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Chemo-enzymatic synthesis of vinyl and L-ascorbyl phenolates and their inhibitory effects on advanced glycation end products

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ABSTRACT

This study successfully established the feasibility of a two-step chemo-enzymatic synthesis of L-ascorbyl phenolates. Intermediate vinyl phenolates were first chemically produced and then underwent *trans*-esterification with L-ascorbic acid in the presence of Novozyme 435® (*Candida Antarctica* lipase B) as a catalyst. Twenty vinyl phenolates and 11 ascorbyl phenolates were subjected to *in vitro* bioassays to investigate their inhibitory activity against advanced glycation end products (AGEs). Among them, vinyl 4-hydroxycinnamate (**17VP**), vinyl 4-hydroxy-3-methoxycinnamate (**18VP**), vinyl 4-hydroxy-3,5-dimethoxycinnamate (**20VP**), ascorbyl 4-hydroxy-3-methoxycinnamate (**18AP**) and ascorbyl 3,4-dimethoxycinnamate (**19AP**) showed 2–10 times stronger inhibitory activities than positive control (aminoguanidine and its precursors). These results indicated that chemo-enzymatically synthesized compounds have AGE inhibitory effect and thus are effective in either preventing or retarding glycation protein formation.

1. Introduction

Glycation is involved in structural and functional alterations of proteins and other cellular components. It is a process that occurs ubiquitously during normal aging, but is accelerated in a diabetic disease state. The final products of the glycation reaction are referred to as advanced glycation end products (AGEs); these fluorescent compounds have been suggested to be a potential cause of diabetes (Abraham, Swamy, & Perry, 1989). The increase in formation of AGEs is a major source of complications related to diabetes (Bonnefont-Rousselot, 2002). Both diabetes and aging are associated with accumulation of AGEs in tissue, increased oxidative stress and decline in antioxidant status. AGE accumulation has been implicated in various conditions such as retinopathy, cataracts, neuropathy, and chronic kidney disease. Intensive treatment to prevent oxidative stress may be a successful strategy for preventing diabetes-related complications.

Polyphenols are secondary plant metabolites and are abundant in fruits, vegetables, and natural beverages; these are thought to reduce the risk of chronic disease. They are an integral part of the human diet and widespread in natural plants and plant products (Pietta, Mauri, Simonetti, & Testolin, 1995). Various derivatives (i.e., phenolic acid, flavonoid, and anthocyanin) have been reported to have beneficial antioxidant effects in diabetes and its complications. The flavonoids isolated from the fruit of *Phellinus linteus* and *Colocasia esculenta* are reported to inhibit AGEs and confer an antioxidant effect (Lee et al., 2008a, 2008b; Li, Hwang, Kang, Hong, & Lim, 2014). Caffeic acid ethylene ester (vinyl caffeate) isolated from *Prunella vulgaris* displayed therapeutic potential in the prevention and treatment of diabetic complications by inhibiting both AGEs and aldose reductase (Li et al., 2012). These results suggest that vinyl phenolates and other phenolate esters could be good candidates for the development of a therapeutic anti-AGE agent.

L-ascorbic acid has long been considered an excellent natural antioxidant in cosmetics as well as a necessary and an important component of diet. It is a water-soluble vitamin and an essential nutrient. L-ascorbic acid is a strong reducing agent and acts as a primary defense against aqueous free radical attack in the blood (Na et al., 2006). Its' function has been widely studied both *in vitro* and *in vivo*, and its role in reducing oxidative stress is of particular interest in various disease states (including diabetes and related complications) (Dracha, Narkiewicz-Michałeka, Sienkiewicza, Szymulaa, & Bravo-Diazb, 2011). Various studies have reported the synthesis of ascorbyl esters catalyzed by lipases in organic solvent systems (Humeau, Girardin, Rovel, & Miclo, 1998) and solid-phase systems (Yan, Bornscheuer, & Schmid,

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1999). For instance, lipase-catalyzed synthesis of L-ascorbyl esters of varied fatty acids is widely reported, although many of the reports have focused specifically on the synthesis of L-ascorbyl-palmitate/linoleate/laurate/oleate. Another area of ongoing research involves L-ascorbyl-palmitate synthesized by *Candida antarctica* and *Bacillus stearothermophilus* SB1 lipase, which may have anticancer effects. This technique has also been successfully used to synthesize derivatives of L-ascorbic acid, with applications in artificial flavoring, cosmetics and as monosaccharides (Claon & Akoh, 1994; Tokiwa, Kitagawa, Raku, Yanagitanib, & Yoshino, 2007; Seino, Seino, Uchibori, Nishitani, & Inamasu, 1984). In this study, we attempted to synthesize phenolate esters, which are the esters of phenolic acids and L-ascorbic acid.

To expand upon previous research, we have attempted chemo-enzymatic *trans*-esterification of phenolic acids with L-ascorbic acid and evaluation using AGEs. To the best of our knowledge, the structure activity relationship (SAR) studies of vinyl and L-ascorbyl phenolates synthesized via chemo-enzymatic transformation of L-ascorbic acid and the AGE-inhibitory activity of phenolic acid have not been reported thus far in the literature. The SAR studies of synthetic compounds were analyzed, in order to assess their potential for further development for use in the food and pharmaceutical industries.

2. Materials and methods

2.1. Apparatus and reagents

Lipase acrylic resin from *Candida antarctica* (Novozyme 435®, 5 U/mg) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). One unit represents the microequivalents of fatty acid hydrolyzed from a triglyceride in 1 h at pH 7.2 at 37 °C. Salicylic acid (**1P**), 4-hydroxybenzoic acid (**2P**), 2,5-dihydroxybenzoic acid (**3P**), vanillic acid (**4P**), syringic acid (**5P**), 3,4-dimethoxybenzoic acid (**6P**), 4-chlorophenylacetic acid (**7P**), 4-hydroxyphenylacetic acid (**8P**), 3-phenylpropanoic acid (**9P**), 4-phenylbutyric acid (**10P**), 5-phenylvaleric acid (**11P**), 3-(4-hydroxyphenyl) propionic acid (**12P**), 3-(3,4-dihydroxyphenyl) propanoic acid (**13P**), cinnamic acid (**14P**), 2-hydroxycinnamic acid (**15P**), 3-hydroxycinnamic acid (**16P**), 4-hydroxycinnamic acid (**17P**), 4-hydroxy-3-methoxycinnamic acid (**18P**), 3,4-dimethoxycinnamic acid (**19P**), 4-hydroxy-3,5-dimethoxycinnamic acid (**20P**), vinyl acetate, molecular sieve (MS), *tert*-butanol (*t*-BuOH), L-ascorbic acid (AA), aminoguanidine hydrochloride (AG), quercetin (QC), methylglyoxal (40% aqueous solution), bovine serum albumin (essentially fatty acid free), D-gluconolactone, *N*-acetyl-glycyl-lysine methyl ester acetate salt (G.K. peptide), D-ribose and silica gel 60 F254 glass plates (0.25 mm thick, 20 × 20 cm) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Sulfuric acid was purchased from Daejung Chemicals & Metals Co., Ltd. (Siheung, Korea). All solvents and other reagents used in this study, unless otherwise specified, were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

2.2. Preparative synthesis of vinyl and ascorbyl phenolates

Twenty vinyl phenolates (**VP**), including vinyl salicylate (**1VP**), vinyl 4-hydroxybenzoate (**2VP**), vinyl 2,5-dihydroxybenzoate (**3VP**), vinyl vanillate (**4VP**), vinyl syringate (**5VP**), vinyl 3,4-dimethxoybenzoate (**6VP**), vinyl 4-chlorophenylacetate (**7VP**), vinyl 4-hydroxyphenylacetate (**8VP**), vinyl 3-phenylpropanoate (**9VP**), vinyl 4-phenylbutyrate (**10VP**), vinyl 5-phenylvalerate (**11VP**), vinyl 3-(4-hydroxyphenyl) propionate (**12VP**), vinyl 3-(3,4-dihydroxyphenyl) propanate (**13VP**), vinyl cinnamate (**14VP**), vinyl 2-hydroxycinnamate (**15VP**), vinyl 3-hydroxycinnamate (**16VP**), vinyl 4-hydroxycinnamate (**17VP**), vinyl 4-hydroxy-3-methoxycinnamate (**18VP**), vinyl 3,4-dimethoxycinnamte (**19VP**) and vinyl 4-hydroxy-3,5-dimethoxycinnamate (**20VP**) were chemically synthesized and purified as previously described (Wang, Hwang, & Lim, 2015). The ascorbyl phenolates were synthesized via a two-step chemo-enzymatic route, wherein vinyl esters were produced as intermediates, which were subsequently esterified with L-ascorbic acid (AA). The vinyl esters (0.5 mM) and 5 mL of the selected organic solvents were added to 10 mL vials for reaction with AA (2 mM); this reaction was catalyzed by immobilized *Candida antarctica* lipase (Novozyme 435®). The 18 organic solvents screened for testing are listed in Table 1. The solutions were incubated at 55 °C with stirring for 48–168 h. The reaction progress was monitored regularly by TLC with methylene chloride/methanol elution (7:3, v/v). At the end of the reaction, the enzyme was filtered and the solvent was evaporated. The resulting solid residue was purified using silica gel chromatography with methylene chloride/methanol (9.7:0.3–8.5:1.5, v/v) as the eluent, to yield 11 ascorbyl phenolates (**APs**).

Table 1
Effects of solvents on conversion yield of L-ascorbyl cinnamate.

Solvent	LogP[a]	Yield (%)[b]
Methnol	−0.74	ND[c]
Acetonitrile	−0.33	6.49
Ethanol	−0.3	ND
Acetone	0.23	8.17
n-Propanol	0.25	ND
Tetrahydrofuran	0.49	10.21
tert-Butanol	0.6	12.12
Ethyl acetate	0.65	3.67
n-Butanol	0.8	4.93
Diethyl ether	0.89	5.54
4-Methyl-2-pentanone	1.31	6.71
n-Pentanol	1.34	4.74
Chloroform	1.97	3.22
Benzene	2.13	2.39
2-Methyl-butane	3.1	3.55
n-Pentane	3.45	4.63
n-Hexane	3.5	7.86
Isooctane	4.37	9.88

*The reactions were carried out at 50 °C. Vinyl cinnamate (0.5 mM) and L-ascorbic acid (2.0 mM) with 30 mg of *Candida Antarctica* (Novozyme 435®) 1500 Unit (60 mg/mL) in different solvents (5 mL) for 24 h.
[a] LogP, where P is the partition coefficient of a given solvent between water and *n*-octanol, is a parameter to describe solvent hydrophobicity.
[b] The conversion yield is the ratio of the amount of product actually obtained to the maximum amount of product possible.
[c] Not determined.

The percentage yield of the reaction was calculated as the ratio of the obtained amount of product to the maximum theoretical amount, multiplied by 100%. The structures of the isolated **APs** were confirmed by ^{1}H and ^{13}C NMR. ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz in $(CD_3)_2SO$, using a Bruker Avance 400 NMR spectrometer (Karlsrube, Germany) operating at 400 and 100 MHz, respectively. Chemical shifts are expressed in ppm, using tetramethylsilane (TMS) as an internal standard.

2.3. Hemoglobin-δ-gluconolactone assay on the amadori compound formation (early stage)

Evaluation of initial stage of protein glycation was determined by δ-gluconolactone assay (Rahbar, Yernini, Scott, Gonzales, & L1alezari, 1999). Briefly, fresh human blood (50 mg/mL) was incubated with glucose (144 mg/mL) in phosphate buffer (pH 7.4) containing 0.2 g/L NaN_3 under sterile, dark conditions at 37 °C for 7 days. In certain experiments, the indicated sample was added to the model system in the concentration range of 0.01–1 mM. Fluorescence of samples was measured at the excitation and