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Concentration of docosahexaenoic and eicosapentaenoic acids by enzymatic alcoholysis with different acyl-acceptors

Lorena Martín Valverde, Pedro A. González Moreno, Luis Esteban Cerdán, Elvira Navarro López, Beatriz Castillo López, Alfonso Robles Medina*

Area of Chemical Engineering, University of Almería, 04120 Almería, Spain

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ABSTRACT

The aim of this work was to produce docosahexaenoic (DHA) and eicosapentaenoic acid (EPA) enriched acylglycerols by alcoholysis of tuna and sardine oils, respectively, using isobutanol and 1-butanol as acyl-acceptors. The alcoholysis reactions were catalyzed by lipases Lipozyme® TL IM from *Thermomyces lanuginosus* and lipase QLG® from *Alcaligenes* sp., because these lipases have shown selectivity towards DHA and EPA, respectively. Studies were made to determine the influence of reaction time, alcohol/oil molar ratio, lipase amount and temperature. In the optimized conditions for the alcoholysis of tuna and sardine oils catalyzed by Lipozyme TL IM and lipase QLG, respectively, the DHA and EPA contents were trebled (from 22 to 69% for DHA, and from 19 to 61% for EPA). The stability of both lipases was also determined. Although Lipozyme TL IM is much more stable in isobutanol than in ethanol, with the former the conversion attained after four reaction cycles was about 40% of the initial conversion. In similar conditions, the conversion obtained with lipase QLG was about 88% of the initial conversion. In addition, the separation of DHA enriched acylglycerols and isobutyl esters from an alcoholysis reaction was studied by liquid–liquid fractionation using the ethanol–water–hexane biphasic system. The DHA enriched acylglycerols obtained were 97.6% pure (64.4% DHA).

1. Introduction

Solid evidences about the fact that n-3 polyunsaturated fatty acids (n-3 PUFAs) are essential nutrients have appeared and also that they may favorably modulate many diseases. Docosahexaenoic acid (DHA) is a vital component of the phospholipids of cellular membranes, especially in the brain and retina [1]. n-3 PUFAs possess potent immunomodulatory activities and have anti-inflammatory properties, and therefore, might be useful in the management of inflammatory and autoimmune diseases. Studies indicate that, unlike n-6 fatty acids, n-3 PUFAs have antithrombotic, antiarrhythmic, hypolipidemic and vasodilatory properties [2,3]. Most published studies have focused mainly on the beneficial effects of fish oils in the prevention and treatment of cardiovascular disease [4] and in the immunomodulation process [5]. In many studies the beneficial effect of fish oils was not clearly attributed to EPA or DHA or the combination of both. In this sense, large-scale studies are conditioned by the insufficient availability of oil with a high concentration of one of these n-3 PUFAs and a low concentration of the other.

Omega-3 PUFAs can be obtained from fish oils and marine microalgae and they have been purified using several procedures: urea complexation, chromatography, distillation, low temperature crystallization, supercritical fluid extraction and enzymatic methods [6,7]. The main advantage of the enzymatic method is that low temperatures and non aggressive reagents are used, which allows to maintain the PUFA structure unchanged. However, stable lipases, which are not deactivated in the reaction conditions, are essential for these processes to be economically viable. Moreover, although there is some debate on this issue, it seems that the bioavailability of EPA + DHA from re-esterified triglycerides was superior to the bioavailability of ethyl esters and free fatty acids [6,8]. Besides, Tamai et al. [9] showed that the hypotriglyceridemic effect and bioavailability of DHA when supplemented in the form of diacylglycerol (DHA-DAG) are essentially equivalent to those of DHA supplemented as triacylglycerol (DHA-TAG) or as a fish oil DHA concentrate (DHA-70). Thus, an appropriate strategy for the production of DHA or EPA concentrates could be the alcoholysis reaction catalyzed by lipases, since this reaction produces EPA and DHA enriched acylglycerols.

* Corresponding author. Tel.: +34 950 015065; fax: +34 950 015484.
E-mail address: arobles@ual.es (A.R. Medina).

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Many of the commercially available lipases discriminate against n-3 PUFAs [10], i.e. they have a lower activity on PUFAs than, for example, on saturated and monounsaturated fatty acids. Thus, most of the saturated and monounsaturated fatty acids of fish oil triacylglycerols are converted into esters, whereas PUFAs are concentrated into a mixture of partial acylglycerols. Other lipases are capable of discriminating between EPA and DHA, and these lipases have been used to separate and concentrate EPA and DHA from fish oil highly rich in both PUFAs [11]. The main problem with using lipases to catalyze alcoholysis and esterification reactions is their stability in the presence of short chain alcohols, because some lipases are deactivated when a short chain alcohol, such as methanol or ethanol, is used [12–14]. This negative effect on lipase stability when methanol or ethanol are used as acyl-acceptors arises due to the low solubility of these alcohols in oils and the fact that lipases are inactivated by contact with insoluble methanol or ethanol [15].

In order to avoid this problem, long chain fatty alcohols have been tested in alcoholysis or esterification reactions. Shimada et al. [16] tested short, medium and long chain alcohols in the esterification of free fatty acids from PUFA rich oil, catalyzed by several lipases. These authors found that long chain fatty alcohols, such as decanol and lauryl alcohol, gave the highest esterification conversions and the highest PUFA (especially DHA) concentration in the esterification of tuna oil catalyzed by *Rhizopus delemar* lipase. This result may be due to the higher stability of lipases in these long chain alcohols, which is due to the higher solubility of these alcohols in oils [15]. In fact, *R. delemar* lipase preserved up to 85% of its original activity after catalyzing 47 batch reactions (of 24 h each) in the alcoholysis of fatty acid ethyl esters with lauryl alcohol [17].

The alcohol structure also affects the selectivity of lipases. Miller et al. [18], for example, studied the effect of the alcohol type (branching, primary or secondary alcohols) on the lipase-catalyzed formation of ester bonds, using as catalyst Lipozyme in an organic solvent medium. They observed that isopropanol gave a much lower initial reaction rate (0.42 mM/h/g) than *n*-propanol (10.3 mM/h/g), i.e., primary alcohols reacted much better than secondary ones. This result has also been observed by other authors, such as Deng et al. [19] and Salis et al. [20]. Haraldsson and Kristinsson [12] also found that in the esterification of free fatty acids from sardine oil (12% DHA) with ethanol, *n*-butanol and *n*-hexanol, catalyzed by *Rhizomucor miehei* lipase, the residual free fatty acid mixture contained 54, 69 and 62% DHA, respectively.

In previous works [13,14], the lipases Lipozyme TL IM, from *Thermomyces lanuginosus*, and QLG, from *Alcaligenes* sp., were chosen to concentrate, respectively, DHA and EPA, by ethanolysis of tuna and sardine oils, respectively. These lipases displayed moderate to high ethanolysis activity and appreciable acyl specificity towards DHA and EPA. Specifically, under optimized conditions and for 56% conversion, the ethanolysis of tuna oil catalyzed by Lipozyme TL IM® produced acylglycerols with 45% DHA concentration, which implies a two-fold increase with respect to the DHA initial content of the oil. Lipozyme TL IM is a sn-1,3 specific lipase and therefore both the positional specificity and acyl-specificity play an important role in the DHA concentration [13]. Similarly, also under optimized conditions and for 59% conversion, the ethanolysis of sardine oil catalyzed by lipase QLG® produced acylglycerols with 39% EPA; again a two-fold increase with respect to the initial EPA content of the oil [14]. In this work it was also observed that although lipase QLG shows the highest selectivity towards EPA, it also shows selectivity towards DHA, which indicated the difficulty of finding lipases that only concentrate EPA, separating it from DHA. Despite this, the selectivity of the lipases selected was quite high. However, the use of ethanol (or methanol) had a negative effect on the stability of these lipases [13,14].

For these reasons the aim of the present work was to concentrate DHA and EPA in the acylglycerol fraction by alcoholysis of tuna (22% DHA) and sardine (19% EPA) oils (Table 1), catalyzed by the previously selected lipases Lipozyme TL IM and QLG, respectively. However in this case the alcoholysis reactions were carried out with 1-butanol and isobutanol, and the results obtained with these alcohols were compared with the obtained with ethanol. In this work studies were carried out to determine the influence of reaction time, temperature and alcohol/oil and lipase/oil ratios on the purity and recovery of DHA and EPA in the acylglycerol fraction. Also, the stability of lipases was tested in the optimal conditions of the alcoholysis reaction. Finally the esters were removed from the DHA and EPA enriched acylglycerols by liquid–liquid fractionation.

2. Materials and methods

2.1. Chemicals and lipases

The chemicals used were tuna and sardine oils (kindly donated by Brudy Technology, Barcelona, Spain), absolute dry ethanol (maximum 0.02% water), isobutanol (2-methyl-1-propanol), 1-butanol (puriss, Sigma-Aldrich, St. Louis, MO, USA) and other reagents of analytical grade (Panreac, S.A., Barcelona, Spain). The complete fatty acid composition of tuna and sardine oils is shown in [14]. Table 1 shows that tuna oil is rich in docosahexaenoic acid (DHA, 22:6n3), while sardine oil is rich in eicosapentaenoic acid (EPA, 20:5n-3). This table shows that both oils have a similar composition in saturated, monounsaturated fatty acids and PUFAs. The lipases used to catalyze the alcoholysis reaction were Lipozyme® TL IM from *T. lanuginosus* (Novozymes A/S, Denmark) and lipase QLG® from *Alcaligenes* sp. (Meito Sangyo Co., Ltd., Tokyo, Japan). Lipozyme® TL IM is immobilized on porous silica granulates. It is a sn-1,3 specific lipase and the manufacturer's recommended temperature range is 20–50 °C. Lipase QLG® is immobilized on granulated diatomaceous earth; it is positionally non-specific and its recommended temperature range is 65–70 °C.

2.2. Alcoholysis reaction

Alcoholysis of tuna and sardine oils with ethanol, isobutanol and 1-butanol was conducted as follows: 5 g of oil (Table 1), 980–2060 μL of alcohol (alcohol/oil molar ratio 2.3–4) and 5 or 20% w/w of Lipozyme TL IM with respect to the oil, or 20 or 40% w/w of lipase QLG with respect to the oil. This mixture was placed in 50 mL Erlenmeyer flasks with silicone-capped stoppers in an inert atmosphere of argon or nitrogen. The mixture was incubated at 35 or 20 °C in an orbital shaking air-bath (Incubator 1000, Unimax 1010 Heidolph, Klein, Germany) at 200 rpm for different reaction times (1–96 h). The reactions were stopped by separation of lipase by filtration (glass plate of porosity 4) under vacuum.

The stability study of both lipases with the three alcohols was carried out in 350 mL glass flasks with lipase contained in a cylindrical polypropylene cartridge filter (pore size 50 μm), attached to a vertical rod placed in the center of the flask and attached to the

Table 1
Contents in saturated and mononunsaturated fatty acids, PUFAs, EPA and DHA of total fatty acids of tuna and sardine oil triacylglycerols (wt%) [14].

Fatty acids	Tuna oil	Sardine oil
Saturated	28.7 ± 0.4	29.1 ± 0.4
Monounsaturated	29 ± 0.3	27 ± 0.5
PUFAs	42.3 ± 0.2	43.9 ± 0.2
20:5n3 (EPA)	9.0 ± 0.3	19.0 ± 1.1
22:6n3 (DHA)	22.0 ± 0.8	9.1 ± 0.4

Determined by GC, Section 2.4.