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Synthesis of omega-3 ethyl esters from chia oil catalyzed by polyethylene glycol-modified lipases with improved stability

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ABSTRACT

Enzymatic synthesis of fatty acid ethyl esters (FAEE) from chia (*Salvia hispanica* L.) oil has been performed with different modified derivatives and compared with commercial immobilized lipases to produce omega-3 rich FAEE. Therefore, the main objective was to synthesize omega-3 esters from chia oil catalysed by polyethylene glycol-modified lipases using a biocatalyst with higher stability than commercial derivatives. Lipase from *Thermomyces lanuginosus* (TLL) was immobilized by hydrophobic adsorption on Sepabeads C-18 followed by a physicochemical coating of lipase surface with a dense layer of PEG. Ethanolysis reactions were carried out using pressurized liquid extracted chia seed oil and with different lipase derivatives to compare the omega-3 FAEE yield and ratio of reaction products, which were analysed by HPLC-ELSD. Furthermore, reutilization of lipase derivatives was studied to evaluate the stability after several reaction cycles to minimize decreasing of catalytic activity and develop a feasible enzymatic process for food industrial applications.

1. Introduction

In recent years, food products enriched with omega-3 fatty acids from new sources have awaken interest for their important role to provide beneficial effects on human health. The main dietary source of omega-3 is fish oil, however, the current demand has led to overfishing certain species with the consequence on the decreased of fish global stocks (Adarme-Vega, Thomas-Hall & Schenk, 2014; Lenihan-Geels & Bishop, 2016). Therefore, sustainable alternatives sources, such as vegetable oils, have been studied in order to provide the worldwide demand for omega-3. Vegetable oils are rich in omega-3 alpha linolenic acid (ALA; 18:3 ω-3) which is converted to eicosapentaenoic acid (EPA; 20:5 ω-3) and docosahexaenoic acid (DHA; 22:6 ω-3) through a series of elongation and desaturation processes in the human body (Baker, Miles, Burdge, Yaqoob & Calder, 2016). Specifically, ALA contributes to the maintenance of normal blood cholesterol levels (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2009), and is also needed for normal growth and development of children (European Food Safety Authority (EFSA), 2008).

Chia oil contains the highest known natural percentage of ALA omega-3, around 65%, and also an important content of antioxidants that give an optimal stability to the oil (Castejón, Luna & Señoráns, 2017). The nutritional benefits and the potential application of chia oil in food industry due to the authorization as novel food by the European Commission (Regulation (EC) No 258/97) have given an emergent interest for its production (Oliveira-Alves et al., 2017; Timilsena, Vongsvivut, Adhikari & Adhikari, 2017). Hence, chia oil is an important raw material to obtain enriched omega-3 food products.

Methods for the production of omega-3 concentrates include the chemical or enzymatic synthesis of fatty acids ethyl esters (FAEE). Enzymatic synthesis is a better alternative due to its mild reaction conditions, minimal undesirable by-products, and less n-3 PUFAs oxidation (Fernández-Lorente, Betancor, Carrascosa & Guisán, 2011, Kamal, Barrow & Rao, 2015, Wang, Li, Wang, Yang & Wang, 2016, Moreno-Perez et al., 2017, He et al., 2017). Nowadays, FAEE derivatives are a common source of omega-3 as functional food ingredient and also, in form of nutraceutical products and nutritional supplements (He, 2009, Rubio-Rodríguez et al., 2010). In addition, synthesis of omega-3 FAEE could be the first stage in the production of structured lipids (Kralovec, Zhang, Zhang & Barrow, 2012; Moreno-Perez, Luna, Señorans, Guisan & Fernandez-Lorente, 2015; Muñío, Robles, Esteban, González, & Molina, 2009).

Lipases, as most enzymes, need to be immobilized for their applications as biocatalysts to facilitate their separation from the reaction

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medium as well as enzyme reuse (Manoel, dos Santos, Freire, Rueda & Fernandez-Lafuente, 2015; Mateo, Palomo, Fernandez-Lorente, Guisan & Fernandez-Lafuente, 2007). The development of stable and active biocatalyst with the possibility of using it for several cycles can significantly reduce process cost (Adlercreutz, 2013; Brady & Jordaan, 2009). For these reasons, new chemical methodologies are being developed in order to improve the stability of the immobilized enzymes. PEGylation, defined as the covalent attachment of polyethylene glycol (PEG) to bioactive substances, is a promising strategy for that purpose (Moreno-Perez et al., 2016), since the viscous layer avoids enzyme alterations. PEG is a non-toxic polymer considered GRAS (generally recognized as safe) by the FDA and used in a wide range of pharmaceuticals products (Pasut & Veronese, 2012; Roberts, Bentley & Harris, 2012; Turecek, Bossard, Schoetens & Ivens, 2016).

Therefore, the main objective of this study was to synthesize omega-3 FAEE from chia oil catalyzed by polyethylene glycol-modified lipases using a biocatalyst with higher stability than commercial derivatives. Lipase from *Thermomyces lanuginosus* (TLL) was immobilized by hydrophobic adsorption on Sepabeads C-18 followed by a physicochemical coating of the lipase surface with a dense layer of PEG. Ethanolysis reactions were carried out using extracted chia seed oil and different lipase derivatives at the same conditions to compare the omega-3 FAEE yield and ratio of reaction products which were analysed by HPLC-ELSD. Furthermore, reutilization cycles of lipase derivatives were studied to evaluate the stability after several reaction cycles to minimize decreasing of catalytic activity.

2. Materials and methods

2.1. Materials

Commercial chia seeds from Bolivia were purchased from Dietica (Cuenca, Spain). Seeds were ground with a particle size less than 500 μm using a grinder (Moulinex-A320R1 700 W) and stored at 4 °C until the oil extraction process. Soluble lipase from *Thermomyces lanuginose* (TLL), *Candida antarctica* B (CAL-B), *Rhizomucor miehei* (RML) and commercial derivative Lipozyme TL IM were kindly donated by Novozymes (Bagsvaerd, Denmark). Commercial TLL immobilized on Immobead 150 was purchased from Sigma-Aldrich. Tested lipases included TLL, RML and CALB that were used as received or modified in the lab as explained below.

Ethanolamine hydrochloride (EDA), p-nitrophenyl butyrate (pNPB), 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC), sodium periodate, dextran 40,000 Da and sodium borohydride were provided by Sigma Chemical Co. (St. Louis, USA). Sepabeads-C18 was kindly donated by Resindion S.R.L. (Rome, Italy). Dialysis Tubing MWCO 12,000–14,000 Da was purchased from Iberlabo S.A. (Madrid, Spain). Methoxypolyethylene glycol amine 2000 Da and 10,000 Da (PEG-NH_2) was from Rapp Polymere (Tuebingen, Germany). Molecular sieves pore size 3 Å (pearl-shaped 2–3 mm) and *n*-hexane was purchased from Scharlau (Barcelona, Spain). The solvents (2,2,4-trimethyl pentane, methyl tert-butyl ether and 2-propanol) used for high-performance liquid chromatography (HPLC) analyses were HPLC-grade and purchased from LABSCAN (Dublin, Ireland). Fatty acid methyl esters standard (Supelco 37 FAME Mix) was from Supelco (Bellefonte, PA, USA). Glyceryl trilinoleate, dioleoylglycerol (mixture of 1,3- and 1,2-isomers), 1-oleoyl-rac-glycerol, oleic acid and ethyl linoleate used as HPLC standards was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and solvents used were of analytical or HPLC grade.

2.2. Immobilization of lipases on Sepabeads-C18 resins

Lipase from *Thermomyces lanuginose* (TLL) was immobilized on Sepabeads-C18 resins by hydrophobic adsorption at low ionic strength (5 mM) in sodium phosphate buffer at 25 °C and pH 7. To follow the immobilization process, the activity of the blank and the supernatant were analysed spectrophotometrically at different times by measuring the absorbance at 348 nm ($\epsilon = 5.150\ M^{-1}\ cm^{-1}$) produced by the release of p-nitrophenol (pNP) by hydrolysis of 0.4 mM p-nitrophenyl butyrate (pNPB) in 25 mM sodium phosphate buffer at pH 7 and 25 °C. After 24 h, immobilization efficiency was 82.5% and enzyme loading of the immobilized preparations was 33 mg of lipase per gram of support.

2.3. Chemical amination of lipases adsorbed on Sepabeads-C18 resins

Chemical amination was performed as previously described (Fernandez-Lorente et al., 2008). Briefly, 1 g of immobilized lipase on Sepabeads-C18 resin was added to 10 mL of 1 M EDA at pH 4.75 under continuous stirring. Solid EDC was added to the suspension to yield a final concentration of 10 mM. After 90 min of gentle stirring at 25 °C, the immobilized-modified lipase preparations were filtered and incubated for 4 h in 0.1 M hydroxylamine at pH 7 and 4 °C (Carraway & Koshland, 1968). Then, the aminated immobilized lipase derivative was filtered and washed with an excess of distilled water.

2.4. Pegylation of lipase derivatives

Immobilized and aminated lipase derivatives were coated with a dense layer of polyethylene glycol. Firstly, dextran with a molecular weight of 40,000 Da was oxidized by the addition of solid sodium periodate in order to increase aldehyde groups. After 90 min of gentle stirring at room temperature, the mixture was dialyzed against distilled water. Secondly, lipase derivatives were added to the dextran-aldehyde solution and reduced with sodium borohydride. Lipase-dextran derivatives were reoxidized and added to a solution with PEG-NH2 overnight. Finally, lipase-dextran-PEG derivatives were washed several times with distilled water. The final modified derivative retained 100% of initial activity when compared with the activity of soluble lipase. The full protocol has been described in Moreno-Perez et al. (2016). Additionally, a scheme of PEGylation process can be seen in Fig. 1 (Supplementary data).

2.5. Drying of lipase derivatives

Lipase derivatives were washed and dried, before their use in enzymatic reactions, with increasing volumes of water, water: acetone and acetone using a sintered glass funnel until derivatives were completely dried.

2.6. Pressurized liquid extraction of chia seed oil

Pressurized liquid extraction was carried out with an ASE 350 DIONEX (Sunnyvale, California) extractor. Oil extraction was performed using 3.00 g of ground chia seeds. Stainless steel extraction cells were used with a volume of 10 mL. Extracts were collected under a nitrogen stream in different vials of 50 mL. Extraction conditions used were performed according to previous studies using hexane as solvent at 90 °C for 10 min of static time achieving a extraction pressure between 1500 and 1600 psi (Castejón et al., 2017; Castejón, Luna & Señoráns, 2018).

2.7. Enzymatic synthesis of fatty acid ethyl esters from chia oil

Enzymatic synthesis of fatty acid ethyl esters was performed as previously described (Moreno-Pérez, Guisan & Fernandez-Lorente, 2013). The reaction mixture was composed by 0.1 g of dried lipase derivative, 0.2 g of molecular sieves pore size 3 Å, ethanol/oil molar ratio of 10:1 and 80% of hexane. The reaction was carried out in glass vials (final volume of 2.5 mL) at 25 °C with constant stirring in an orbital shaker at 200 rpm (Unimax 1010, Heidolph, Germany) under dark conditions. A negative control without lipase derivative was carried out at the same described conditions. The synthesis of fatty acid ethyl esters