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Concentration of docosahexaenoic acid by enzymatic alcoholysis with different acyl-acceptors, using *tert*-butanol as reaction medium

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ABSTRACT

The aim of this work was to produce docosahexaenoic acid (DHA)-enriched acylglycerols through the alcoholysis of tuna oil (24–26% DHA) using ethanol, dodecanol and isobutanol as the acyl-acceptors. The alcoholysis reaction was catalyzed using *Thermomyces lanuginosus* lipase (Lipozyme TL IM) and was carried out both in solvent-free medium and using *tert*-butanol to increase lipase stability. Studies were carried out to determine the influence of the reaction time and the lipase/oil ratio. The tuna oil's DHA content was trebled (from 26 to 78 wt%) using isobutanol as the acyl acceptor while carrying out the alcoholysis reactions in solvent-free medium. However, under these conditions, the lipase was partially deactivated given that the conversion rate decreased by around 80% after catalyzing six successive reactions using the same lipase batch. With *tert*-butanol as the reaction medium, the lipase was more stable but the resulting DHA concentrations were lower: 56% with isobutanol and dodecanol (59% with dodecanol in solvent-free medium) and 42.5% with ethanol (47.5 % without solvent).

1. Introduction

Omega-3 polyunsaturated fatty acids (n-3 PUFAs), especially eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), have a significantly positive influence on human health, including on atherosclerosis, coronary heart disease, inflammatory disease and behavioural disorders [1,2]. It is not clear if both EPA and DHA are similarly important in cardiovascular protection because both species coexist in most oils. Some data obtained from humans subjects suggest that DHA is more effective in lowering blood pressure and improving vascular function [3]. DHA is a vital component in the phospholipids of cellular membranes, especially in the brain and retina. There is considerable consensus on its consumption benefits for proper nervous system development during pregnancy and early life stages [4–6]. For this reason, it is used as a dietary supplement during pregnancy and in infant formula manufacture. Other studies suggest that DHA might be useful in the prevention and treatment of certain mental disorders such as attention deficit hyperactivity disorder [5].

Omega-3 PUFAs can be obtained from fish oils and marine microalgae. They have been purified using several procedures, such as urea complexation, chromatography, distillation, low temperature crystallization, supercritical fluid extraction, and enzymatic methods [6–8]. The main advantage of the enzymatic method is that low temperatures and non-aggressive reagents are used, which leave the DHA structure unchanged. Many commercially-available lipases discriminate against n-3 PUFAs [9]; that is to say, they attack PUFAs at a lower velocity than they do, for example, saturated and monounsaturated fatty acids. Thus, if lipase-catalyzed ethanolysis is carried out on triacylglycerols, most of the saturated and monounsaturated fatty acids of the fish oil triacylglycerols are converted into ethyl esters whereas PUFAs remain concentrated in the acylglycerol fraction. Martín Valverde et al. [10] showed that Lipozyme TL IM, Lipozyme RM IM and lipase from *Thermomyces lanuginosus* are able to concentrate DHA in the acylglycerol fraction. The main problem with using lipases to catalyze alcoholysis and esterification reactions is their low stability in the presence of short-chain alcohols (methanol and ethanol), since these alcohols present low solubility in oils and the lipases are deactivated on contact with the non-solubilized short-chain alcohols [8,10–13]. In order to avoid this problem, several alternatives have been put forward such as the step addition of methanol [14], lipase immobilization [15], utilizing solvents such as *tert*-butanol [16] as the reaction medium, and the use of long-chain fatty alcohols in alcoholysis or esterification reactions. Shimada et al. [17] tested short, medium and long-chain alcohols in the esterification of free fatty

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acids from tuna oil, catalyzed by *Rhizopus delemar* lipase. These authors found that long-chain fatty alcohols, such as decanol and lauryl alcohol, provided the highest esterification conversions and the highest PUFA (especially DHA) concentration. This may be due to the greater stability of lipases in these long-chain alcohols resulting from the higher solubility of these alcohols in oils [13]. Haraldsson and Kristinsson [11] 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 [8,10], the *T. lanuginosus* lipase (Lipozyme TL IM) was chosen to concentrate DHA through tuna oil alcoholysis with ethanol, butanol and isobutanol. In solvent-free medium, isobutanol and 1-butanol deactivate the lipase less than ethanol does, and for this reason, higher conversions and DHA concentrations were obtained with butanol and isobutanol than they were with ethanol. Thus, under optimized conditions and for a 74% conversion, the isobutanolysis of tuna oil catalyzed by Lipozyme TL IM trebled the tuna oil's DHA concentration (22% DHA), otaining acylglycerols with 69% DHA, along with a DHA recovery of almost 78%. However, even though Lipozyme TL IM is much more stable using isobutanol than ethanol, with the former, the conversion achieved after catalyzing four consecutive alcoholysis reactions was about 40% of the initial rate [8].

For the above-mentioned reasons, the aim of the present work was to concentrate DHA in the acylglycerol fraction through tuna oil (24–26 % DHA) alcoholysis catalyzed using the previously selected Lipozyme TL IM lipase. However, once the conditions for concentrating DHA have been achieved, it is important to maintain lipase activity for as long as possible; in many cases, this means changing the prior optimal conditions for concentrating DHA. Consequently, in this work, we sought a compromise in the conditions between that which is optimal in concentrating DHA and that providing greater lipase stability. In this respect, the alcoholysis reactions were carried out with ethanol, dodecanol and isobutanol, whilst using *tert*-butanol as the reaction medium to avoid (or minimize) lipase deactivation.

2. Materials and methods

2.1. Chemicals and lipases

The chemicals used were tuna oil (kindly donated by Brudy Technology, Barcelona, Spain), absolute dry ethanol (maximum 0.02% water), isobutanol (2-methyl-1-propanol) (puriss; Sigma–Aldrich, St.Louis, MO, USA), 1-dodecanol (reagent grade, 98%; Sigma–Aldrich, St.Louis, MO, USA), tert-butanol (2-methyl-2-propanol) and other reagents of analytical grade (Panreac, S.A., Barcelona, Spain). The complete fatty acid composition of tuna oil is shown in [12]. This oil contains between 24.3 and 26.2% docosahexaenoic acid (DHA). The lipase used to catalyze the alcoholysis reaction was Lipozyme® TL IM from *T. lanuginosus* (Novozymes A/S, Denmark). This lipase is immobilized on porous silica granulates. It is an sn-1,3 specific lipase and the manufacturer's recommended temperature range is 20–50 °C.

2.2. Alcoholysis reaction

We conducted the tuna oil alcoholysis using ethanol, isobutanol and 1-dodecanol, with or without *t*-butanol as the co-solvent, as follows: 5 g of tuna oil, 980–2060 μL of alcohol (alcohol/oil molar ratio 2.3–6), 3.3 mL of t-butanol (0.66 mL/g oil) and 5 or 20 wt% of Lipozyme TL IM with respect to the oil. This mixture was placed in 50 mL Erlenmeyer flasks with silicone-capped stoppers in an inert argon or nitrogen atmosphere. The mixture was incubated at 35 °C in an orbital shaking air-bath (Incubator 1000, Unimax 1010Heidolph, Klein, Germany) at 200 rpm for different reaction times (1–72 h). The reactions were stopped for lipase separation by filtration (a glass plate with porosity level 4) inside a vacuum.

We carried out the lipase stability study with the three alcohols tested in 350 mL glass flasks, with the 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 fixed to the flask cap. This filter allowed reactants and products to pass but retained the immobilized lipase inside the cartridge. In this case the reaction mixture consisted of 100 g of tuna oil, a 2.3:1 alcohol/oil molar ratio, 65.5 mL of *t*-butanol (0.655 mL/g oil) and 5 g of Lipozyme TL IM (5%w/w, with respect to the oil). The reaction was carried out at 35 °C in the orbital shaking air-bath, at 200 rpm for 24 h. The same lipase batch contained in the cartridge filter was used to catalyze successive reactions, and between turns, the lipase was washed with hexane and dried in nitrogen stream.

As all the reactions and their corresponding analyses were carried out in duplicate, each datum is the arithmetic mean of four experimental data sets; SDs were always below 15%.

2.3. Product analysis and determination of DHA purity and yield in the acylglycerol fraction

The alcoholysis reaction mixtures contained acylglycerols (mono-, di- and triacylglycerols) and ethyl, isobutyl or dodecyl esters. We carrier out the qualitative identification of these species using thin-layer chromatography (TLC) following the procedure described in [18]. Using this technique, we found that free fatty acids either did not appear, or appeared in negligible amounts with respect to the other compounds present in the sample. We carried out the quantitative determination of fatty acids in acylglycerols using the procedure described in [12]. Firstly, we analyzed 30 μL of non-methylated samples using gas chromatography (GC); and, therefore, in this case, only the ethyl, isobutyl or dodecyl esters were quantitatively analyzed, since acylglycerols are not sufficiently volatile to be analyzed using this technique. Another test tube containing 0.4 mL of sample, 0.4 mL of hexane and 0.2 mL of internal standard solution was methylated by direct transesterification with acetyl chloride/methanol (1:20 v/v) following the method of Rodríguez Ruiz et al. [19]. In this case, we quantitatively determined all the fatty acids as methyl esters. Thus, the amount of each fatty acid present in the sample as acylglycerols was determined from the difference between the amount of fatty acid in the methylated sample and that in the esters (the non-methylated sample).

We analyzed methyl, ethyl, isobutyl and dodecyl esters in all these samples with an Agilent Technology 6890 gas chromatograph (Avondale, PA, USA) using the procedure described in [12]. The oven temperature program for methyl and ethyl esters was as follows: 150 °C for 3 min, increasing from 150 to 240 °C at 7.5 °C/min, and finally 240 °C for 12 min. The program for the isobutyl esters was: 150 °C for 3 min, increasing from 150 to 240 °C over 5 °C/min, and finally 240 °C for 19 min. The program for the dodecyl esters was: 150 °C for 3 min, increasing from 150 to 240 °C at 7.5 °C/min, and finally 240 °C for 55 min. Matreya (Pleasant Gap, PA) n-3 PUFA standard (catalog number 1177) was used for the qualitative determination of fatty acids. We calculated the fatty acid masses as indicated in [12].

The alcoholysis reaction conversion was expressed as the weight ratio:

$$\text{Conversion} = \frac{\text{fatty acid amount in esters}}{\text{total amount of fatty acids}} \quad (1)$$