). The high cost of these biocatalysts, approx. \$900/kg [16], makes it difficult their application to food processes. The lipases from *B. stearothersophilus* [17] and *Candida* sp. [14] also catalyze the esterification of L-ascorbic acid with fatty acids; however, these lipases are not commercially available in immobilized form at large scale.

Most of the processes developed up to now are based on esterification strategies with different fatty acids, whereas transesterification with alkyl and vinyl esters have been also successfully employed. We have found only one report using triglycerides (in particular, palm oil) as acyl donor [18]. An interesting two-step route for the synthesis of ascorbyl esters from oils has been also described, i.e. their transformation into biodiesel followed by the transesterification of the corresponding methyl esters with ascorbic acid [19].

In order to develop an efficient and affordable strategy to synthesize ascorbyl fatty acid esters, we have assessed the use of less expensive biocatalysts than those based on lipase B from *C. antarctica*. In particular, we have focused on the silica-granulated lipase from *Thermomyces lanuginosus*, Lipozyme TL IM [20, 21], whose price is 8 and 10 times lower than other immobilized lipases such as Lipozyme RM IM and Novozym 435, respectively [22]. The hydrolytic activity of Lipozyme TL IM (2,490 U/g),

measured in the hydrolysis of tripropionin, is slightly higher than that of Novozym 435 (1,725 U/g). On the other hand, we have explored the use of triglycerides and oils as acyl donors, which could represent another improvement to the cost of the process.

Synthesis of Ascorbyl Esters Using Fatty Acids, Alkyl and Vinyl Esters

The low solubility of ascorbic acid (hydrophilic) in non-polar solvents is the major hurdle for its acylation with fatty acids. Different solvents such as 2-methyl-2-butanol (*tert*-amyl alcohol), *t*-butanol and acetone have been selected for the enzymatic synthesis of ascorbyl esters of fatty acids [8]. We used *tert*-amyl alcohol as reaction medium since ascorbic acid is sufficiently soluble in this solvent and most of the lipases are stable and active [23]. Compared with other polyhydroxylated compounds such as carbohydrates, the lower hydrophilicity of ascorbic acid made not necessary to add cosolvents like dimethyl sulphoxide [20].

In order to explore the applicability of Lipozyme TL IM in these processes, the synthesis of ascorbyl palmitate was attempted by esterification with palmitic acid and by transesterification with different palmitic acid donors (ethyl, vinyl) (Fig. 1). A 3/1 M ratio acyl donor/ascorbic acid was selected. It is important to consider that the hydrolysis of the alkyl or vinyl ester to fatty acid catalyzed by the lipase is an undesirable but unavoidable reaction (water can also act as nucleophile) yielding free fatty acid.

As shown in Fig. 1, the activated vinyl ester gave a faster reaction and a higher yield than the rest of acyl

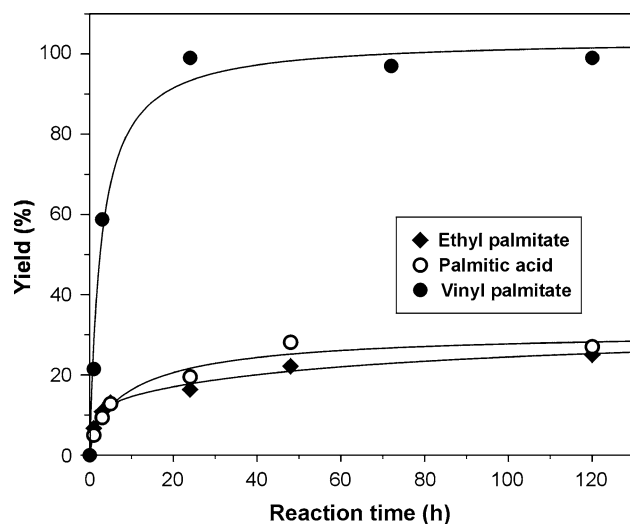


Fig. 1 Time course of enzymatic synthesis of L-ascorbyl palmitate in 2-methyl-2-butanol catalyzed by Lipozyme TL IM using different acyl donors. Experimental conditions: 100 mM L-ascorbic acid, 300 mM acyl donor, 25 mg/ml biocatalyst, 40 °C

donors. It is well reported that the transesterification of hydroxylated compounds with vinyl esters is about 20–100 times faster than with alkyl esters [24]. In addition, the vinyl alcohol formed during the process tautomerizes to the low-boiling-point acetaldehyde, shifting the equilibrium towards the ester formation thus increasing yield [23]. In fact, the monoester yield passed from 20% with palmitic acid and ethyl palmitate to 100% employing the vinyl ester (Table 1).

We also studied the transesterification with methyl oleate. The yield was significantly higher than the obtained with ethyl palmitate (50 vs. 20%, see Table 1). We confirmed by mass spectrometry and NMR (data not shown) that the major products obtained with Lipozyme TL IM were the monoesters at hydroxyl 6-OH, as occurs with the lipase from *C. antarctica*.

Potential Use of Natural Oils as Substrates for the Synthesis of Ascorbyl Esters

Vinyl esters give rise to the fastest reactions and highest yields. However, from the economical point of view, vinyl esters of long-chain fatty acids are still too expensive [25, 26]. Oils, which are mainly composed of triglycerides, offer an alternative route for the synthesis of ascorbyl esters. Acylation of ascorbic acid directly using oils usually yields a mixture of ascorbyl fatty esters with acyl chains of different length depending on the oil composition [8]. Burham et al. [18] reported the synthesis of mixed ascorbyl esters using palm oil and Novozym 435.

In a first attempt, we assayed tripalmitin and triolein as acyl donors (in a 100 mM equimolar ratio with L-ascorbic acid, which is equivalent to threefold excess of fatty acid with respect to vitamin C) using Lipozyme TL IM (Fig. 2). As shown, reaction is slightly faster with triolein, probably due to its higher miscibility with the substrates, yielding approx. 64% of 6-*O*-ascorbyl oleate in 140 h. Figure 3 illustrates a typical chromatogram obtained with triolein, in which the formation of mono- and di-glycerides as by-products is clearly observed. As the formed mono- and diacylglycerols could also serve as emulsifiers [27], it would not be necessary to remove them from the final product. As illustrated in Fig. 2, Novozym 435 produced a higher yield of 6-*O*-ascorbyl oleate than Lipozyme TL IM (84 vs. 64%). Different concentrations of L-ascorbic acid and triolein were tested (Table 1). Using 300 mM triolein and 100 mM L-ascorbic acid (which represents a 9/1 M ratio of oleic acid to L-ascorbic acid), the yield was not improved significantly. Interestingly, when assaying 300 mM triolein and 300 mM L-ascorbic acid the yield obtained with Lipozyme TL IM was 70.5%, which is equivalent to a theoretical concentration of 6-*O*-ascorbyl oleate of nearly 97 g/l.

Table 1 Effect of the nature of acyl donor, molar ratio donor/acceptor and biocatalyst on the yield of fatty acid ascorbyl esters

Ascorbic acid (mM)	Acyl donor	Acyl donor (mM)	Biocatalyst	Yield (%)
100	Ethyl palmitate	300	Lipozyme TL IM	20
100	Palmitic acid	300	Lipozyme TL IM	20
100	Vinyl palmitate	300	Lipozyme TL IM	100
100	Methyl oleate	300	Lipozyme TL IM	50
100	Tripalmitin	100	Lipozyme TL IM	50
100	Triolein	100	Lipozyme TL IM	64
100	Triolein	100	Novozym 435	84
100	Triolein	300	Lipozyme TL IM	71
100	Triolein	300	Novozym 435	82
300	Triolein	300	Lipozyme TL IM	70.5
100	Olive oil	88 mg/ml ^a	Lipozyme TL IM	27
100	Olive oil	88 mg/ml ^a	Novozym 435	33

^a The amount of olive oil was the same as the employed in the experiments with 100 mM triolein, on a weight basis

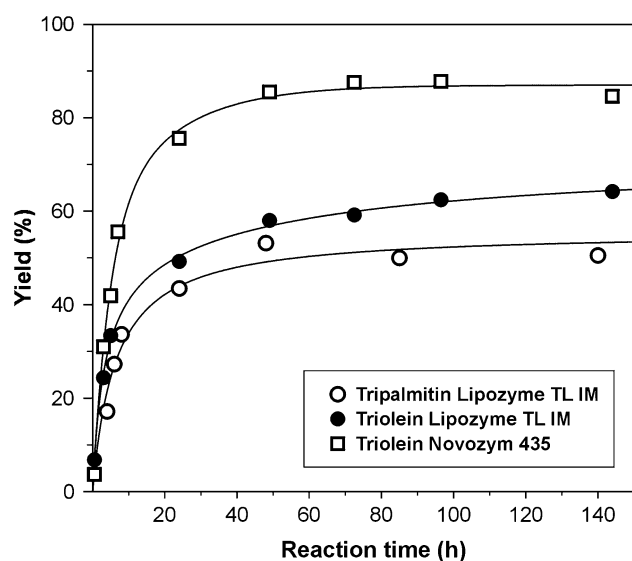


Fig. 2 Kinetics of acylation of L-ascorbic acid in 2-methyl-2-butanol using triolein and tripalmitin catalyzed by Lipozyme TL IM and Novozym 435. Experimental conditions: 100 mM L-ascorbic acid, 100 mM triglyceride, 25 mg/ml biocatalyst, 40 °C

Olive oil is the main source of fat in the Mediterranean diet and is associated to a low incidence of cardiovascular disease [28]. The composition of the virgin olive oil depends on the variety and the grade of the olive. The saponifiable fraction (96–98%) is constituted mainly by triglycerides formed of different fatty acids with oleic acid as the major component (60–80%), and variable amounts of palmitic, palmitoleic, stearic and linoleic acids. We tested virgin olive oil as acyl donor for L-ascorbic acid acylation. We observed in the HPLC chromatograms (data not shown) the presence of other monoesters apart from the monooleate, as expected considering the typical composition of the oil. We determined the formation of 6-O-ascorbyl oleate with both Lipozyme TL IM and Novozym 435 lipases (Fig. 4). The differences between the two biocatalysts were

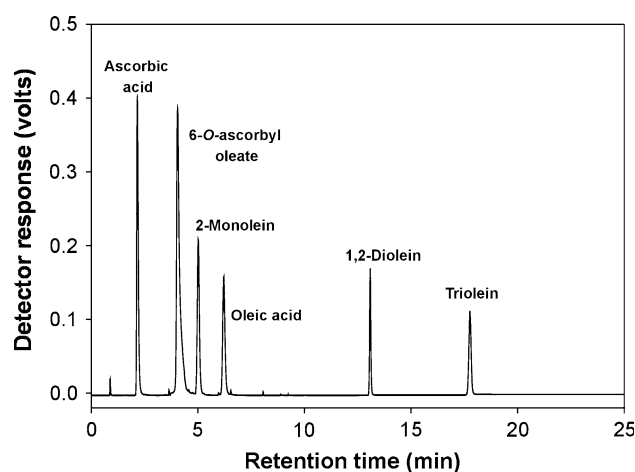


Fig. 3 HPLC chromatogram showing the different products formed in the synthesis of 6-O-ascorbyl oleate from L-ascorbic acid and triolein. Analytical conditions described in the “Experimental” Section

not very significant, with Novozym 435 giving a higher yield in 168 h (33 vs. 27%).

CMC and Surface Tension of Ascorbyl Esters

For a surfactant to act as an emulsifier, it must show good surface activity and generate a low interfacial tension in the particular system in which it is to be used. This means that it must have a tendency to migrate to the interface, rather than to remain dissolved in either one of the bulk phases [29]. Few data are available characterizing the surfactant properties of ascorbyl esters [30]. Figure 5 illustrates the variation of surface tension as a function of concentration of the ascorbyl esters synthesized in this work.

The synthesized 6-O-ascorbyl derivatives displayed excellent surface-active properties. The minimum surfactant concentration needed to form micelles, the called

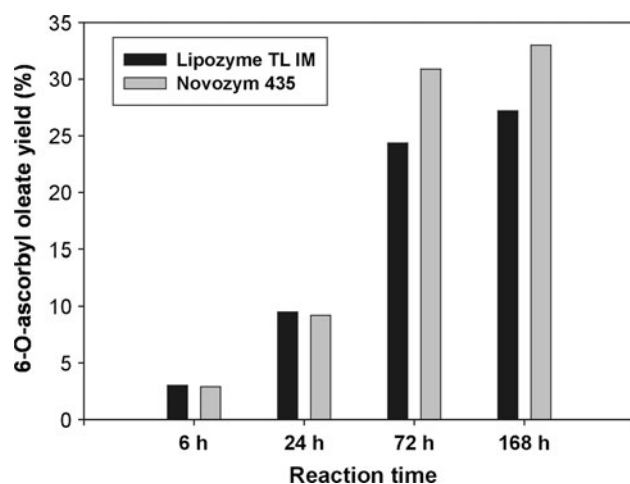


Fig. 4 Comparative kinetics of acylation of L-ascorbyl acid using olive oil in 2-methyl-2-butanol catalyzed by Lipozyme TL IM and Novozym 435. Experimental conditions: 100 mM L-ascorbic acid, 100 mM olive oil, 25 mg/ml biocatalyst, 40 °C

critical micelle concentration (CMC), was determined graphically from the inflexion appearing in the plots of surface tension versus logarithm of concentration. When considering 6-*O*-ascorbyl oleate, the characteristic inflection point at the critical micelle concentration was slightly distorted by the minimum appearing in the graph. This is usually related to the presence of byproducts, even in small traces. However, the estimated CMC value (0.1–0.3 mM) was in the same range as that reported previously for the same compound at 25 °C (0.22 mM) [30]. Although the CMC of 6-*O*-ascorbyl palmitate (0.1 mM) was closely similar to that of the oleyl derivative, the palmitoyl ester solubility in water is much lower than for the unsaturated ester, corroborating that a C16 saturated alkyl chain is more insoluble than a C18 unsaturated one. On the other hand, the surface tension at CMC of the palmitoyl ester was 32 mN/m, higher than the obtained with oleic acid ester (26–27 mN/m), which indicates the higher effectiveness of the oleyl derivative.

The amphiphilic character of fatty acid ascorbyl esters may serve to solubilize and stabilize drug molecules. Thus, the hydrophobic drug stays in the inner hydrophobic core of the micelle, whereas the antioxidant nature of the hydrophilic shell protects the drug against oxidation. In this context, Palma et al. [31] reported the stabilization and solubilization of several lipophilic molecules in micelles of 6-*O*-ascorbyl octanoate.

Antioxidant Activity

In physiological systems, fatty acid ascorbyl esters are hydrolyzed to vitamin C and fatty acids—without forming any hazardous components [8]. However, for their

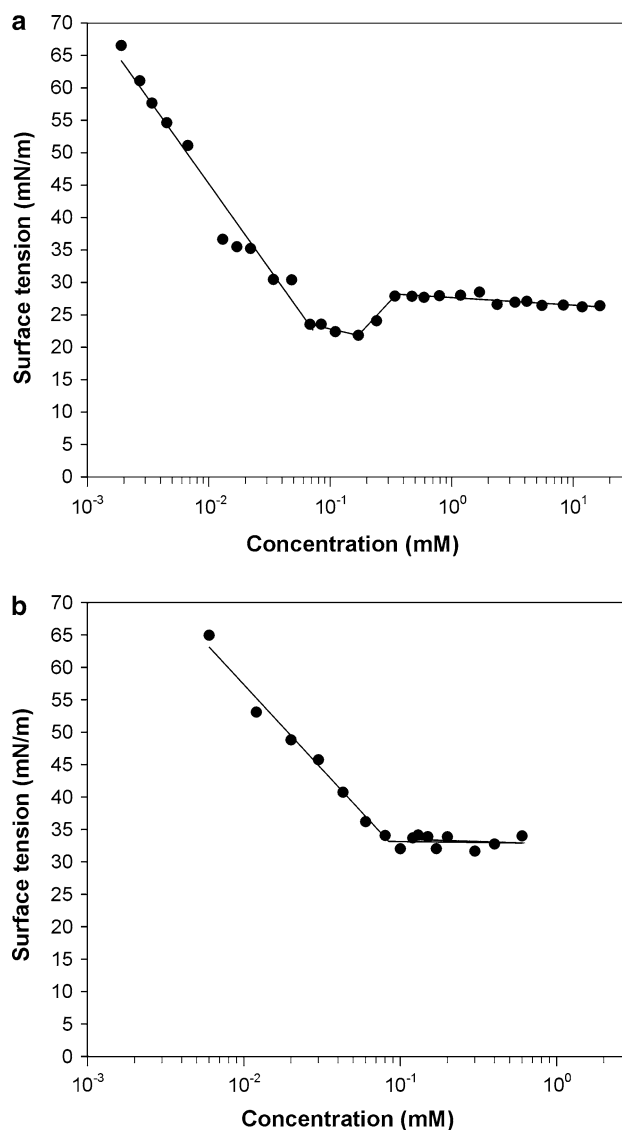


Fig. 5 Variation of surface tension as a function of concentration for 6-*O*-ascorbyl oleate (a) and 6-*O*-ascorbyl palmitate (b)

applications as antioxidants in lipophilic food products, it is interesting to determine their antioxidant activity. In this work, the antioxidant activity of the synthesized derivatives was analyzed using the TEAC assay. The results of the experiments are represented in Fig. 6. We observed that the addition of an acyl chain in the position 6-OH of L-ascorbic acid caused a loss of antioxidant activity. The TEAC values, calculated from the slopes in Fig. 6, are presented in Table 2. 6-*O*-ascorbyl palmitate and 6-*O*-ascorbyl oleate showed 84 and 71% efficiency compared with L-ascorbic acid.

It seems that the selection of the assay to measure antioxidant activity may render different conclusions. In this context, Lo Nostro et al. [32, 33] reported that the addition of an acyl chain to the L-ascorbic acid in the 6-position, retained or even enhanced its physiological and

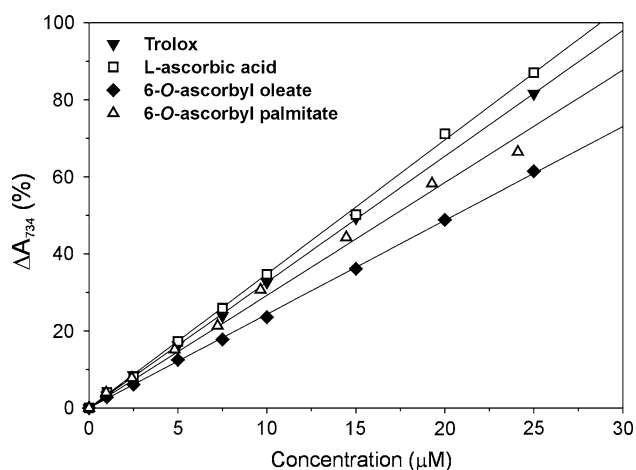


Fig. 6 Effect of antioxidant concentration on the decrease of ABTS⁺⁺ absorbance for Trolox, L-ascorbic acid and its fatty acid esters

Table 2 Antioxidant properties of fatty acid ascorbyl esters

Sample	TEAC	Hours Rancimat	Stability increase, Rancimat (%)
Control	1.06	6.83	–
6- <i>O</i> -Ascorbyl palmitate	0.89	8.27	21.1
6- <i>O</i> -Ascorbyl oleate	0.75	8.22	20.4

antioxidant activity measured by DPPH (α,α -diphenyl- β -picrylhydrazyl) method. Song et al. [33] demonstrated that 6-*O*-ascorbyl oleate had a better protective effect on human umbilical cord vein endothelial cells than other ascorbyl derivatives.

The accelerated test Rancimat was applied to evaluate the oxidative stability of soybean oil in presence of the fatty acid ascorbyl esters (Table 2). The results indicated that both ascorbyl oleate and palmitate produce stabilization of nearly 20% towards peroxide formation. In this context, Viklund et al. [34] compared the efficiency of ascorbyl oleate and palmitate to retard peroxide development in rapeseed oil; they concluded that the oleate derivative was more effective than the palmitate in a 18-week period.

Conclusion

We have demonstrated the applicability of immobilized *Thermomyces lanuginosus* lipase (Lipozyme TL IM) to synthesize fatty acid ascorbyl esters using triglycerides and natural oils as acyl donors. Although yields are slightly lower than those obtained with immobilized *Candida antarctica* lipase preparations, its tenfold lower price may compensate the reduced efficiency. Ascorbyl oleate presents

a good miscibility with oils, an excellent surfactive behavior and maintains a substantial level of antioxidant activity, which facilitates its use in foods and also as a micellar vehicle to solubilize and stabilize hydrophobic drugs and other substances.

Acknowledgments We thank Prof. Manuel Bernabé (Instituto de Química Orgánica, CSIC, Madrid, Spain) for help with NMR analysis. We thank Ramiro Martínez (Novozymes A/S, Madrid, Spain) for supply of lipase samples and for suggestions. We are grateful to Comunidad de Madrid for a research contract to P. Torres and to CONACYT for grant 020256 to D. Reyes. This research was supported by the Spanish CSIC (Project 200680F0132) and by the Latin-American Science & Technology Development Programme (CYTED Project 108RT0346).

References

- Torres P, Kunamneni A, Ballesteros A, Plou FJ (2008) Enzymatic modification for ascorbic acid and alpha-tocopherol to enhance their stability in food and nutritional applications. *Open Food Sci J* 2:1–9
- Stamatis H, Sereti V, Kolisis FN (2001) Enzymatic synthesis of hydrophilic and hydrophobic derivatives of natural phenolic acids in organic media. *J Mol Catal B Enzym* 11:323–328
- Bonrath W, Netscher T (2005) Catalytic processes in vitamins synthesis and production. *Appl Catal A Gen* 280:55–73
- Torres P, Reyes-Duarte D, Lopez-Cortes N, Ferrer M, Ballesteros A, Plou FJ (2008) Acetylation of vitamin E by *Candida antarctica* lipase B immobilized on different carriers. *Process Biochem* 43:145–153
- Alcalde M, Ferrer M, Plou FJ (2007) Environmental biocatalysis: from remediation with enzymes to novel green processes. *Biocatal Biotransform* 25:113
- Wohlgemuth R (2007) Modular and scalable biocatalytic tools for practical safety, health and environmental improvements in the production of speciality chemicals. *Biocatal Biotransform* 25:178–185
- Kochhar SP, Rossell JB (1990) Detection, estimation and evaluation of antioxidants in food systems. In: Hudson BJF (ed) *Food antioxidants*. Elsevier, Essex, pp 19–64
- Karmee SK (2009) Biocatalytic synthesis of ascorbyl esters and their biotechnological applications. *Appl Microbiol Biotechnol* 81:1013–1022
- Valentin HE, Qi QG (2005) Biotechnological production and application of vitamin E: current state and prospects. *Appl Microbiol Biotechnol* 68:436–444
- Humeau C, Girardin M, Coulon D, Miclo A (1995) Synthesis of 6-*O*-palmitoyl L-ascorbic-acid catalyzed by *Candida antarctica* lipase. *Biotechnol Lett* 17:1091–1094
- Yan YC, Bornscheuer UT, Schmid RD (1999) Lipase-catalyzed synthesis of vitamin C fatty acid esters. *Biotechnol Lett* 21:1051–1054
- Park S, Viklund F, Hult K, Kazlauskas RJ (2003) Ionic liquids create new opportunities for nonaqueous biocatalysis with polar substrates: acylation of glucose and ascorbic acid. In: Rogers RD, Seddon KR (eds) *Ionic liquids as green solvents: progress and prospects*. ACS Symposium Series, American Chemical Society, vol 856, pp 225–238
- Jun HK, Bae KM, Kim YH (1998) Identification of L-ascorbic acid 2-*O*-alpha-glucoside, a stable form of ascorbic acid, in kimchi. *J Microbiol Biotechnol* 8:710–713

14. Song QX, Wei DZ (2002) Study of vitamin C ester synthesis by immobilized lipase from *Candida* sp. *J Mol Catal B Enzym* 18:261–266
15. Ferrer M, Plou FJ, Fuentes G, Cruces MA, Andersen L, Kirk O, Christensen M, Ballesteros A (2002) Effect of the immobilization method of lipase from *Thermomyces lanuginosus* on sucrose acylation. *Biocatal Biotransform* 20:63–71
16. Borole AP, Davison BH (2007) Techno-economic analysis of biocatalytic processes for production of alkene epoxides. *Appl Biochem Biotechnol* 137:437–449
17. Bradoo S, Saxena RK, Gupta R (1999) High yields of ascorbyl palmitate by thermostable lipase-mediated esterification. *J Am Oil Chem Soc* 76:1291–1295
18. Burham H, Gafoor RA, Rasheed A, Noor NM, Badruddin S, Sidek H (2009) Enzymatic synthesis of palm-based ascorbyl esters. *J Mol Catal B Enzym* 58:153–157
19. Hsieh HJ, Chen JW, Giridhar R, Wu WT (2005) Synthesis of mixed esters of ascorbic acid using methyl esters of palm and soybean oils. *Prep Biochem Biotech* 35:113–118
20. Ferrer M, Soliveri J, Plou FJ, Lopez-Cortes N, Reyes-Duarte D, Christensen M, Copa-Patino JL, Ballesteros A (2005) Synthesis of sugar esters in solvent mixtures by lipases from *Thermomyces lanuginosus* and *Candida antarctica* B, and their antimicrobial properties. *Enzyme Microb Technol* 36:391–398
21. Reyes-Duarte D, Polaina J, Lopez-Cortes N, Alcalde M, Plou FJ, Elborough K, Ballesteros A, Timmis KN, Golyshin P, Ferrer M (2005) Conversion of a carboxylesterase into a triacylglycerol lipase by a random mutation. *Angew Chem Int Edit* 44:7553–7557
22. Awang R, Basri M, Ahmad S, Salleh AB (2005) *Thermomyces lanuginosus* lipase-catalyzed esterification of 9,10-dihydroxystearic acid and monohydric alcohol. *J Oleo Sci* 54:305–309
23. Plou FJ, Cruces MA, Ferrer M, Fuentes G, Pastor E, Bernabe M, Christensen M, Comelles F, Parra JL, Ballesteros A (2002) Enzymatic acylation of di- and trisaccharides with fatty acids: choosing the appropriate enzyme, support and solvent. *J Biotechnol* 96:55–66
24. Wang YF, Lalonde JJ, Momongan M, Bergbreiter DE, Wong CH (1988) Lipase-catalyzed irreversible transesterifications using enol esters as acylating reagents—preparative enantioselective and regioselective syntheses of alcohols, glycerol derivatives, sugars, and organometallics. *J Am Chem Soc* 110:7200–7205
25. Cao LQ, Bornscheuer UT, Schmid RD (1999) Lipase-catalyzed solid-phase synthesis of sugar esters. Influence of immobilization on productivity and stability of the enzyme. *J Mol Catal B Enzym* 6:279–285
26. Reyes-Duarte D, Lopez-Cortes N, Ferrer M, Plou FJ, Ballesteros A (2005) Parameters affecting productivity in the lipase-catalysed synthesis of sucrose palmitate. *Biocatal Biotransform* 23:19–27
27. Plou FJ, Barandiaran M, Calvo MV, Ballesteros A, Pastor E (1996) High-yield production of mono- and di-oleoylglycerol by lipase-catalyzed hydrolysis of triolein. *Enzyme Microb Technol* 18:66–71
28. Brenes M, Garcia A, Dobarganes MC, Velasco J, Romero C (2002) Influence of thermal treatments simulating cooking processes on the polyphenol content in virgin olive oil. *J Agric Food Chem* 50:5962–5967
29. Ferrer M, Comelles F, Plou FJ, Cruces MA, Fuentes G, Parra JL, Ballesteros A (2002) Comparative surface activities of di- and trisaccharide fatty acid esters. *Langmuir* 18:667–673
30. Adamczak M, Bornscheuer UT (2009) Improving ascorbyl oleate synthesis catalyzed by *Candida antarctica* lipase B in ionic liquids and water activity control by salt hydrates. *Process Biochem* 44:257–261
31. Palma S, Manzo RH, Allemandi D, Fratoni L, Lo Nostro P (2002) Solubilization of hydrophobic drugs in octanoyl-6-*O*-ascorbic acid micellar dispersions. *J Pharm Sci* 91:1810–1816
32. LoNostro P, Capuzzi G, Pinelli P, Mulinacci N, Romani A, Vincieri FF (2000) Self-assembling and antioxidant activity of some vitamin C derivatives. *Colloid Surf A Physicochem Eng Asp* 167:83–93
33. Song QX, Wei DZ, Zhou WY, Xu WQ, Yang SL (2004) Enzymatic synthesis and antioxidant properties of L-ascorbyl oleate and L-ascorbyl linoleate. *Biotechnol Lett* 26:1777–1780
34. Viklund F, Alander J, Hult K (2003) Antioxidative properties and enzymatic synthesis of ascorbyl FA esters. *J Am Oil Chem Soc* 80:795–799