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Production and Scale-up of phosphatidyl-tyrosol catalyzed by a food grade phospholipase D

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ABSTRACT

Highly purified phosphatidyl-tyrosol was obtained by using a food grade phospholipase D from *Actinamadure* sp. transphosphatidylation in a GRAS (Generally Recognized As Safe) biphasic medium. The reaction medium, comprised of an aqueous phase and ethyl butyrate, has been considered as an alternative to other biphasic systems previously reported utilizing more harmful organic solvents. The purpose of the present study was to purify phosphatidyl-tyrosol from a transphosphatidylation reaction mixture by using a procedure readily scalable to obtain a new valuable food ingredient. Initially, phosphatidyl-tyrosol was purified via semi-preparative HPLC equipment to be used as analytical standard. The best results of the transphosphatidylation reaction were obtained for two different PC concentrations, namely 83 and 166 mmol/L, with PC conversion of ca. 97 and 94% (w/w) and a final phosphatidyl-tyrosol concentration of 81 and 157 mmol/L, respectively. Finally, the procedure was scaled-up and 40 g of highly purified phosphatidyl-tyrosol (97% (w/w)) were readily purified by centrifugation without involving the utilization of organic solvents.

Keywords: Transphosphatidylation; Phospholipase D; Purification; Tyrosol; Lipophilization; Phospholipid

1. Introduction

Biocatalysis is being intensively studied to produce ingredients that provide health benefits beyond normal nutritional functions, including prevention against illness and chronic and degenerative conditions. Phospholipids (PLs) are recognized as important contributors to beneficial effects on human health, since several biological functions in cell signaling and regulation have been identified (Espinosa et al., 2011). On the other hand, PLs can be also used as emulsifiers and vehicles for transporting bioactive compounds.

Tyrosol is the most abundant phenol in extra virgin olive oil, which protect Caco-2 cells against the cytotoxic/apoptotic effects of oxidized LDL, inhibit the activity of the leukocyte 5-lipoxygenase, and improves the intracellular antioxidant defense systems. Although, tyrosol is a phenolic compound with a chemical structure unsuitable for strong antioxidant activity, it has been shown to exert powerful protective effects against oxidative injuries in cell systems when the capabilities to spare glutathione (GSH) and reinforce intracellular antioxidant defenses are considered (Di Benedetto et al., 2007). However, it should be noted that the effectiveness of tyrosol in some biological systems is related to its capability to penetrate the cells (Weitkamp et al., 2008). Moreover, in pharmaceuticals as well as in food preparations, limitations occur due to its weak solubility and stability in a lipophilic environment.

Some studies have shown that the antioxidant effects of phenolic-based antioxidants in oil matrices can be improved by lipophilization (Morales et al., 2007). Hence, lipophilic derivatives of flavonoids when esterification with aliphatic molecules increase their lipophilicity and improve their cell permeability (Fragopoulou et al., 2007). Synthesis of tyrosyl oleate as a food ingredient has also been described wherein the synthesis was catalyzed by two immobilized lipases from *Candida antarctica* in the absence of

Abbreviations: PLs, phospholipids; PT, phosphatidyl-tyrosol; PLD, phospholipase D; PC, phosphatidylcholine.

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organic solvents. Lipophilization of tyrosol slightly improved the antioxidant activity of this phenolic compound compared to free tyrosol in oil matrices (Fernández et al., 2012).

Phospholipase D (PLD) has been extensively used as a catalyst in the transphosphatidylation of PLs in a wide variety of fields such as the production of fine chemicals and functional foods (Servi, 1999; Ulbrich-Hofmann et al., 2005). Heterogeneous reaction systems for PLD catalysis are commonly used in processes involving water-insoluble reactants. To solubilize the substrate and product at reasonable concentrations without additional surfactants, biocatalytic reactions with PLD are mostly performed in emulsion systems. In these reaction systems, the enzyme is initially dissolved in the water phase containing buffer and ions as activators (Koo and Turk, 1977; Ulbrich-Hofmann et al., 2005) with alcohol (if it is water-soluble), while the organic phase will contain the PLs and eventually the alcohol (if it is water insoluble).However, common processes make use of organic solvents that can be hazardous to human health and the environment, such as diethyl ether or ethyl acetate. Transphosphatidylation with organic solvents, up to 20 mM PC and several phenolic compounds, such as phenylalkanols, monoterpenes and other aromatic phenols, has been described (Takami et al., 1994; Yamamoto et al., 2008a, 2011). When transphosphatidylation of several primary alcohols with 2:1 (v/v) water:organic solvent ratio is employed, efficient reaction can be obtained with 200 mM PC concentration in the organic phase, but the purification of these compounds is not straightforward (D'Arrigo et al., 1996). Although there are bio-catalysis methodologies, which avoid using toxic solvents or generation of toxic byproducts, they yield low amounts of the modified PLs (Juneja et al., 1992). In this sense, transphosphatidylation of other alcohols in a solvent free medium has been already investigated (Iwasaki et al., 2003). In this mentioned work, an aqueous suspension system comprised of a solution containing serine, PLD, and lecithin adsorbed on silica or calcium sulfate powder at 40 °C was utilized. The reactant concentration was PC 10 mM, serine 1.48 M in the presence of 3 Units of a PLD from a recombinant strain of *Escherichia coli* bearing the PLD gene of *Streptomyces antibioticus* (One unit of PLD activity was defined as the amount of enzyme that hydrolyzes 1 mol PC in 1 min at 37 °C). This reaction generated more than 80% of phosphatidylserine in 24 h. However the concentration of PC adsorbed on silica is quite low, thus this methodology is only able to produce low concentrations of phosphatidylserine. Alternatives to the emulsion system are membrane reactors, use of immobilized enzymes, and an aqueous phase for the production of phosphatidylglycerol (Servi, 1999).

Regarding isolation of the new PL synthesized directly from the reaction system, temperature and salt concentration of the reaction mixture can affect phase behavior and consequently the partitioning coefficient of remaining substrates, products and protein. It is noteworthy that traditionally PL isolation has been accomplished by using acetone precipitation and solvent fractionation in combination with column chromatography.

Therefore, the enzymatic transphosphatidylation avoiding the use of harmful organic solvents for foods purposes to produce modified PLs containing a molecule of tyrosol in the polar head becomes relevant. Two main objectives need to be fulfilled: (1) an adequate procedure readily scalable to produce phosphatidyl-tyrosol (PT); and (2) an appropriate methodology for purifying the PT produced. For that matter, the present study shows an easily scalable methodology to produce highly purified PT. In addition, transphosphatidylation was carried out in the presence of a food grade PLD commercially available. Finally, in an attempt to avoid using any additional solvent in the isolation step, the purification of PT from the product mixture was attained by semi-continuous centrifugation. A schematic representation of the proposed phosphatidylcholine transphosphatidylation with tyrosol is shown in Fig. 1 and a complete flow sheet diagram of the process is depicted in Fig. 2.

2. Materials and methods

2.1. *Materials*

Hydrogenated phosphatidylcholine, 90% (PC 90H), was purchased from Lipoid (Cham, Switzerland). Powdered preparation of *Actinamadure* sp. phospholipase D (EC 3.1.4.4) was acquired from Meito Sangyo CO (Nikko-Cho, Fuchu, Tokyo, Japan). According to the vendor specifications PLD contained 1500 U/mg (1 PLD Unit is defined as the amount of enzyme producing 1 μmol/h of choline from L-a-phosphatidylcholine when the enzyme solution is reacted at pH 8 at 30 °C). Tyrosol (98.0% w/w purity) was purchased from TCI Europe (Zwijndrecht, Belgium). Anhydrous calcium chloride (PRS Grade), formic acid (98%), ethyl butyrate (99.5%), squalene (97%) triethylamine (99.5%), and sodium acetate trihydrate (99%) were acquired from Sigma–Aldrich (St. Louis, MO, USA). Alpha tocopherol was purchased from BTSA (Madrid, Spain), lysophosphatidylcholine (1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine) was acquired to Avanti Polar Lipids. Inc.

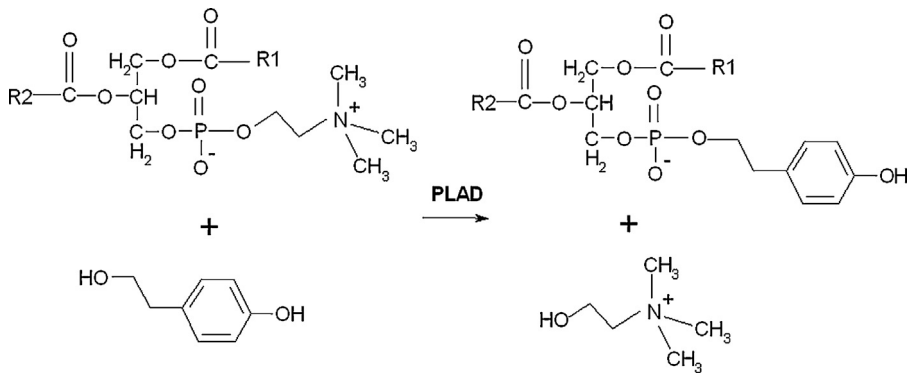

Fig. 1 – Schematic representation of phospholipase D (PLD) transphosphatidylation reaction.