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Enzymatic synthesis and antioxidant properties of L-ascorbyl oleate and L-ascorbyl linoleate

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Abstract

L-Ascorbyl oleate and L-ascorbyl linoleate were synthesized by an immobilized lipase from *Candida antarctica* with yields of 38% and 44%, respectively. L-Ascorbyl oleate was stable in sterile culture medium over 12 h at 37 °C but L-ascorbyl linoleate degraded by 17%. Ascorbyl oleate had a better protective effect on human umbilical cord vein endothelial cells treated with H_2O_2 than of L-ascorbic acid-2-phosphate-6-palmitate (Asc2P6P).

Introduction

L-Ascorbic acid and its derivatives have attracted considerable attention in recent years for preventing oxidative stress related diseases (Leung *et al.* 1993). L-Ascorbic acid has many biological functions and is a scavenger of reactive oxygen species (ROS) (Kiyatkin & Rebec 1998). Its instability and poor liposolubility, however, limit its application diminishing the ROS levels *in vivo*. To cope with this problem, many derivatives of L-ascorbic acid have been synthesized; for example, ascorbic acid-2-phosphate-6-palmitate (Asc2P6P) which was more stable than L-ascorbic acid and thus can better decrease the intracellular ROS concentration (Du *et al.* 2003, Fan *et al.* 2004).

Modification of L-ascorbic acid via esterification or transesterification with aliphatic molecules, such as fatty acids, is a useful way to alter its solubility in oil-based formulae and emulsion. L-Ascorbyl derivatives, such as L-ascorbyl laurate and L-ascorbyl palmitate, were synthesized using immobilized lipase (Humeau *et al.* 1995, Bradoo *et al.* 1999, Yan *et al.* 1999, Thierry *et al.* 2000). Unsaturated fatty acids are regarded as having more beneficial effects on human nutrition than saturated fatty acids (Shlomo *et al.* 2002) and, as alternatives to a saturated fatty acid, we have synthesized two L-ascorbic acid derivatives, L-ascorbyl oleate and L-ascorbyl linoleate, using an immobilized lipase (see also Song & Wei 2002). The biological protection properties of these esters have then been evaluated on human umbilical cord vein endothelial cells.

Materials and methods

Materials

Novozym 435 (Type B lipase from *Candida antarctica*, immobilized on a macroporous acrylic resin) was kindly provided by Novo Nordisk. The purity of oleic acid and linoleic acid was over 90% and L-ascorbic acid was over 99%. L-Ascorbic acid-2-phosphate-6-palmitate (Asc2P6P) was synthesized in our laboratory (Du *et al.* 2003). All of the solvents were HPLC grade.

HPLC analysis

Reactants and products were analyzed by HPLC using a reversed-phase column (Hewlett Packard XDB-C18, 250 mm × 4 mm, 5 μm) with methanol/water/H_3PO_4 (85:15:0.1, by vol.) as eluant at 1 ml min^{-1}. The eluant was monitored at 280 nm.

Procedure for the enzymatic synthesis and purification

A thermostatted tank reactor (60 mm × 40 mm) was continuously stirred with a magnetic bar (20 mm × 5 mm) at 200 rpm. L-Ascorbic acid, 4 mmol, was reacted with 4 mmol oleic acid or linoleic acid in the presence of 300 mg Novozym 435 in 10 ml *t*-amyl alcohol and with 500 mg molecular sieve (nominal pore diameter 4 Å). At the end of the reaction, the biocatalyst was removed by filtration and the reaction mixture was evaporated under reduced pressure. The residue was purified by HPLC using a reversed-phase column (300 mm × 15 mm, filled with Lichroprep RP-18, 40–63 μm, Merck, Germany). Methanol/water (80:20 v/v) was used as the eluant at 2 ml min^{-1} and monitored at 280 nm.

Cell culture and cell viability assays

Human umbilical cord vein endothelial (ECV-304) cells were purchased from Cell Bank of Chinese Academic of Science and were cultured in RPMI Medium 1640 supplemented with 10% (v/v) dialyzed heat-inactivated bovine serum (BS) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

ECV-304 cells were seeded in 96-well plates and incubated in RPMI-1640 medium for 12 h. After being grown to confluence, the cells incubated with 150 μM H_2O_2 for 3 h. The cells were incubated for further 12 h in the medium supplemented with different concentrations of antioxidants. Cell viability was determined by MTT method (Hensen *et al.* 1989, Vladimir *et al.* 1996).

Intracellular reactive oxygen species (ROS) level assays

The cells were first treated with 150 μM H_2O_2 for 3 h. The medium was then changed to serum-free RPMI-1640 medium containing different concentrations of antioxidants. After 12 h, the cultures were rinsed three times with PBS (pH 7.4) and incubated with PBS solution containing 10 μM CDCFH (6-carboxy-2′,7′-dichlorodihydrofluorescein) solution for 4 h. CDCFH taken up into cells was hydrolyzed to become membrane impermeable, and oxidized to the highly fluorescent CDCF primarily by H_2O_2, hydroxyl radicals and diverse peroxides. The supernatant was discarded and PBS (pH 7.4) was added to the plate wells for measurement of the intracellular oxidative stress. The fluorescence intensity was measured with a fluorescence plate reader (Ascent software) with excitation and emission wavelengths of 485 nm and 538 nm, respectively.

Statistical analysis

The data was analyzed by pair *t*-test. The criterion of statistical significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Results shown are means ± SD.

Results and discussion

Enzymatic synthesis and purification of L-ascorbyl unsaturated fatty acid ester

The enzymatic synthesis of unsaturated fatty acid esters of L-ascorbic acid is shown in Figure 1. Maximum yields were after 12 h. The purity of isolated products was determined as over 95% by HPLC and the identities of the products were confirmed by IR, mass spectrometry and ^{1}H-NMR (Song & Wei 2002).

Stability of L-ascorbic acid derivatives

The stability of L-ascorbic acid derivatives is presented in Figures 2 and 3. L-Ascorbyl linoleate and L-ascorbyl oleate were stable in RPMI-1640 medium at 4 °C but not at 37 °C. L-Ascorbyl oleate was stable for 12 h at 37 °C, but L-ascorbyl linoleate decreased to 83% of its initial concentration over this time indicating that the experiment methods used to determine the biological function was not applied to L-ascorbyl linoleate. Asc2P6P is very stable and could be kept in RPMI-1640 medium for 4 days (Fan *et al.* 2004).

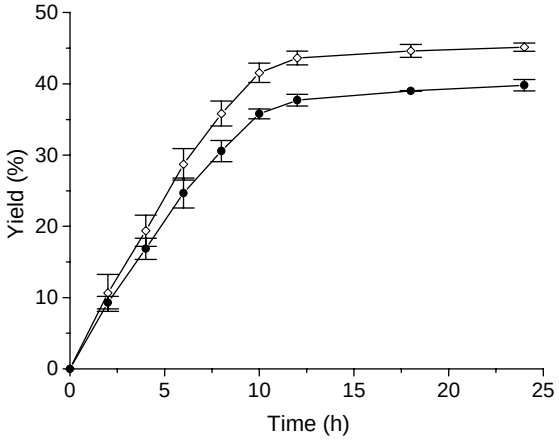

Fig. 1. Time course of enzymatic synthesis of L-ascorbyl linoleate and L-ascorbyl oleate. The reaction was carried out with 0.7 g L-ascorbic acid and 1.13 g oleic acid (●) or 1.12 g linoleic acid (◇), in the presence of 300 mg of Novozym 435 in 10 ml *t*-amyl alcohol and with 500 mg molecular sieves. Data from four independent experiments is shown.

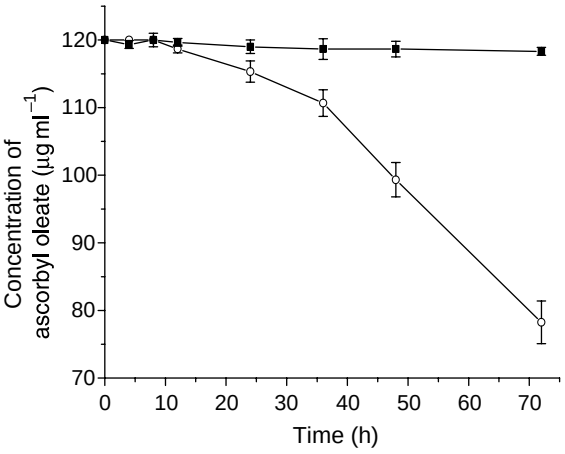

Fig. 2. Stability of L-ascorbyl oleate in RPMI-1640 medium. L-Ascorbyl oleate was dissolved in RPMI-1640 medium at 120 μg ml^{-1}, then incubated at 37 °C, under 5% CO_2 and 95% humidity conditions (○), or placed at 4 °C (■). Its concentration was determined by HPLC. Data shown was each conducted in fourfold, the SD of which is represented.

Protective effects of L-ascorbyl oleate and Asc2P6P on ECV-304 cells treated with H_2O_2

As shown in Figure 4, both L-ascorbyl oleate and Asc2P6P could protect ECV-304 cells against the H_2O_2-induced cytotoxicity. Under the experimental conditions, Asc2P6P did not show any toxicity within the tested concentration range; its protective effect increased with increasing concentration. L-Ascorbyl oleate could protect the cells against

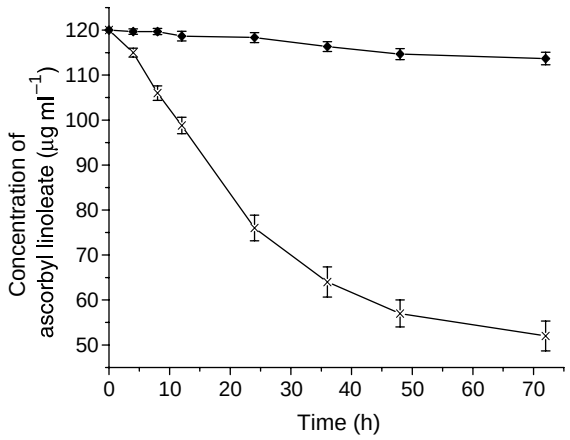

Fig. 3. Stability of L-ascorbyl linoleate in RPMI-1640 medium. L-Ascorbyl linoleate was dissolved in RPMI-1640 medium at 120 μg ml^{-1}, then incubated at 37 °C, under 5% CO_2 and 95% humidity conditions (×), or placed at 4 °C (♦). Its concentration was determined by HPLC. Data shown was each conducted in fourfold, the SD of which is represented.

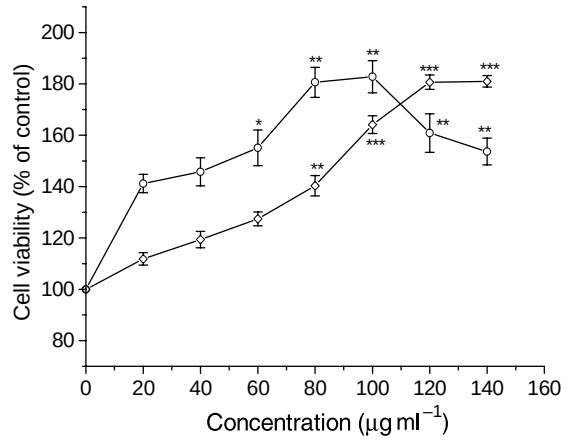

Fig. 4. Effect of antioxidants on the cell viability of the human umbilical cord vein endothelial cells (ECV-304). Confluent cultures in 96-well were treated with 150 μM H_2O_2 for 3 h, then the cells were cultured in RPMI-1640 medium containing different concentrations of L-ascorbyl oleate (○) or Asc2P6P (◇) for 12 h, then measured with MTT method. Data shown was each conducted in fourfold, the SD of which is represented by the vertical bar. The criterion of statistical significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

H_2O_2-induced cytotoxicity at less than 100 μg ml^{-1} and increased cell viability but above 100 μg ml^{-1}, the cell viability declined. L-Ascorbyl oleate above 100 μg ml^{-1} may thus be cytotoxic. Cell viability reached the same maximum (180% of the control) with Asc2p6p at 120 μg ml^{-1} and L-ascorbyl oleate at 80 μg ml^{-1}, thereby suggesting that the antioxidant effect of L-ascorbyl oleate is better than that of Asc2P6P.

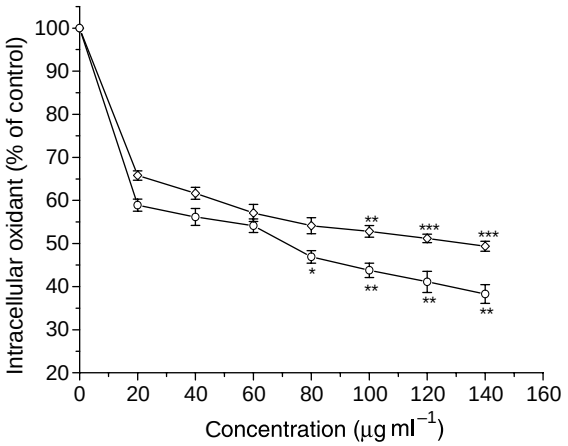

Fig. 5. Effect of antioxidants on the scavenging ROS of the human umbilical cord vein endothelial cells (ECV-304). Confluent cells in 96-well were treated with 150 μM H_2O_2 for 3 h, then the cells were cultured in serum-free RPMI-1640 medium containing different concentrations of L-ascorbyl oleate (○) or Asc2P6P (◇) for 12 h, then measured with CDCFH method. Data shown was each conducted in fourfold, the SD of which is represented by the vertical bar. The criterion of statistical significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Fluorographic analysis of L-ascorbyl oleate and Asc2P6P scavenging ROS

To examine the influence of the antioxidants on its ROS level in ECV-304, CDCFH was quantified as a redox indicator by fluorometry. As shown in Figure 5, the study showed that both L-ascorbyl oleate and Asc2P6P could scavenge ROS. When the cells were treated with L-ascorbyl oleate or Asc2P6P, even or low concentrations, ROS levels decreased but more so with L-ascorbyl oleate than Asc2P6P.

L-Ascorbic acid esters of unsaturated fatty acids are a new category of antioxidants. At present, there are few reports on their enzymatic synthesis or antioxidant effects. Our previous study showed that the yield of L-ascorbyl linoleate was higher than that of L-ascorbyl oleate in the enzymatic synthesis. However, L-ascorbyl oleate was more stable than L-ascorbyl linoleate at 37 °C. The study of protective effects on ECV-304 cells treated with H_2O_2 showed that Asc2p6p at 120 μg ml^{-1} and L-ascorbyl oleate at 80 μg ml^{-1} have similar maximum protection effect suggesting that, on an equal height basis, the antioxidant effect of L-ascorbyl oleate is better than Asc2P6P.

Acknowledgement

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