Food Chemistry 138 (2013) 1438–1447

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Phytosteryl sinapates and vanillates: Chemoenzymatic synthesis and antioxidant capacity assessment

Zhuliang Tan, Fereidoon Shahidi *

Department of Biochemistry, Memorial University of Newfoundland, St. John's, NL, Canada A1B 3X9

ARTICLE INFO

Article history:
Received 13 July 2012
Received in revised form 10 September 2012
Accepted 8 October 2012
Available online 12 November 2012

Keywords:
Chemoenzymatic synthesis
Phytosteryl sinapates and vanillates
ORAC
Cooked muscle foods system
Antioxidant capacity

ABSTRACT

Phytosterols and their derivatives have attracted much attention because of their health benefits to humans and are widely used in food, pharmaceuticals, and cosmetics in the past decades. While most of the research has focused on free phytosterols and phytosteryl esters of fatty acids, few researches reported on phytosteryl phenolates, the esters of phytosterols with phenolic acids. Two novel group phytosteryl phenolates, namely phytosteryl sinapates and vanillates, were successfully chemoenzymatically synthesised in this work and their structures confirmed. Fourier transform infrared (FTIR) and high performance chromatography-mass spectrometry/mass spectrometry (HPLC–MS/MS) using atmospheric pressure chemical ionisation (APCI) under both positive and negative ion modes were employed for this purpose. High antioxidant capacity of phytosteryl sinapates was observed using both oxygen radical absorbance capacity (ORAC) assay and cooked ground meat model system. Although phytosteryl vanillates showed lower antioxidant capacity than phytosteryl sinapates, they were stronger antioxidants than vanillic acid and vinyl vanillate in both assays employed. Conjugation of phytosterols with sinapic or vanillic acid rendered higher antioxidant capacity. Further studies on health benefits of phytosteryl sinapates and vanillates are necessary.

1. Introduction

Phytosterols and their derivatives occur in plants in the free and conjugated forms, including phytosteryl esters of fatty or phenolic acids, phytosteryl glycosides, and acylated phytosteryl glycosides (Piironen, Lindsay, Miettinen, Toivo, & Lampi, 2000). These products have attracted much attention because of their benefits to human health and are widely used in food, pharmaceuticals, and cosmetics (Moghadasian, 2000). Phytosteryl esters have higher solubility in oils and lower melting points compared with free phytosterols. The addition of phytosterols to the diet has been shown to decrease the low-density lipoprotein (LDL) plasma cholesterol levels without significant effects on the level of high-density lipoprotein (HDL) cholesterol in both animal and human models (Miettinen, Puska, Glyying, Vanhanen, & Vartiainen, 1995; Moghadasian & Frohlich, 1999). In addition, they also show the ability to prevent and delay the development of atherosclerotic lesions in animal models (Tan, Le, Moghadasian, & Shahidi, 2012). However, most research has focused on phytosteryl esters of fatty acids through either chemical or enzymatic synthesis pathways, but only a few are related to phytosteryl phenolates, which are the phytosteryl esters of phenolic acids.

Sinapic acid possesses 3,5-dimethoxy and 4-hydroxyl substitution in the phenyl group of cinnamic acid. It is a widely prevalent substance in the plant kingdom and is present in various sources such as rye, fruits, and vegetables (Andreasen, Landbo, Christensen, Hansen, & Meyer, 2001). Sinapic acid, one of the main phenolic compounds of canola and rapeseed, constitutes over 73% of their free phenolic acids. Various biological activities of sinapic acid have been reported; these include antioxidative potential (Wanasundara, Amarowicz, & Shahidi, 1996; Zou, Kim, Kim, Choi, & Chung, 2002), anxiolytic-like effects (Yoon et al., 2007), anti-inflammatory properties (Yun et al., 2008) and antibacterial activity (Tesaki et al., 1998). Sinapic acid is also known to have peroxynitrite ($ONOO^-$)-scavenging activity (Niwa, Doi, Kato, & Osawa, 1999) and can be utilised for protection of the cellular defence activity against diseases involving $ONOO^-$ (Zou et al., 2002). Vanillic acid is a hydroxybenzoic acid, which is generally used as a flavouring agent. It is an intermediate in the production of vanillin from bioconversion of ferulic acid. The root of *Angelica sinensis*, commonly used as traditional Chinese medicine, has been reported to contain the highest amount of vanillic acid (Duke, 1992). Various studies have provided evidence of the effectiveness of vanillic acid in the management of immune or inflammatory responses. Chiang, Ng, Chiang, Chang, and Lin (2003) reported that vanillic acid enhanced the activity of human lymphocyte proliferation and secretion of interferon-gamma in human peripheral blood mononuclear cells.

* Corresponding author. Tel.: +1 709 864 8552; fax: +1 709 864 4000.
E-mail address: fshahidi@mun.ca (F. Shahidi).

0308-8146/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2012.10.093

Another study has shown that vanillic acid renders a hepatoprotective effect through its suppression of immune-mediated liver inflammation in concanavalin A-induced liver injury (Itoh et al., 2009). Vanillic acid has also shown beneficial effect on dextran sulphate sodium-induced ulcerative colitis, so it can be used to regulate the chronic intestinal inflammation (Kim, Kim, Um, & Hong, 2010).

We have previously reported successful preparation of phytosteryl ferulates and caffeates by a chemoenzymatic pathway (Tan & Shahidi, 2011, 2012). As part of an on-going research in our laboratory, the present work was aimed at exploring the possibility of chemoenzymatic production of phytosteryl sinapates and vanillates and evaluating their antioxidant capacity. To the best of our knowledge, chemoenzymatic synthesis of phytosteryl sinapates and vanillates has not yet been reported in the literature.

2. Materials and methods

2.1. Materials

Phytosterols were procured from Forbes Medi-Tech Inc. (Vancouver, BC, Canada). The sample used contained 75.6% sitosterol, 12.2% sitostanol, 8.1% campesterol, and 4.1% other minor phytosterols. Ten lipases were obtained from different commercial sources. Novozyme 435 (lipase acrylic resin from *Candida antarctica*) and *Mucor miehei* (Lipozyme-IM) were procured from Novo Nordisk (Franklinton, NC, USA). Three lipases were purchased from Sigma–Aldrich Canada (Oakville, ON, Canada), which were: Amano lipase from *Pseudomonas fluorescens*, Amano lipase PS from *Burkholderia cepacia* and lipase from *Candida rugosa* type VII. The other five lipases, which were: lipoprotein lipase (LPL) 311, LPL 314, cholesterol esterase (COE) 301, COE 311 and COE 313, were obtained from Toyobo Co., Ltd. (Osaka, Japan), AAPH (2,2′-azobis(2-aminopropane)dihydrochloride, anhydrous monosodium phosphate, anhydrous disodium phosphate, fluorescein sodium salt, randomly methylated cyclodextrin (RMCD), sinapic acid, trolox, and vanillic 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 (Oakville, ON, Canada).

2.2. Chemical synthesis of vinyl sinapate and vanillate and structural confirmation by ^{1}H-NMR

Vinyl sinapate and vanillate were chemically synthesised via the vinyl interchange reaction of vinyl acetate and sinapic acid or vanillic acid according to the method described by Tan and Shahidi (2011, 2012) without any modification. Column chromatography was employed for purification of vinyl sinapate and vanillate 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 pre-coated flexible TLC sheets (2.5 × 7.5 cm, J.T. Baker, Phillipsburg, NJ, USA) with mobile phase hexane/ethyl acetate (4:1, v/v). Products were visualised under UV light at 254 nm using Spectroline® (Spectronics Corporation, New York, NY, USA). ^{1}H-NMR analysis of purified vinyl sinapate and vanillate was carried out in order to confirm the formation of vinyl esters. NMR spectra of pure sinapic acid or vanillic acid and isolated vinyl sinapate or vanillate were recorded on a Bruker AVANCE 500 spectroscopy (Bruker Biospin Co., Billerica, MA, USA). Proton spectra were recorded on a 500 MHz equipment using a solvent field lock. The samples were dissolved in perdeuterated dimehtyl sulphoxide (DMSO-d_6) containing tetramethylsilane (TMS) as internal standard. Signal processing and interpretation were performed with the software *Topspin* 1.3 (Bruker Biospin Co., Billerica, MA, USA) 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 sinapate or vanillate with that of the parent sinapic acid or vanillic acid.

2.3. Enzyme screening for synthesis of phytosteryl sinapate and vanillate

Vinyl sinapate or vanillate was placed into a test tube together with 0.06 g of phytosterol mixture (mole ratio of vinyl sinapate or vanillate to phytosterol mixture, 1:2) and different lipases (8% of the total weight of both substrates). Three millilitres of solvent mixture (hexane/2-butanone, 9:1, v/v) were added to the test tube which was then flushed with nitrogen before being sealed with a screw cap. The reaction mixture was shaken in a gyrotory water bath shaker at 200 rpm and 45 °C (New Brunswick Scientific Co., Inc., New Brunswick, NJ, USA). Ten enzymes as listed in Section 2 were tested for the best enzyme for the synthesis of phytosteryl sinapate and vanillate. The reaction was monitored by using Baker-flex® silica gel IB-F pre-coated flexible TLC sheets (2.5 × 7.5 cm, J.T. Baker, Phillipsburg, NJ, USA) with an eluent mixture of hexane/ethyl acetate (4:1, v/v). After 10 days, the reaction was stopped by placing the tubes under running tap water. The enzyme was filtered through a P5 Fisherbrand filter paper. Solvent was evaporated with rotary evaporator at 40 °C and the solid residue was subjected to column chromatography. The structures of the phytosteryl sinapates and vanillates were then elucidated using FTIR and HPLC–MS/MS analysis.

2.4. Column chromatographic separation of phytosteryl sinapates and vanillates

Five grams of reaction mixture were 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 (reaction mixture/silica gel, 1:40, w/w) 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 pre-coated flexible TLC sheets (2.5 × 7.5 cm, J.T. Baker, Phillipsburg, NJ, USA) were used to monitor the different fractions. Mobile phase for TLC plate was *n*-hexane/ethyl acetate (4:1, v/v).

2.5. FTIR and HPLC–MS/MS analysis of phytosteryl sinapates and vanillates

The purified phytosteryl sinapates and vanillates were characterised using a Fourier-transform infrared (FTIR) spectroscopy. The IR spectra were recorded using FTIR Bruker Tensor 27 spectroscopy (Bruker Optik GmbH, Ettlingen, Germany), which 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 both liquid and solid samples.

HPLC–MS/MS with atmospheric pressure chemical ionisation (APCI) using both negative ion (NI) and positive ion (PI) modes was employed to confirm the identities of phytosteryl sinapates and vanillates. The analysis was carried out on an Agilent 1100 HPLC–MSD system (Palo Alto, CA, USA) with an on-line solvent degasser, binary solvent delivery system, autosampler and UV–Vis diode array detector (DAD) as explained elsewhere (Tan & Shahidi, 2011). Separation was achieved on a C18 column (250 mm length, 4.6 mm I.D., 5 μm particle size, Sigma–Aldrich Canada Ltd., Oakville, ON, Canada) coupled with a Supelpguard™ guard column (Supelco, Bellfonte, PA). Phytosteryl sinapates and vanillates were eluted using an isocratic solvent system containing methanol/water (95:5, v/v) at a flow rate of 1.0 ml/min. Fifty