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Chemoenzymatic Synthesis of Phytosteryl Ferulates and Evaluation of Their Antioxidant Activity

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ABSTRACT: The feasibility of a two-step chemoenzymatic synthesis of phytosteryl ferulates was successfully established in this work. An intermediate vinyl ferulate was first chemically produced and subsequently esterified with phytosterols through alcoholysis with *Candida rugosa* as a catalyst. The structures of phytosteryl ferulates were confirmed by Fourier transform infrared (FTIR) and high-performance chromatography−mass spectrometry/mass spectrometry (HPLC-MS/MS) using atmospheric pressure chemical ionization (APCI) under both positive and negative ion modes. The antioxidant activity of phytosteryl ferulates was higher than that of the starting material and the intermediate in the assays employed. The results indicated that phytosteryl ferulates had a good potential to be used as food antioxidants and may also serve as cholesterol-lowering agents.

KEYWORDS: chemoenzymatic synthesis, phytosteryl ferulates, antioxidant activity, ORAC

INTRODUCTION

There is increasing interest in research for antioxidants, especially naturally existing ones, due to their noteworthy health benefits in preventing damage from reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive chlorine species (RCS) in the human body as well as concerns over possible carcinogenic effects of synthetic antioxidants in foods.[1] In this relation, various phytosterols have attracted the attention of food and medical scientists due to their perceived benefits in reducing blood cholesterol. Phytosterols occur in plants as free and conjugated forms, that is, phytosteryl esters of fatty or phenolic acids, phytosteryl glycosides, and acylated phytosteryl glycosides.[2] Most of the research has focused on free phytosterols and phytosteryl esters of fatty acids; little is related to the phytosteryl esters of phenolic acids.

Ferulic acid is a ubiquitous plant constituent found naturally in plant cell walls, leaves, and seeds. Oats, brown rice, whole wheat, peanuts, apples, pineapples, and some other fruits and herbs are good sources of ferulic acid and its derivatives. It is the most abundant hydroxycinnamic acid derivative in the plant kingdom and occurs in both free and conjugated forms. Much of the ferulic acid occurs as esters in many plants. It is covalently conjugated with mono- and disaccharides, plant cell wall polysaccharides, glycoproteins, lignins, β-cyanins, and other insoluble cell wall carbohydrate biopolymers. Ferulic acid exhibits a wide range of therapeutic effects against various diseases such as cancer, diabetes, and cardiovascular and neurodegenerative ailments.[3] A wide spectrum of beneficial activities for human health has been advocated for this phenolic compound, at least in part because of its strong antioxidant activity. Due to its phenolic nucleus and an extended side-chain conjugation, it readily forms a resonance-stabilized phenoxyl radical, which accounts for its potent antioxidant potential. Anticarcinogenic activity of ferulic acid has also been reported.[4]

Phytosteryl ferulates, as a major component of γ-oryzanol, have been extensively studied and shown several biological activities, including antioxidant, serum cholesterol lowering, anti-inflammatory, and antitumor properties. Chemical processes have successfully been developed to synthesize phytosteryl ferulates. However, these involve using harsh chemicals, isolating highly reactive intermediate products, and protection and deprotection. Kondo et al.[5] found that *trans*- and *cis*-feruloyl phytosterols were the major ovulatory active compounds in crops of Job's tears and successfully isolated *trans*-feruloyl stigmastanol and *trans*-feruloyl campestanol. In the same study, they also reported a method to synthesize *trans*-feruloyl stigmastanol using 4-*O*-acetylferuloyl chloride as the acylating agent. The method has also been described in a U.S. patent, "fertility drug and method of producing the same", disclosed by Kondo et al.[6] However, the limitations of this method are (1) the required preparation of *trans*-4-*O*-acetylferuloyl chloride, which is difficult to purify and handle due to its high reactivity, and (2) the deprotection step that uses sodium borohydride to remove the protective acetyl group on the *trans*-feruloylated product. An improved method reported by Condo et al.,[7] instead of employing highly reactive *trans*-4-*O*-acetylferuloyl chloride, used *trans*-4-*O*-acetylferulic acid by acetylation of ferulic acid with acetic anhydride/pyridine, followed by condensation of *trans*-4-*O*-acetylferulic acid with phytostanol in the presence of *N*,*N*-dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine. Finally, selective deacetylation of the feruloyl acetate was achieved. Both methods used harsh chemicals and required isolation of highly reactive intermediates and protection and deprotection of the hydroxyl group in ferulic acid. To avoid these problems, enzymatic methods that use lipase may be developed to make the synthesis of this group of compounds more environmentally friendly, economical, and workable, especially for possible industrial production. Guyot and co-workers[8] reported, for the first time, enzymatic esterification of phenolic acids and alcohols with lipase from *Candida antartica* lipase B. Meanwhile, Buisman et al.[9] studied the esterification of cinnamic acid and some

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benzoic acid derivatives with fatty alcohols with various chain lengths of 4−12 carbon atoms (C4−C12). Vinyl esters were selected as acyl donors in this study because they have previously been shown to be useful for lipase-catalyzed preparation of enantiomerically pure compounds.[10] The vinyl alcohol formed during the transesterification reaction tautomerizes to acetaldehyde, thus making the process irreversible.[11] Vinyl esters have been reported as acylating agents for enzymatic synthesis of menthyl acetate and primary terpenyl esters.[12,13]

To the best of our knowledge, chemoenzymatic synthesis of phytosteryl ferulates has not yet been reported in the literature. The objective of this work was to develop a novel chemoenzymatic method for the synthesis of phytosteryl ferulates and to evaluate their antioxidant activity.

MATERIALS AND METHODS

Chemicals. Phytosterols used in this study were from Forbes Medi-Tech Inc. (Vancouver, BC, Canada). They contained 75.6% sitosterol, 12.2% sitostanol, 8.1% campesterol, and 4.1% other minor phytosterols. Ten lipases from different commercial sources were used in this study. Novozyme 435 (lipase acrylic resin from *C. antarctica*) and *Mucor miehei* (Lipozyme-1M) were procured from Novo Nordisk (Franklinton, NC). Three lipases were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada), which included Amano lipase from *Pseudomonas fluorescens*, Amano lipase PS from *Burkholderia cepacia*, and lipase from *Candida rugosa* type VII. Five lipases were obtained from Toyobo Co., Ltd. (Osaka, Japan), including lipoprotein lipase (LPL) 311, LPL 314, cholesterol esterase (COE) 301, COE 311, and COE 313. Randomly methylated anhydrous monosodium phosphate, anhydrous disodium phosphate, randomly methylated cyclodextrin (RMCD), Trolox, fluorescein sodium salt, 2,2′-azobis(2-aminopropane) dihydrochloride (AAPH), and ferulic acid were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Ground pork was purchased from a local supermarket. All other chemicals and solvents, unless otherwise specified, were purchased from Sigma-Aldrich Canada.

Chemical Synthesis of Vinyl Ferulate. Vinyl ferulate was chemically synthesized via the vinyl interchange reaction of vinyl acetate and ferulic acid according to the method described by Swern and Jordan[14] and Gao et al.[15] Ferulic acid (0.01 mol), vinyl acetate (0.16 mol, 15 mL), mercury acetate (4%, w/w), and tetrahydrofuran (THF, 10 mL) were added into a 125 mL Erlenmeyer flask. The mixture was stirred with a magnetic stirrer under a blanket of nitrogen for 30 min, and then 2 μL of sulfuric acid (0.04 mmol) was added to start the reaction. The reaction mixture was flushed again with nitrogen and then placed into a 40 °C shaking water bath (200 rpm). After 12 h, an excess of sodium acetate (20 mg) was added to neutralize the sulfuric acid and to stop the reaction. The solvent and the excess of vinyl acetate were removed, using a rotary evaporator (Büchi, Flawil, Switzerland) at 40 °C. The residue obtained was subjected to column chromatography on silica gel using an isocratic elution with hexane/ethyl acetate (4:1, v/v). Thin layer chromatography (TLC) was carried on Baker-flex silica gel IB-F (2.5 × 7.5 cm) precoated flexible TLC sheets (J. T. Baker, Phillipsburg, NJ). Products were visualized under UV light at 254 nm using Spectroline (Spectronics Corp., New York, NY).

NMR Analysis of Ferulic Acid and Vinyl Ferulate. Proton nuclear magnetic resonance (^{1}H NMR) analysis of purified vinyl ferulate was carried out to confirm the formation of vinyl esters. NMR spectra of pure ferulic and isolated vinyl ferulate were recorded using a Bruker Avance 500 spectrometer (Bruker Biospin Co., Billerica, MA). Proton spectra were recorded at 500 MHz using a solvent field lock. The samples were dissolved in deuterated DMSO (DMSO-d_6) containing tetramethylsilane (TMS) as internal standard. Signal processing and interpretation were performed with the software Topspin 1.3 (Bruker Biospin Co.) and MestRe Nova (Mestrelab Research SL, Santiago De Compostela, Spain). Chemical shifts were expressed in δ (parts per million) values relative to TMS as internal reference. Structure elucidation was accomplished by comparing the chemical shifts of vinyl ferulate with those of the parent ferulic acid.

Screening of Enzymes for Synthesis of Phytosteryl Ferulates. Vinyl ferulate (0.015 g) was placed into a test tube together with 0.06 g of phytosterol mixture (mole ratio of vinyl ferulate to phytosterol mixture, 1:2) and different lipases (8% of the total weight of both substrates). Three milliliters of solvent (hexane/2-butanone, 9:1, v/v) was added to the test tube, which was then flushed with nitrogen before being sealed with a screw cap. The reaction mixture was then shaken in a gyrotory water bath shaker at 200 rpm and 45 °C (New Brunswick Scientific Co., Inc., New Brunswick, NJ). The 10 enzymes tested for screening of the best enzyme for the synthesis of phytosteryl ferulates are listed under Chemicals. The reaction was monitored by using Baker-flex silica gel IB-F (2.5 × 7.5 cm) precoated flexible TLC sheets (J. T. Baker) with eluents of hexane/ethyl acetate (80:20, v/v). After 10 days, the reaction was stopped by placing the tubes under the running tap water. The enzyme was filtered through a filter paper (Fisherbrand P5). Solvent was evaporated using a rotary evaporator (Büchi) at 40 °C, and the solid residue was subjected to column chromatography. The structures of the phytosteryl ferulates were then elucidated by using Fourier transform infrared (FTIR) and high-performance chromatography−mass spectrometry/mass spectrometry (HPLC-MS/MS).

Colum Chromatographic Separation of Phytosteryl Ferulates. In a typical example, reaction mixture (5 g) was dissolved in a minimum amount of *n*-hexane/ethyl acetate (4:1, v/v) and applied to a column (40 cm × 5 cm i.d.) packed with silica gel (Selecto Scientific, Suwannee, GA) as a slurry in *n*-hexane. The column was subsequently eluted with *n*-hexane/ethyl acetate (4:1, v/v). The fractions were collected in test tubes. Baker-flex silica gel IB-F (2.5 × 7.5 cm) precoated flexible TLC sheets (J. T. Baker) were used to monitor the different fractions.

HPLC-MS/MS Analysis of Phytosteryl Ferulates. The identities of phytosteryl ferulates were confirmed by HPLC-MS/MS with atmospheric pressure chemical ionization (APCI) using both negative ion (NI) and positive ion (PI) modes. The analysis was performed using an Agilent 1100 HPLC-MSD system (Agilent, Palo Alto, CA) with an online solvent degasser, binary solvent delivery system, autosampler, and UV−vis diode array detector (DAD). The MS detector has atmospheric pressure chemical ionization capability with a mass range of m/z 50−3000.

Separation was achieved on a C18 column (250 mm length, 4.6 mm i.d., 5 μm particle size, Sigma-Aldrich Canada Ltd.) coupled with a guard column. Phytosteryl ferulates were eluted using an isocratic solvent system containing methanol/water (95:5, v/v) at a flow rate of 1.0 mL/min. Fifty microliters of sample was injected into the system. Phytosteryl ferulates were detected at 325 nm by UV detection. LC flow was analyzed online by a mass selective detector system (LC-MSD-Trap-SL, Agilent) with both positive and negative ion APCI. The operating conditions used were 121 V for the fragmentor voltage, 350 °C for drying temperature, 400 °C for APCI temperature, 60 psi for the nebulizer pressure, and 7 L/min for the drying gas flow.

FTIR Analysis of Phytosteryl Ferulates. The above purified phytosteryl ferulates were characterized using a Fourier transform infrared spectrometer. The IR spectra were recorded using a FTIR Bruker Tensor 27 spectrometer (Bruker Optik GmbH, Ettlingen, Germany). This spectrometer has a spectral range of 7500−370 cm^{-1} and is equipped with a MIRacle attenuated total reflectance (ATR) accessory allowing rapid and easy analysis of liquid and solid samples.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The ORAC assay for lipophilic antioxidant described by Madhujith and

Shahidi[16] was adapted with modifications. The determination of ORAC was carried out using a Fluostar Optima plate reader (BMG Labtech, Durham, NC) equipped with an incubator and injector pump. Fluorecsein was used as the probe, and AAPH was employed as the radical generator. The reaction was carried out in a 96-well Costar black plate (Corning Inc., Corning, NY) with a final reaction mixture of 275 μL. Twenty microliters of sample was dissolved in 7% (w/v) randomly methylated β-cyclodextrin (RMCD) in acetone/water (1:1, v/v), and 200 μL of fluorescein (0.11 mM in distilled water) was injected manually into each well. The mixture was incubated for 20 min at 37 °C in the built-in incubator, and subsequently 75 μL of AAPH solution (63.4 mM in PBS), equilibrated at 37 °C, was rapidly injected into the wells using the injector pump. The plate was shaken for 4 s after each addition at 4 mm shaking width. Other conditions for Fluostar Optima plate reader were set as follows: cycle no., 25; cycle time, 210 s; position delay, 0.3 s; shaking, 8 s; orbital, 4 mm width; shaking before each cycle; injection, pump 1; and speed, 420 μL/s. To optimize signal amplification to obtain maximum sensitivity, gain adjustment was performed at the beginning of each measurement. No more than 36 wells of the 96-well plate were used due to increased cycle time. Fluorescence was determined and recorded, and the antioxidant activity of test compounds was calculated as Trolox equivalents using a standard curve prepared in the concentrations range of 6.25−50 μM Trolox in 7% (w/v) RMCD in acetone/water (1:1, v/v) and blank 7% (w/v) RMCD in acetone/water (1:1, v/v), fluorescein, and AAPH. Filters with an excitation wavelength of 485 nm and an emission wavelength of 520 nm were used.

Antioxidant Activity in Cooked Ground Meat Model System. A cooked ground meat model system was employed for assessing the antioxidant activity of phytosteryl ferulates. Fresh ground pork (40 g) and deionized water (10 mL) were mixed in a 200 mL Mason jar as described by Shahidi and Alexander.[17] Samples and the reference antioxidant compound (butylated hydroxyanisole, BHA), dissolved in ethanol, were added to meat at a level of 100 μmol/kg. A control without any antioxidant was also prepared. The mixture was thoroughly homogenized with a glass rod and cooked at 80 °C in a thermostated water bath for 40 min with intermittent stirring. The cooked meat was cooled to room temperature and homogenized with a glass rod again. The homogenate was then transferred into plastic bags and stored at 4 °C for 14 days. The meat samples were taken on days 0, 3, 5, 7, and 14 for measurement of thiobarbituric acid reactive substances (TBARS) values as an indicator of the formation of secondary oxidation products.

TBARS values were determined as described by Shahidi and Pegg.[18] A series of 1,1,3,3-tetramethoxypropane (TMP) standard solutions at different concentrations was mixed with thiobarbituric acid (TBA) in screw-capped tubes and heated in boiling water bath for 45 min. After cooling to room temperature, the absorbance at 532 nm was recorded and a standard curve was constructed (absorbance versus concentration). For determination of TBARS in the cooked meat model system, 2 g of meat was mixed with 5 mL of trichloroacetic acid (TCA, 10% in deionized water) in a centrifuge tube, followed by the addition of 5 mL of the TBA reagent (0.02 M in deionized water). The mixture was centrifuged at 3000*g* for 10 min, and the supernatant was filtered using a Fisher brand P5 filter paper. The filtrate was heated in a boiling water bath for 45 min, and the absorbance was read at 532 nm using a Hewlett-Packard 8452A diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) after cooling to room temperature. TBARS values in meat samples were calculated using the standard curve as micromoles of malonaldehyde equivalents per kilogram of pork. All experiments were carried out in triplicate.

Statistical Analysis. One-way analysis of variance (ANOVA) with comparisons for all pairs using Turkey−Kramer HSD were used to determine differences in mean values on the basis of data collected from replication of various experiments. Significance was determined at a 95% level of probability. All statistical analyses were carried out using software JMP version 6.0.0 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Preparation of Vinyl Ferulate. The enzymatic synthesis of vinyl ferulate using ferulic acid and vinyl acetate as substrates and employing different lipases as catalysts was attempted in this study (data not shown). Ten commercially available lipases were used; however, none of them led to successful tranesterification between ferulic acid and vinyl acetate. This may be due to the inhibition of enzyme activity by both ferulic acid and vinyl acetate. Therefore, synthesis of vinyl ferulate was achieved through a chemical pathway (Figure 1). The yield of the product after column chromatographic separation of vinyl ferulate was 46%. The low yield of vinyl ferulate may be due to formation of byproduct and some loss of the product during flash column chromatographic separation. A similar yield of vinyl ferulate, at 45−61%, was reported by Gao et al.[15] and Nyaradzo et al.[19] The yield of vinyl ferulate may be enhanced by optimization of the reaction and purification conditions. However, the primary objective of this work was to assess the feasibility of enzymatic synthesis of phytosteryl ferulate rather than to maximize the yield of the intermediate. The R_f values of ferulic acid and vinyl ferulate were 0.07 and 0.27, respectively. The synthesized vinyl ferulate had a relatively higher hydrophobicity compared with ferulic acid as indicated by its higher R_f value.

It has been reported that the electron-donating effect of the *p*-hydroxyl group leads to a reduction of the reactivity of the electrophilic carbon center of carboxylic group, especially when the side chain on the aromatic ring is unsaturated.[9,20,21] Modification of ferulic acid and its conversion to the corresponding vinyl esters was favored in the alcoholysis reactions with phytosterols. This may be explained by facile access of vinyl esters to the active site of the enzyme and formation of the intermediate adduct, which favors alcoholysis with phytosterols. In contrast, the carboxylic acid group in the original ferulic acid together with the hydroxyl groups on the aromatic ring may inhibit enzyme activity for their reaction with phytosterols. The modification may also render them different chemical and biological properties, for example, antioxidant activity.

The structure of the synthesized vinyl ferulate was confirmed by ^{1}H NMR spectroscopy after its purification by flash column chromatography. DMSO-d_6 was used as a solvent for NMR studies as it is an excellent solvent for ferulic acid and vinyl ferulate and the chemical shift of phenolic hydroxyl protons in this solvent is characteristic and proton exchange is slow. To observe the phenolic hydroxyl proton signals by ^{1}H NMR it is required that their rate of exchange with the solvent be sufficiently slow. This can be achieved by using an organic solvent such as DMSO-d_6.[22] The strong hydrogen bonds formed between DMSO-d_6 and phenolic hydroxyl protons can retard the exchange and thus give isolated and narrow NMR signals for phenolic hydroxyl protons.[23] On studies of lignin, the chemical shift for phenolic hydroxyl proton was mainly at δ 8.0−9.4 when DMSO-d_6 was used as a solvent.[24] The ^{1}H NMR data for ferulic acid and vinyl ferulate are presented below.

Ferulic Acid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.11 (1H, COO<u>H</u>), 9.53 (1H, O<u>H</u>), 7.50 (1H, O═C−CH═C<u>H</u>−), 7.28 (1H, ferulic acid Ar), 7.08 (1H, ferulic acid Ar), 6.80 (1H, ferulic acid Ar), 6.38(1H, O═C—C<u>H</u>═CH−), and 3.82(3H, −O—C<u>H</u>$_3$).

Vinyl Ferulate. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.71 (1H, O<u>H</u>), 7.72 (1H, O═C−CH═C<u>H</u>−), 7.38 (1H, ferulate Ar), 7.34 (1H, C—O—C<u>H</u>═CH$_2$), 7.17, 6.81 (1H, ferulate Ar), 6.54 (1H, ferulate Ar), 6.46 (1H, O═C—C<u>H</u>═CH−), 4.95 (1H,

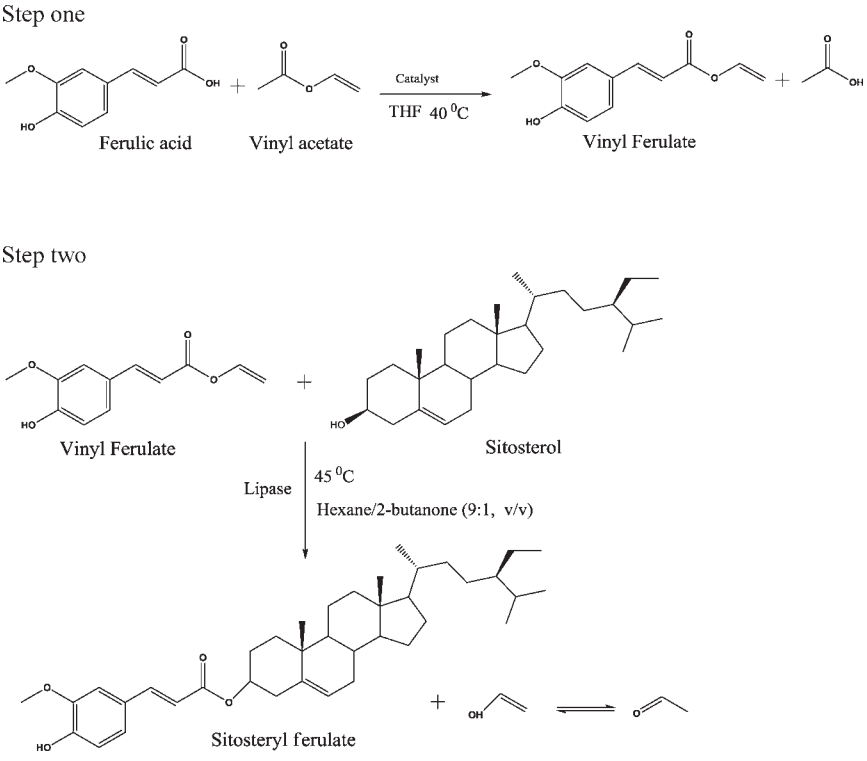

Figure 1. Two-step chemoenzymatic synthesis of phytosteryl ferulates.

C—O—CH=C$\underline{H}_2$), 4.69 (1H, C—O—CH=C$\underline{H}_2$), and 3.83 (3H, −O—C$\underline{H}_3$).

The NMR data for vinyl ferulate are in agreement with those reported by Gao et al.[15] The ^{1}H NMR spectrum of vinyl ferulate showed three vinyl proton signals, two of which were at δ 4.69 and δ 4.95; the chemical shift value for the third one was δ 7.34. The peak at δ 12.11 in the ferulic acid represents the proton from the carboxylic acid group (COOH), which disappeared in vinyl ferulate. A broad peak at δ 9.71 indicated that the hydroxyl group in ferulic acid was not derivatized during the chemical synthesis of vinyl ferulate. All proofs indicated that vinyl ferulate was successfully synthesized.

Preparation of Phytosteryl Ferulates. Ten enzymes, as listed under Materials and Methods, were initially screened for their ability in catalyzing the alcoholysis reaction between phytosterols and vinyl ferulate in milligram scale in test tubes. Lipase from *C. rugosa* was the only enzyme that successfully catalyzed the alcoholysis reaction of the esters formed between phytosterols and vinyl ferulate (Figure 1). It was then used for the remaining alcoholysis reactions for phytosteryl ferulates in this research work.

Hydrophobic solvents generally support higher lipase activity than the hydrophilic ones.[25,26] However, the solubility of ferulic acid in hydrophobic solvents was limited. Therefore, a binary organic solvent system consisting of hexane and 2-butanone has been used. This mixture is an unreactive and nontoxic solvent and has been successfully employed in other enzymatic reactions.[27] Furthermore, the ratio of these two solvents is an important factor for biosynthesis reactions as it may affect the rate of enzymatic reactions and the bioconversion yield as well as reaction selectivity.[28] The hexane/2-butanone solvent mixture at a ratio of 85:15 (v/v) was reported as the appropriate reaction medium for production of phenolic lipids with a high bioconversion yield.[29] Therefore, the hexane/2-butanone mixture at a ratio of 85:15 (v/v) was used as an effective solvent in this study.

The R_f values of the synthesized phytosteryl ferulates was 0.38. The higher R_f value of phytosteryl ferulates compared with its corresponding intermediate products and ferulic acid indicated their higher hydrophobicity. The synthesis of phytosteryl ferulates was also successfully scaled up from milligram in test tubes to grams in flasks. The yield of phytosteryl ferulates was 90%.

Structural Analysis of Phytosteryl Ferulates. The phytosteryl ferulates were purified by flash column chromatography and then subjected to different analyses to confirm their structures. Because phytosterols are relatively lipophilic with few polar functional groups, they are difficult to ionize through conventional electrospray methods.[30,31] Although atmospheric pressure chemical ionization (APCI) is not the most sensitive method, it is widely used for the analysis of phytosterols. APCI can be easily coupled with an HPLC system and has successfully been employed for the identification of phytosterols in soybean oil,[32] for the characterization of phytosterols in spelt,[30] for the determination of ergosterol levels in bulrush,[33] and for the measurement of cholesterol oxides in different food supplies.[34] To illustrate the structures of the synthesized phytosteryl ferulates, both negative-ion (NI) and positive-ion (PI) APCI-MS modes have been employed. The test compounds showed different ionization pathways under these two ionization modes, and the MS data are presented below.

The retention times for three components in phytosteryl ferulates were 18.77, 20.45, and 22.88 min for campesteryl ferulate, sitosteryl ferulate, and sitostanyl ferulate, respectively. The same elution order and very close retention times under similar HPLC conditions have been reported for the HPLC separation of rye bran extract.[35] In the study of Hakala et al., the first eluting compound was campesteryl ferulate (17.5 min),

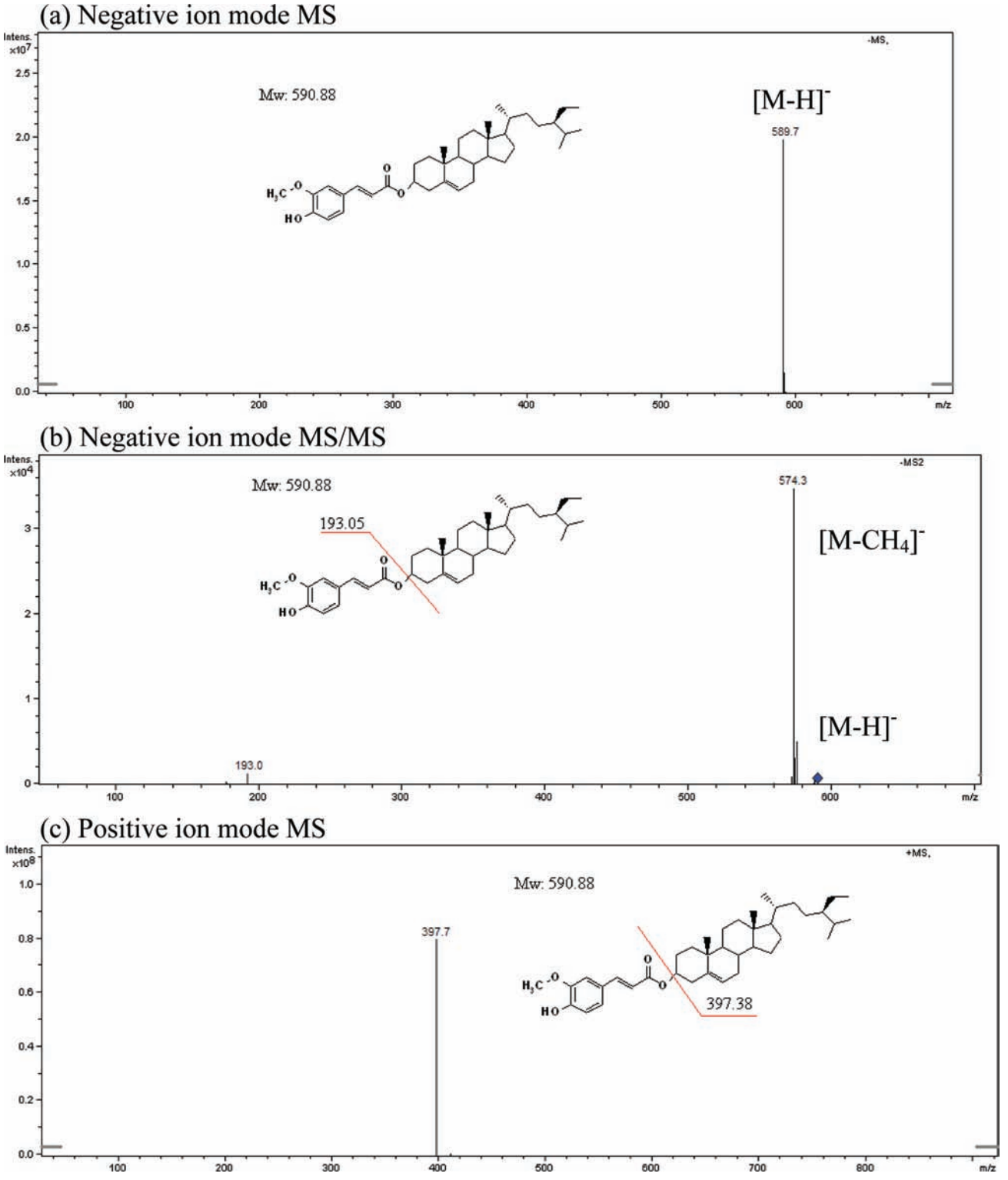

Figure 2. Mass spectra of sitosteryl ferulate (compound **2**): (a) negative-ion mode MS; (b) negative-ion mode MS/MS; (c) positive-ion mode MS.

followed by sitosteryl ferulate (19.5 min) and then sitostanyl ferulate (22 min).[35]

Representative APCI-MS spectra of phytosteryl ferulates as indicated by sitosteryl ferulate are shown in Figure 2. Under the NI-APCI-MS, compounds **1**, **2**, and **3** yielded base peaks at m/z 577.6, 589.7, and 591.7, respectively, which match the molecular weight of campesteryl, sitosteryl, and sitostanyl ferulates. These base peaks represented the molecular ion $[M - H]^-$ of the three components of interest. Lack of fragments corresponding to phytosterols means that phytosterol moieties were not ionized when the negative-ion mode APCI-MS was used. As the MS/MS is known to be specific and selective, the molecular ions were

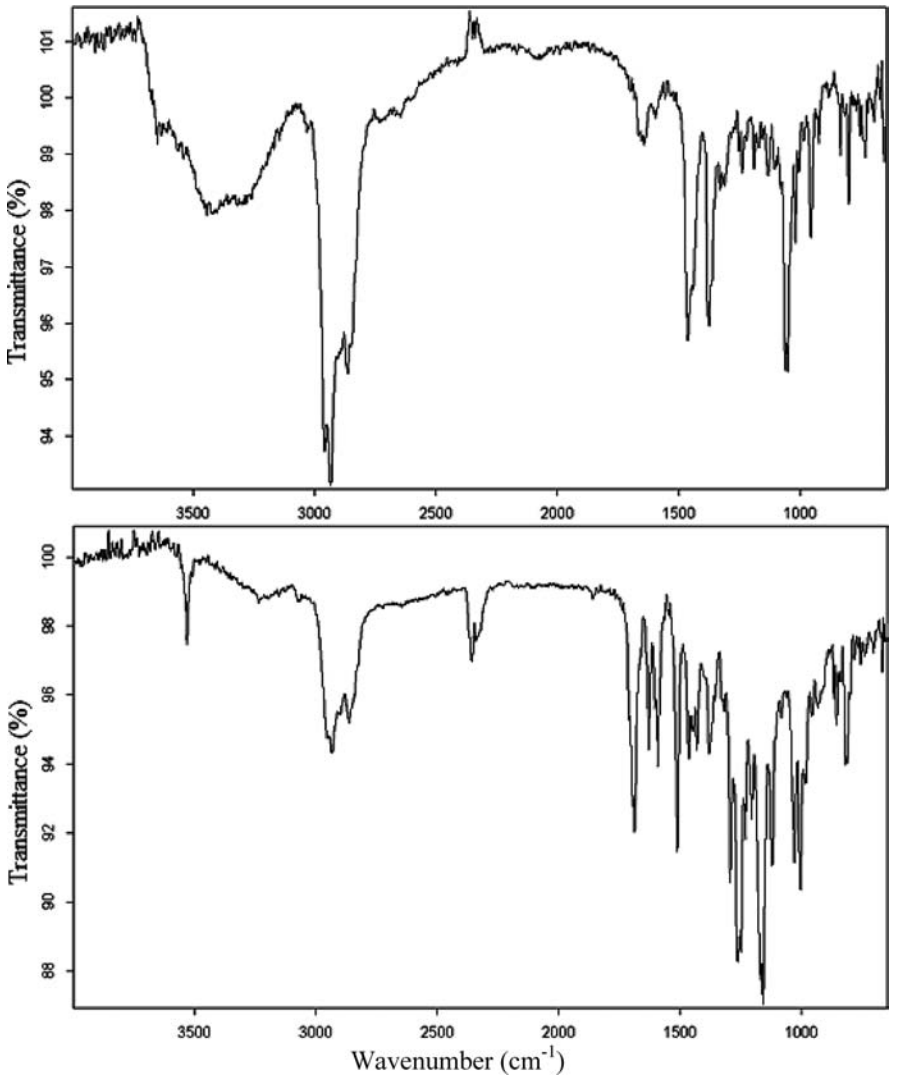

Figure 3. IR spectra of free phytosterols (top) and phytosteryl ferulates (bottom).

then chosen as parent ions to run the second MS. As shown in Figure 2b, sitosteryl ferulate yielded a $[M - CH_4]^-$ peak as well as a peak at m/z 193.0, which represented the feruloyl moiety in sitosteryl ferulate. Sitostanyl ferulate yielded a base peak at m/z 576.5, which represents the ion $[M - CH_4]^-$. Peaks with very low intensity for the feruloyl moiety in sitostanyl ferulate were observed. The characteristic ions for the feruloyl moiety include a ferulic acid at m/z 194.2 and a deprotonated ferulic acid at m/z 193.3, as well as fragments at m/z 178, 177, and 175, which are derived from deprotonated ferulic acid. The positive-ion mode was further used to confirm the identification of compounds. In PI-APCI-MS, the base peaks of compounds **1**, **2**, and **3** were the typical fragments for the intact moieties of phytosterols. These ions were $[M + H -$ ferulic acid$]^+$ with m/z at 383.9, 397.9, and 399.3 for campesteryl ferulate, sitosteryl ferulate, and sitostanyl ferulate, respectively. This characteristic fragmentation pathway for campesteryl ferulate and sitosteryl ferulate has also been reported from positive ion chemical ionization−mass spectroscopy (CI-MS),[36] positive-ion electrospray ionization−mass spectroscopy (ESI-MS),[37] and positive-ion APCI-MS.[35,38] Sitostanyl ferulate also showed a characteristic ion of the protonated molecular ion $[M + H]^+$ as well as protonated feruloyl moiety at m/z 194.9. These MS data confirmed that the synthesized products were campesteryl ferulate (compound **1**), sitosteryl ferulate (compound **2**), and sitostanyl ferulate (compound **3**).

The IR spectra of free phytosterols and the synthesized phytosteryl ferulates were compared, as shown in Figure 3. These spectra provide evidence that the chemoenzymatically synthesized products were phytosteryl esters. The free phytosterols had a strong absorption at 1053 cm^{-1} that was assigned to the C−O vibration via participation of oxygen atom of the hydroxyl group. A broad band around 3300−3600 cm^{-1} corresponds to the O−H vibration of the hydroxyl group of phytosterols. The IR spectrum of phytosteryl ferulates (Figure 3, bottom) showed strong sharp bands at 1689 and 1159 cm^{-1}, which indicate the functional groups C═O and C—O—C, respectively. A sharp band at 3531 cm^{-1} in the phytosteryl ferulates also indicates the presence of a hydroxyl group in the ferulic acid moiety.

Ultraviolet (UV) spectroscopic study of neutral methanolic solutions of sitostanyl ferulate extracted from corn fiber oil and ferulic acid showed UV maxima at 325 and 321 nm, respectively.[39] UV spectra of the synthesized phytosteryl ferulates are displayed in Figure 4. Our results are in agreement with the above findings.

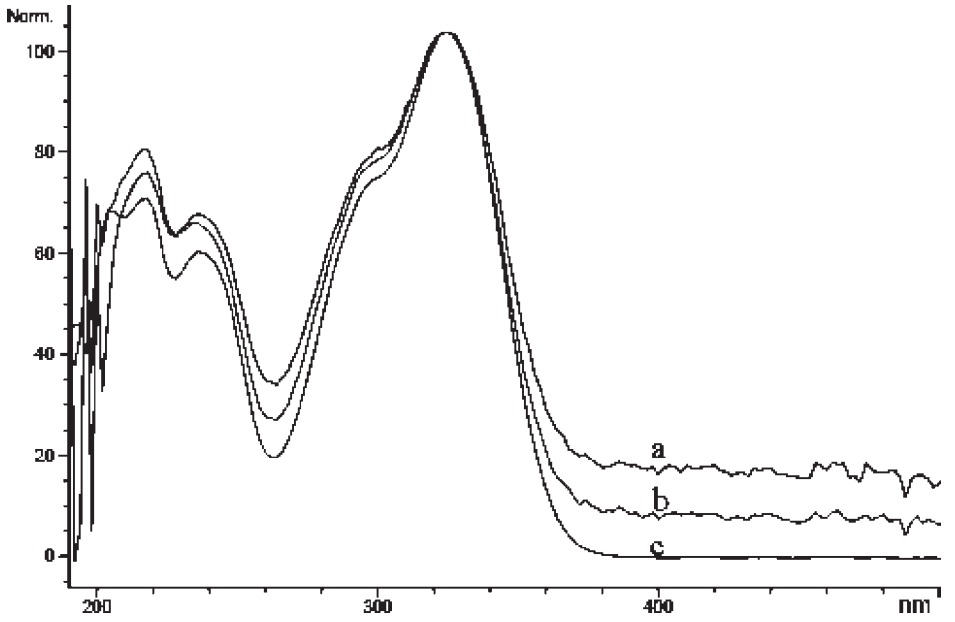

Figure 4. Ultraviolet spectra of HPLC peaks for the phytosteryl ferulates (a, campesteryl ferulate; b, sitostanyl ferulate; c, sitosteryl ferulate). Spectra were taken by a diode array detector (DAD) during HPLC separation.

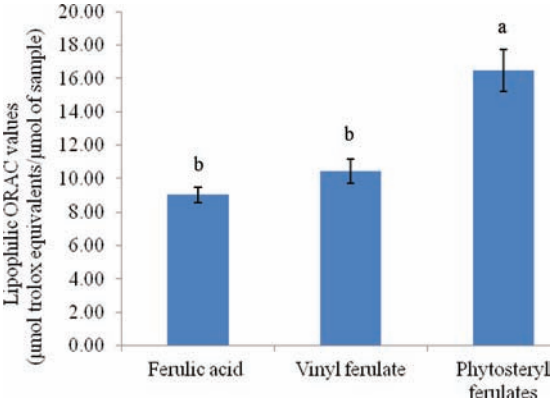

Figure 5. Lipophilic ORAC values of ferulic acid, vinyl ferulate, and phytosteryl ferulates as Trolox equivalents. Means in each column sharing the same letter are not significantly ($P > 0.05$) different from one another.

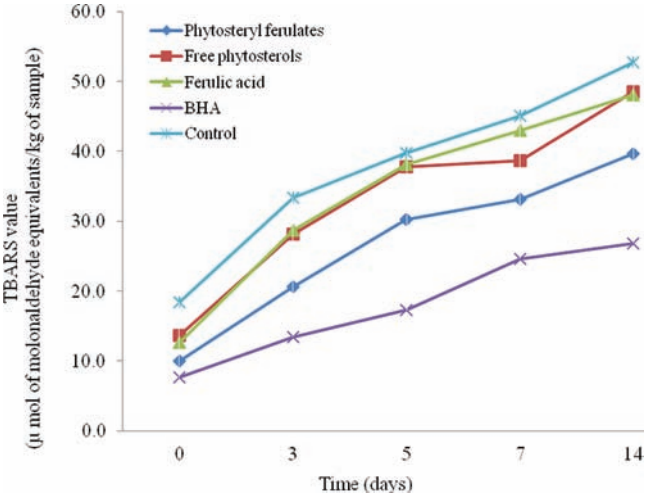

Figure 6. Thiobarbituric acid reactive substance (TBARS) values in cooked pork as affected by free phytosterols, ferulic acid, phytosteryl ferulates, and butylated hydroxyanisole (BHA).

The above information collectively demonstrates that the synthesized products were phytosteryl ferulates.

ORAC Assay. To have a better understanding of the free radical scavenging capacity of phytosteryl ferulates in biological environments, they were evaluated together with the starting materials and intermediates for their ability to scavenge peroxyl radicals, measured as ORAC. The ORAC assay utilizes a biologically relevant radical source and has been established as a standard method for assessing the activity of hydrophilic antioxidants. ORAC values, expressed as micromoles of Trolox equivalents per micromole of sample, are given in Figure 5. Phytosteryl ferulates showed the highest antioxidant activity, which was 16 times that of Trolox, as a reference antioxidant. The esterification process had a positive effect on phytosteryl ferulates as there were significant differences between starting ferulic acid and its corresponding phytosteryl esters. The ORAC values of phytosteryl ferulates were around 2 times higher than that of their corresponding starting ferulic acid, which indicates their greater hydrogen atom donating ability under the test conditions employed. The enhanced antioxidant activity may be partially explained by the increased hydrophobicity of vinyl ferulate and phytosteryl ferulates compared with ferulic acid.

Antioxidant Activity in Cooked Ground Meat Model System. Cooked ground muscle foods provide an excellent model for assessing the effectiveness of antioxidants in thermally processed whole or modified tissue foods, in which heating causes rapid oxidation of lipid and development of "warmed-over flavor".[40] The antioxidant activity of phenolic acids and their derivatives has previously been evaluated in a muscle food model system. In a cooked pork model system, phenolic acids exhibited a higher antioxidant activity than typical food antioxidants butylated hydroxytoluene (BHT) and α-tocopherol.[17]

In the work reported here, the antioxidant activity of phytosteryl ferulates was determined in a cooked pork model system by monitoring the formation of TBARS as affected by test compounds and BHA as a reference antioxidant. During storage at 4 °C, all meat samples showed increased content of TBARS with time as a result of lipid oxidation (Figure 6). The samples with added phytosteryl ferulates had lower TBARS values than the control over the entire storage period. The antioxidants added to fresh meat exerted an inhibitory effect against oxidation during cooking of the meat prior to storage, which explains the significantly higher TBARS value in the control sample on day 0. The antioxidant activity of test compounds followed the trend BHA > phytosteryl ferulates > free phytosterols ≈ ferulic acid. Phytosteryl ferulates showed moderate antioxidant activity compared with that of BHA, which indicate that they could be used as alternative food antioxidant to replace the synthetic ones. The lower TBARS value of free phytosterols indicates their lower antioxidant activity in the model system employed.

Conclusions. A simple two-step enzymatic synthesis of phytosteryl ferulates was successfully developed. The antioxidant activity of synthesized phytosteryl ferulates was evaluated using assays such as ORAC and a cooked ground meat model system. Results indicated that the synthesized phytosteryl ferulates possess higher antioxidant activity than ferulic acid. Thus, phytosteryl ferulates could be potentially used as alternative food antioxidant with cholesterol-lowering properties to replace the synthetic ones. The availability of the synthetic approach for the preparation of these complex molecules would greatly facilitate the further investigation of their biological properties. Investigation of other health benefits of vinyl ferulate and phytosteryl ferulates is currently underway.

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