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Enzymatic production of bioactive docosahexaenoic acid phenolic ester

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ABSTRACT

Docosahexaenoic acid (DHA) is increasingly considered for its health benefits. However, its use as functional food ingredient is still limited by its instability. In this work, we developed an efficient and solvent-free bioprocess for the synthesis of a phenolic ester of DHA. A fed-batch process catalyzed by *Candida antarctica* lipase B was optimised, leading to the production of 440 g/L vanillyl ester (DHA-VE). Structural characterisation of the purified product indicated acylation of the primary OH group of vanillyl alcohol. DHA-VE exhibited a high radical scavenging activity in acellular systems. *In vivo* experiments showed increased DHA levels in erythrocytes and brain tissues of mice fed DHA-VE-supplemented diet. Moreover, *in vitro* neuroprotective properties of DHA-VE were demonstrated in rat primary neurons exposed to amyloid-β oligomers. In conclusion, DHA-VE synergized the main beneficial effects of two common natural biomolecules and therefore appears a promising functional ingredient for food applications.

1. Introduction

Omega-3 polyunsaturated fatty acids from fish oils promote well-established health and anti-aging benefits that justify their use as functional ingredients in dietary supplements, healthy foods and nutraceutical products (Swanson, Block, & Mousa, 2012). Among them, eicosapentaenoic (EPA, C20:5) and docosahexaenoic acids (DHA, C22:6) continue to receive particular attention because of their numerous biological properties and positive effects on human health (Lorente-Cebrian et al., 2013). In addition to recognised benefits for the prevention of cardiovascular diseases, EPA and DHA were reported to protect from inflammation-mediated disorders including obesity and diabetes, Alzheimer's and related neurodegenerative diseases (Calder, 2013; Farooqui, 2012).

Human metabolism exhibits limited ability to synthesise ω3 PUFAs. Dietary supply of preformed compounds therefore appears an essential alternative. However, the practical use of such lipids as food ingredients is often limited by their high susceptibility to oxidation, which is responsible for the undesirable off-flavour and odour of rancid oils, associated with a loss of nutritional value (Albert, Cameron-Smith, Hofman, & Cutfield, 2013). Various solutions can be implemented to minimise these degradation pathways and the most commonly used by manufacturers is addition of antioxidants (Wang et al., 2011). Intensive research has been pursued on natural phenolic antioxidants issued from plants. Many studies reported their high efficiency to protect ω3-enriched food products from oxidation (Huber, Rupasinghe, & Shahidi, 2009; Sekhon-Loodu, Warnakulasuriya, Rupasinghe, & Shahidi, 2013). In addition, some works pointed out the interest of formulations mixing ω3 PUFAs and phenolic compounds for the prevention of

Abbreviations: Aβ, amyloid-β; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); BHT, butylated hydroxytoluene; CALB, *Candida antarctica* lipase B; DHA, docosahexaenoic acid; DHA-EE, DHA-ethyl ester; DHA-VE, DHA-vanillyl ester; DPPH, 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl; EPA, eicosapentaenoic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TEAC, trolox equivalent antioxidant capacity; VA, vanillyl alcohol.

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Alzheimer's disease and the treatment of obesity through lipid-lowering effects (Cole et al., 2005; Radler et al., 2011). Another approach consists in bringing together ω3 lipids and phenolic compounds into a single entity as described by few authors (Mbatia, Kaki, Mattiasson, Mulaa, & Adlercreutz, 2011; Torres de Pinedo, Penalver, Rondon, & Morales, 2005; Ying Zhong & Shahidi, 2011). Such an association was shown to improve the stability of highly oxidizable fatty acids while facilitating the solubilisation of phenolic compounds in lipid phases. Additional effects could be an increased bioavailability of phenols as well as cumulative and even synergistic biological activities (Tan, Le, Moghadasian, & Shahidi, 2012; Wahle, Brown, Rotondo, & Heys, 2010). In addition, PUFA phenolic esters were reported to exhibit higher antimicrobial, antioxidant and anti-inflammatory activities than native phenols (Mellou et al., 2005; Zhong, Chiou, Pan, & Shahidi, 2012).

Fatty-acid phenolic esters are preferentially produced by enzymatic bioprocesses that exhibit high selectivity towards polyfunctional substrates and mild reaction conditions comparing with chemical synthesis pathways. Most often, esterification reactions were carried out in dry organic solvent, but few studies reported the possibility to process without any solvent (Hong, Ma, Kim, Seo, & Kim, 2012; Weitkamp, Weber, & Vosmann, 2008). Moreover, most of these studies only intended to make the proof of concept, but ignored upscale potential.

The objective of the present work was to develop an efficient and environment-friendly solvent-free bioprocess for the synthesis of DHA vanillyl ester (DHA-VE) in sufficient quantities to allow further *in vitro* and *in vivo* experiments as well as potential applications. DHA was chosen because of its major protective potential, while vanillyl alcohol (VA) is a phenolic compound commonly found as antioxidant in foodstuffs. DHA-VE bioavailability was evaluated in mice, as well as its neuroprotective properties *in vitro*, in comparison with those of DHA and VA used alone or in combination.

2. Materials and methods

2.1. Chemicals and enzyme

Candida antarctica lipase B (CALB) immobilized on a macroporous acrylic resin (Novozym 435®, Novo Industry) was used to catalyze acylation reactions. Docosahexaenoic-acid ethyl ester (DHA-EE, 95% pure) was supplied by KD-Pharma (Bexbach, Germany). Solvents of analytic or HPLC grade were from Merck. Vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol, VA) and all other chemicals were purchased from Sigma–Aldrich Chemicals.

2.2. Enzymatic synthesis of DHA vanillyl ester (DHA-VE)

2.2.1. Enzymatic synthesis of DHA-VE in organic medium

Enzymatic acylation reactions were achieved in organic solvent, under atmospheric pressure. Reaction media were prepared by solubilising VA (100 mM, 15.4 g/L) and DHA-EE (200 mM) in water-free acetonitrile (water activity below 0.1). Reactions were performed under orbital shaking and initiated by adding 20 g/L of Novozym 435®. This protocol was further referred as solvent system.

2.2.2. Synthesis of DHA-VE in molten media

Reactions were performed under either atmospheric or reduced pressure. For syntheses achieved under atmospheric pressure, reaction media were prepared by solubilising VA (200 mM, 30.8 g/L, which corresponds to the maximal solubility of the substrate at 50 °C) in a large excess of DHA-EE as acyl donor (2 mL), at 50 °C. Reactions were initiated by adding 20 g/L of Novozym 435®. After 72 h of reaction, the supernatant was taken after enzyme particle decantation. This protocol led to reaction system A.

Syntheses achieved under reduced pressure were performed at 37 °C and 500 mbar in a rotary evaporator, so that the by-product of the reaction, *i.e.* ethanol, could be continuously eliminated while avoiding VA evaporation. Reaction media were prepared by solubilising VA (162 mM, 25 g/L that corresponds to the substrate solubility at 37 °C) in 10 mL of DHA-EE. Reactions were started by adding 20 g/L of Novozym 435®. After 72 h, the enzyme was eliminated by filtration. This protocol led to reaction system B.

2.2.3. Process intensification

The production of DHA-VE was intensified by increasing VA intakes. VA (50 g/L) was introduced under a nitrogen stream (fed batch process) in 10 mL of DHA-EE. Syntheses were performed as described in Section 2.2.2. After the first 4 h of synthesis, the reaction medium was filtered and transferred to another flask. A new supply of VA (50 g/L) was then carried out. Once the phenolic compound was totally solubilised, the reaction was restarted by adding fresh enzymatic preparation, thereby maintaining optimal activity. This protocol was repeated twice. This protocol led to reaction system C.

2.2.4. Kinetic following of the syntheses

Kinetic following of the reactions was performed by HPLC, using a Shimadzu Class-VP system equipped with a computer-controlled system (Class-VP 6.1 software). Separations were carried out on a reversed-phase Altima C18 column (150 × 2.1 mm, 5 μm, Grace-Alltech). VA and DHA-VE were detected at 280 nm on a multichannel photodiode-array detector (SPD-M10A VP). DHA-EE and free DHA were followed by evaporative light scattering detection (ELSD), using nebulizer and evaporator temperatures of 35 °C and 45 °C, respectively. Analyses were carried out with compressed air as ELSD gas at a pressure of 1.5 bars. Elution was performed using a gradient of solvent A [methanol/water 70/30 (v/v)] and B [methanol (100%)], at a flow rate of 0.2 mL/min. Elution protocol was as follows: 0–5 min: 100–0% A, 5–25 min: 100% B, 25–35 min: 100–0% B, 35–45 min: 100% A. Calibrations were made using analytical standard compounds. During synthesis, aliquots were withdrawn from each reactor at predetermined times and then diluted 100 times in solvent A. All samples were filtered through a 0.2-μm membrane before injection.

2.3. Purification of DHA-VE by flash chromatography

DHA-VE was purified on glass columns KONTES CHROMAFLEX packed with silica gel 60 (particle size of 40–63 μm). Gradient system was generated by 2 pumps Gilson model 306. Cyclohexane and ethyl acetate were used as mobile phase. The elution gradient was as follows: 0–40 min: 5–15% ethyl acetate, 40–60 min: 15–40% ethyl acetate. The flow rate was 20 mL/min. A sample of 20 mL was collected every minute for proper identification. The presence of the ester was detected by thin-layer chromatography on precoated silica gel $60F_{254}$ TLC plates, referring to the pure molecule as standard. The plates were visualised under UV light at 254 nm and then sprayed with a solution of sulphuric acid 20% in methanol. Fractions containing the ester were combined and the solvent was evaporated.

2.4. Structural analyses

2.4.1. Liquid chromatography–mass spectrometry (LC–MS)

The structure of the product was determined by HPLC-MS. The mass spectra were obtained using a binary solvent delivery pump and a linear ion trap mass spectrometer (LTQ-MS, Thermo