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RESEARCH PAPER

Lipase Catalyzed Reaction of L-ascorbic Acid with Cinnamic Acid Esters and Substituted Cinnamic Acids

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Abstract To perform the lipase-catalyzed synthesis of L-ascorbic acid derivatives from plant-based compounds such as cinnamic and ferulic acid under mild reaction conditions, the activities of immobilized *Candida ntarctica* lipase with different cinnamic acid esters and substituted cinnamic acids were compared. As a result, immobilized *C. ntarctica* lipase was found to prefer vinyl cinnamic acid to other esters such as allyl-, ethyl-, and isobutyl cinnamic acids as well as substituted cinnamic acids such as *p*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid. Based on these results, large-scale synthesis of 6-*O*-cinnamyl-L-ascorbic acid ester was performed using immobilized *C. ntarctica* lipase in dry organic solvent, resulting in 68% yield (493 mg) as confirmed by ^{13}C-NMR.

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1. Introduction

L-ascorbic acid is one of the most famous and important chemicals since its discovery in the late 1920s. It is very well known as an antioxidant in cosmetics and a beneficial ingredient in foods [1-3]. However, it has shown very low stability in aqueous solution as it easily becomes oxidized. For these reasons, many researchers, especially in the cosmetic industry, have attempted to make stable and soluble L-ascorbic acid derivatives [4,5]. As a result, some successful derivatized molecules, such as 6-*O*-palmitoyl-2-*O*-isopropyloxy carbonyl-L-ascorbic acid and 2-*O*-isopropyloxycarbonyl-L-ascorbic acid, have been synthesized. L-ascorbic acid has even sometimes demonstrated an additive effect [6]. These reactions could be used to increase not only the stability and solubility but also the usability and efficacy of L-ascorbic acid in various human body parts.

In an effort to identify a derivatization partner, we considered cinnamic acid and its substituted compounds, including *p*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid [7]. Since cinnamic acid (CA) and its substituted compounds are well known components in flavoring, perfumes, synthetic indigo, pharmaceuticals, and cosmetics, the sugar ester of CA and its derivatives also have been investigated in medical and pharmaceutical applications [8,9]. It was also reported that CA derivatives have many efficacies in the human body and can be obtained easily and cheaply from natural products such as lignin, which is the second most abundant polymer on earth

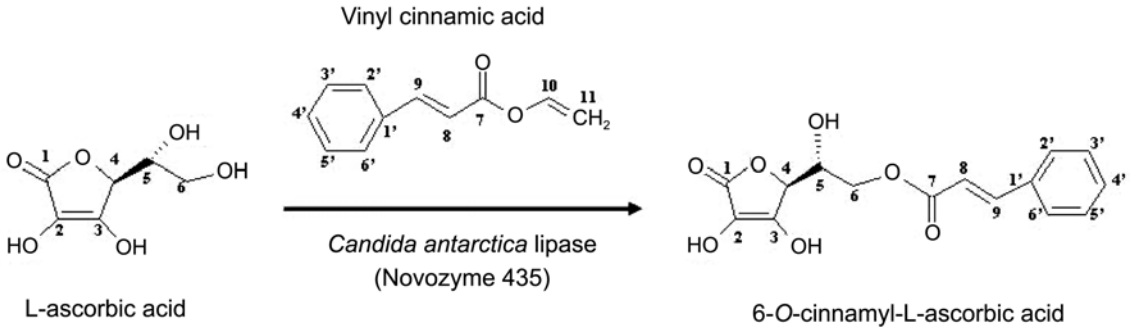


Fig. 1. Synthesis of 6-*O*-cinnamyl-L-ascorbic acid ester with L-ascorbic acid and vinyl cinnamic acid.

[10,11].

In the present study, lipase catalyzed synthesis of L-ascorbic acid derivatives with various CA esters and substituted cinnamic acids were examined. From the results, immobilized *C. ntarctica* lipase (Novozyme 435) with L-ascorbic acid and vinyl CA in dry organic solvents was used to produce 6-*O*-cinnamyl-L-ascorbic acid ester. We show that the resulting product is another good example of a L-ascorbic acid derivative.

2. Materials and Methods

2.1. Materials

Immobilized lipase B from *C. ntarctica* (Novozym 435) was purchased from Novozyme. L-ascorbic acid, vinyl cinnamic acid (CA), 0.4 nm molecular sieves (8 ~ 12 mesh), *tert*-butanol, other esters such as allyl-, ethyl-, and isobutyl cinnamic acids as well as substituted cinnamic acids such as *p*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid were purchased from Sigma (USA).

2.2. Analytical methods

The chemical structures of 6-*O*-cinnamyl-L-ascorbic acid were determined by ^{13}C-NMR (Jeol Ltd.: JNM-EX270 (67.8 MHz)). TLC was used for qualitative analysis of L-ascorbic cinnamic ester on silica gel 60F plates from E. Merck Darmstadt with ethyl acetate/methanol/water (17:2:1, v/v). The spots were detected with UV at 210 nm. CA ester was measured by HPLC using a Waters Symmetric C18 column (250 × 4.6 mm, 5 ìm particle size) with a mobile phase of 40% acetonitrile in water at a flow rate of 1 mL/min using a HPLC diode array detector was set at 210 nm.

2.3. Enzymatic synthesis of 6-*O*-cinnamyl-L-ascorbic acid

The reactions were initiated by the addition of 600 mg of Novozym 435 to 60 mL of 100% *tert*-butanol containing 40 mM ascorbic acid, 100 mM vinyl CA, and 600 mg of molecular sieves [12]. The suspension was stirred at 130 rpm for 6 days at 30ºC, after which the reactions were terminated by filtering off the enzyme. The solvents were then evaporated, and formation of the products was confirmed by TLC. The product was separated by silica gel chromatography with eluent consisting of chloroform/methanol (7:1, v/v) [13].

3. Results and Discussion

3.1. Relative enzyme activities in the presence of L-ascorbic acid and various cinnamic acid esters

Our efforts focused on the derivatization of labile L-ascorbic acid. Initially, the combination of ascorbic acid and cinnamic acid ester was postulated from a previous report on the synthesis of 6-cinnamyl-D-glucuronide and 6-*O*-phenylbutyroyl-L-ascorbic acid using Novozym 435 [9]. Although the synthesis of either compound did not result in high yield (15%), it suggested possible combinations of L-ascorbic acid and cinnamic acid esters. Based on this idea, initial enzyme activity with L-ascorbic acid and vinyl cinnamic ester was determined by TLC with UV irradiation

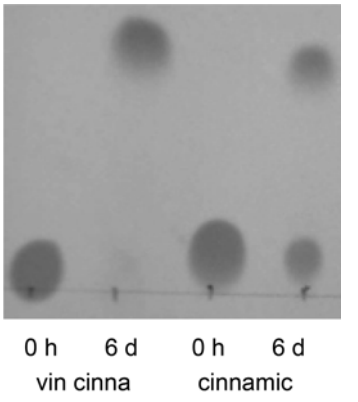
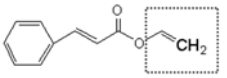
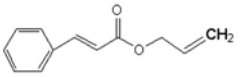
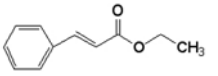
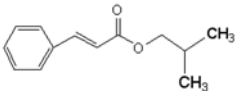


Fig. S1. Synthesis of 6-*O*-cinnamyl-L-ascorbic acid ester monitored by TLC (bottom: L-ascorbic acid, top: 6-*O*-cinnamyl-L-ascorbic acid ester).

Table 1. Relative activities with various cinnamic acid esters

Compound	Structure	Relative activity* (%)
Vinyl cinnamic acid		100%
Allyl cinnamic acid		70%
Ethyl cinnamic acid		32%
Isobutyl cinnamic acid		25%

* Relative activities were compared with that of vinyl cinnamic acid. The conversion ratio from ascorbic acid to each ascorbic ester was determined based on the concentrations of ascorbic acid and products in the reaction mixture. Cinnamic vinyl ester was measured by HPLC using a Waters Symmetric C18 column (250 × 4.6 mm, 5 μm particle size) with a mobile phase of 40% acetonitrile in water at a flow rate of 1 mL/min using a HPLC diode array detector set at 210 nm.

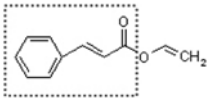
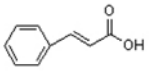
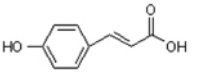
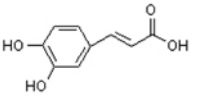
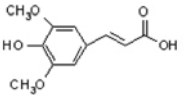
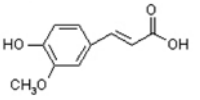
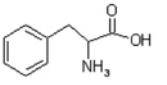
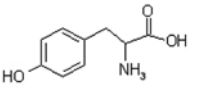
(Supplementary Fig. S1). A spot with R_f = 0.32 was observed between L-ascorbic acid and cinnamic acid (data not shown), which were separated by the conditions explained in the Materials section. The spot was identified as 6-*O*-cinnamyl-L-ascorbic acid by ^{13}C NMR. This compound has never been reported previously. Considering that both compounds are widely applied in the medical and pharmaceutical industries, it could have applications in cosmetics.

To determine the effect of the ester group, four compounds, including vinyl cinnamic acid, allyl cinnamic acid, ethyl cinnamic acid, and isobutyl cinnamic acid, were examined. Vinyl cinnamic acid resulted in the highest activity with L-ascorbic acid, whereas allyl cinnamic acid resulted in the second highest (Table 1). Interestingly, enzyme activity with ethyl cinnamic acid was less than that with allyl cinnamic acid, which has more double bonds. This suggests that length was less a factor than double bonding. Isobutyl cinnamic acid with longer branched chains showed a negative effect, whereas cinnamic acid without an ester group was better than ethyl and isobutyl cinnamic acid in the synthesis of 6-*O*-cinnamyl-L-ascorbic acid (Table 2).

3.2. Relative enzyme activities in the presence of hydroxyl cinnamic acid and substituted cinnamic acids with L-ascorbic acid

Relative enzyme activities with various cinnamic acid derivatives and L-ascorbic acid were compared in order to examine the efficacies of other partners with L-ascorbic acid. Based on the structure of vinyl cinnamic acid, cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, phenylalanine, and tyrosine were examined as candidates (Table 2). As expected, the results for vinyl cinnamic acid and cinnamic acid showed that the vinyl group was very crucial for the reaction. A steep decrease in activity was observed without the vinyl group.

Table 2. Relative activities with substituted cinnamic acids and amino acids

Compound	Structure	Relative activity[a] (%)	R_f of products
Vinyl cinnamic acid		100%	0.32
Cinnamic acid		44%	0.32
p-Coumaric acid		14%	0.32
Caffeic acid		< 5%	0.23
Ferulic acid		9%	0.22
Sinapic acid		0%	N.D[b]
Phenylalanine		< 5%	0.30
Tyrosine		< 5%	0.31

[a] Relative activities were compared with that of vinyl cinnamic acid. The conversion ratio from ascorbic acid to each ascorbic ester was determined based on the concentrations of ascorbic acid and products in the reaction mixture. TLC spots were compared and analyzed by Imagemaster 2D millennium (Amersham Bioscience).
[b] N.D.: not detected.

From the results for cinnamic acid and *p*-coumaric acid, substitution of a hydroxyl group at the *para*-site seemed to greatly affect enzyme activity. Moreover, second and third substitutions also slightly affected enzyme activity, as observed in the results for caffeic and ferulic acid. For synaptic acid, no spot was found. Interestingly, a comparison of cinnamic acid and phenylalanine, which differ only in a C-C double bond and amine group, revealed a large difference in enzyme activity. This might have been partially due to the low solubility of phenylalanine in solvent. However, this low activity was probably due to the enzyme itself when considering early reported results using *Rhizomucor miehei* lipase, which showed great preference for amino acids [14]. R_f values of the products were between 0.2 and 0.35 in comparison with L-ascorbic acid (R_f value below

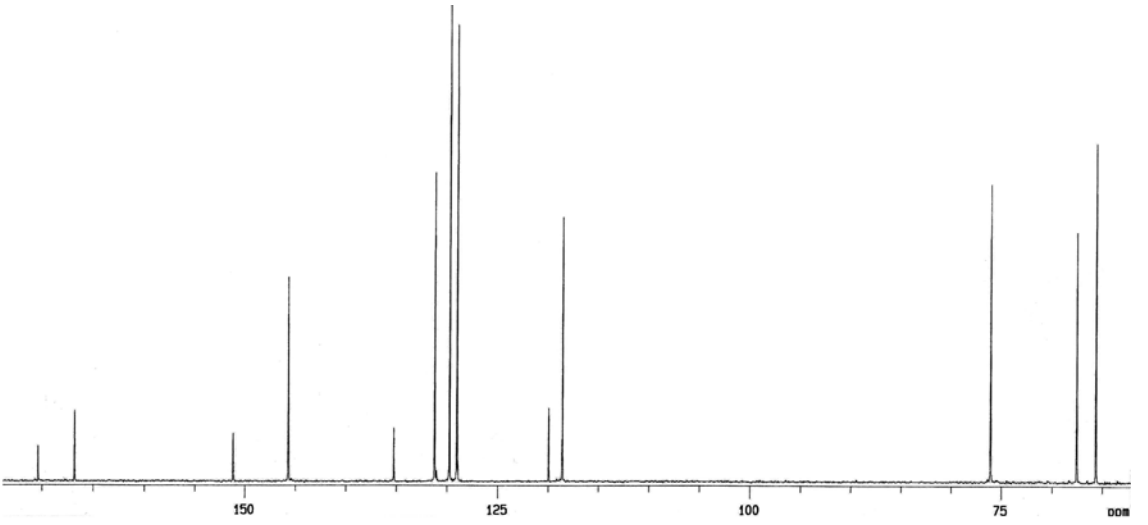


Fig. S2. ^{13}C-NMR of 6-*O*-cinnamyl-L-ascorbic acid.

Table 3. Chemical shifts of ^{13}C-NMR of ascorbic acid and *6-O*-cinnamyl-L-ascorbic acid

	Carbon numbers	L-ascorbic acid	*6-O*-cinnamyl-L-ascorbic acid
L-ascorbic acid	1	170.57	170.24
	2	117.98	117.74
	3	152.89	152.19
	4	74.71	75.11
	5	68.49	65.61
	6	62.09	64.83
Vinyl moiety	10		
	11		
Cinnamic acid moiety	7		165.81
	8		118.11
	9		144.73
	1’		133.87
	2’		128.27
	3’		128.84
	4’		130.4
	5’		128.84
	6’		128.27

0.1) and cinnamic acid (R_f over 0.7). These values were quite different, and the reaction mixture was easily purified by a simple separation method.

3.3. Synthesis of 6-*O*-cinnamyl-L-ascorbic acid

The reaction conditions have already been explained in the Materials and Methods section. The yield was calculated based on the amount of L-ascorbic acid. After the solvent was evaporated off, the product was purified by silica gel chromatography. TLC was used to monitor the purification, which resulted in a yellowish-colored powder. The purified product had a yield of 68% (493 mg) based on the amount of ascorbic acid. ^{13}C-NMR results are shown in Table 1 and Supplementary Fig. S2. In all these reactions, the low solubility of L-ascorbic acid was a problem. Especially, synthesis of 6-*O*-cinnamyl-L-ascorbic acid was carried out even though L-ascorbic acid remained in solution. This type of partial dissolution, which can reduce the reaction time and amount of solvent required, has already been applied to the production of 6-*O*-acylglucose esters [15]. However, before large-scale production and high concentration reactions can be carried out, further studies on the solubility and exact functionality of 6-*O*-cinnamyl-L-ascorbic acid are needed.

4. Conclusion

We initiated to overcome the instability of L-ascorbic acid and give synergistic effects by combining various antioxidants. As a result, the enzyme-catalyzed synthesis of 6-*O*-cinnamyl-L-ascorbic acid with L-ascorbic acid and vinyl cinnamic acid was performed in organic solvent with good yield, and the substrate specificities of cinnamic acid derivatives and amino acids were examined. This constitutes another good example of the functional combination of L-ascorbic acid.

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