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Synthesis of 2-monoacylglycerols rich in polyunsaturated fatty acids by ethanolysis of fish oil catalyzed by 1,3 specific lipases

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ABSTRACT

This paper studies the synthesis of 2-monoacylglycerols (2-MAG), rich in polyunsaturated fatty acids (PUFAs) by alcoholysis of fish oils with ethanol, catalyzed by 1,3 specific lipases. Cod liver and tuna oils were used as PUFA rich substrates, and the influence of the following variables was studied: (i) ethanol purity (commercial, 96% (v/v); absolute, 0.5% water, and absolute dry, 0.02% water), (ii) the lipase used (Novozym 435 from *Candida antarctica*; lipase D from *Rhizopus oryzae* and lipase EU O93 or Rd, from *Rhizopus delemar*), (iii) ethanol/oil molar ratio, (iv) treatment intensity (TI = lipase amount × reaction time/oil amount), and (v) solvent/oil ratio. High yields in 2-MAG (90.3%) were obtained with 96% ethanol and lipase Novozym 435, which behaves as 1,3 specific when a great excess of ethanol is used. Nevertheless, the water accompanying ethanol produces free fatty acids (FFA), which make the posterior separation of 2-MAG difficult. 2-MAG yield of 75% was reached with lipases D and Rd immobilized on Accurel MP-1000, using absolute ethanol; in these conditions free fatty acid formation and acyl-migration were not observed. This yield was obtained by using an ethanol/oil molar ratio of 11 and 6 mL of acetone per gram of oil; the alcoholysis rate reached a minimum value when no solvent (acetone) was used; nevertheless, the 2-MAG yield at equilibrium did not seem to be influenced by the acetone/oil ratio.

The separation of 2-MAG from ethyl esters was carried out by silica gel chromatography and solvent extraction. The first method obtained 2-MAG with 96% purity and 85% yield while smaller values were obtained by the second method (89% purity and 77% yield). Nevertheless, much higher amounts of 2-MAG can be obtained by the latter using less volume of solvents.

Finally, the positional analysis of the two initial oils revealed that all the PUFAs that were initially located in position 2 of triacylglycerols stay in this position in the final 2-MAG. This revealed that lipases D and Rd are completely 1,3 specific and that no acyl-migration occurred.

1. Introduction

The important role of *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs) in human health has been widely recognized. Eicosapentaenoic acid (EPA, 20:5*n*3) can affect the circulatory system and can help to prevent arteriosclerosis and thrombosis [1], and docosahexaenoic acid (DHA, 22:6*n*3) is particularly important for brain development; a normal adult brain contains more than 20 g of DHA [2]. *n*-3 PUFAs from both seafood and plant sources may reduce coronary heart disease risk [3] and exert ischemic anti-arrhythmic effects [4].

Quantitatively speaking, triacylglycerols (TAG) are the most important lipid components of the Western adult human diet. The structure and fatty acids composition of TAG affect their absorption and the distribution of fatty acids in the organism [5]. The structured triacylglycerols (STAG) contain medium chain fatty acids (M) located at positions 1 and 3 of the glycerol backbone and functional long chain polyunsaturated fatty acids (L) located at position 2 (MLM). These STAG are claimed to benefit the immune function and to help improve lipid clearance from the bloodstream [6].

MLM TAG with EPA and DHA in position 2 were more readily absorbed sources of these PUFAs than other TAG with similar fatty acid compositions but with a random fatty acid distribution [7]. STAG may have the potential to prevent hypertriglyceridemia and obesity caused by consumption of a high-fat diet [8]. It was suggested that the daily intake of STAG in the diet could result in weight loss and less accumulation of fats, as well as a reduction in serum cholesterol [9].

The simplest and most direct route for the synthesis of MLM type STAG is acidolysis between long-chain TAG and medium-

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chain FFA, catalyzed with a 1,3 specific lipase [10–16]. Lipases offer high catalytic efficiency, specificity and selectivity by incorporation of the required acyl-group into a specific position of the native TAG. Nevertheless, acyl-migration is a major problem in the synthesis of STAG in batch reactors, causing a decrease in yield. The high substrate/enzyme ratio requires long reaction times to reach equilibrium, and consequently results in acyl-migration.

In order to avoid this problem, one of the alternatives is a process in two-steps [17–21]. First, 2-monoacylglycerols (2-MAG) are obtained from oils rich in functional fatty acids by enzymatic alcoholysis using 1,3 specific lipases. Then, medium-chain fatty acids are added in the extreme positions of 2-MAG by enzymatic esterification also using 1,3 specific lipases. For example, this procedure was also used for the synthesis of MLM type STAG, with oleic and linoleic acid (from peanut oil) in position 2, and caprylic acid in positions 1 and 3, using *Rhizomucor miehei*, *Rhizopus delemar* and *Rhizopus javanicus* lipases [18]. The STAG obtained from peanut oil contained more than 90% caprylic acid in positions 1 and 3, whereas position 2 consisted of 98.5% long chain fatty acids (oleic and linoleic acid). The best results were obtained with methyl-*tert*-butyl ether (MTBE) as solvent and the resulting mixture was purified in a chromatographic column. For these authors, the support used for the immobilization of lipase seems to be related with the phenomenon of acyl-migration. The immobilized *Candida antarctica* lipase (Novozym 435), which only behaves as 1,3 specific with a great excess of ethanol, presented high activity toward the polyunsaturated TAG [19]; starting with a heterogeneous substrate (bonito oil) and absolute ethanol to carry out the alcoholysis, an intermediate stage of 2-MAG purification was included to eliminate the esters formed prior the later esterification of the 2-MAG [20].

Other authors [22] used Lipozyme IM and lipase D as catalysts for the alcoholysis of TAG and the results obtained were both qualitatively and quantitatively dependent on the lipase used. When using Lipozyme the process was controlled by acyl-migration whereas with lipase D no acyl-migration occurred.

The present work outlines the results of the research to synthesize 2-MAG using different lipases and substrates. This process was first developed in dispersion reactors, the final objective being to apply the optimal operational conditions to produce 2-MAG in a continuous reactor as an intermediate stage of producing STAG for human consumption. The recovery and purification of 2-MAG is also studied by using low toxicity substrates and solvents.

2. Materials and methods

2.1. Oils

Cod liver oil (provided by Acofarma, Barcelona, Spain) and tuna oil (donated by Brudy Technologies, S.L., Barcelona, Spain) were used as substrates for the enzymatic reactions. Table 1 shows the fatty acid composition of these oils. Verification by thin layer chromatography showed that neither of them contained partial acylglycerols.

2.2. Lipases and chemicals

The lipases used were Novozym 435 (N-435) from *C. antarctica* (donated by Novozymes, Denmark), lipase D from *Rhizopus oryzae* (Amano Pharmaceutical Co., Nagoya, Japan), lipase Rd or lipase EU 093 from *R. delemar* (Europe Bioproducts, Cambridge, United Kingdom).

Analytical grade ethanol (96%, v/v), ethanol absolute (0.5% water) and ethanol absolute dry (0.02% water) were used as substrates of alcoholysis and analytical grade acetone (Panreac S.A., Barcelona, Spain) was used as solvent. All other chemicals (of analytical grade or better) were also obtained from commercial sources.

2.3. Immobilization of lipases

Lipases D and Rd were immobilized on Accurel MP 1000 (Akzo Nobel Faser, Obernburg, Germany) following the procedure described by Soumanou et al. [18], modified by Hita et al. [16]. 1 g of lipase powder was dissolved in 25 mL of phosphate buffer (pH 6.0, 20 mM); the solution was added at room temperature to a mixture of 1.5 g support and 5 mL ethanol; after shaking for 8 h at 150 rpm (Inkubator 1000, Unimax 1010 Heidolph, Klein, Germany), 5 mL acetone was added at −24 °C. The immobilized lipase was recovered by filtration, washed three times with phosphate buffer (pH 6.0, 20 mM) at 0 °C, dried under vacuum for 48 h and stored at 5 °C until use.

2.4. Alcoholysis reaction

In this reaction the oil (TAG) reacts with ethanol in the presence of solvent and a 1,3 specific lipase, to give MAG, diacylglycerols (DAG) and ethyl esters. In the final reaction mixture there are also ethanol and TAG which have not reacted.

$$\text{Oil(TAG)} + \text{Ethanol} \xrightarrow[\text{Solvent}]{sn-1,3\ \text{specificlipase}} \text{MAG} + \text{DAG} + \text{ethyl esters}$$

A reaction mixture consisted of 500 mg oil, 500 mg ethanol, 3 mL acetone (6 mL/g oil) and 60 mg immobilized lipase (D or Rd on MP-1000). This was placed in 50-mL Erlenmeyer flasks with silicone-capped stoppers under inert atmosphere. The mixture was incubated at 37 °C and agitated in an orbital shaker, with temperature controlled by air, at 200 rpm (Inkubator 1000, Unimax 1010 Heidolph, Klein, Germany). When lipase N-435 was used the reaction mixture contained 750 mg oil,

Table 1
Fatty acid composition of cod liver and tuna oils and fatty acid composition in position 2 (% of total fatty acid weight) of TAG of these oils determined by the pancreatic lipase and the Shimada et al. [29] methods

Fatty acids	Cod liver oil			Tuna oil		
	Oil	Position 2 pancreatic lipase	Position 2 Novozym-435	Oil	Position 2 pancreatic lipase	Position 2 Novozym-435
14:0	3.1	7.3	6.9	4.6	7.2	6.9
16:0	10.0	18.2	16.1	19.5	21.1	19.3
16:1*n*7	7.5	7.4	7.5	6.8	6.8	6.9
16:2*n*4	0.5	0.9	1.0	1.2	1.1	1.4
16:3*n*4	0.3	0.5	n.d.	0.3	0.3	0.7
18:0	2.3	0.5	n.d.	5.6	1.6	1.6
18:1*n*9	17.3	9.4	9.6	14.6	10.6	9.3
18:1*n*7	5.4	1.4	1.2	2.8	1.8	1.7
18:2*n*6	1.3	2.1	1.5	1.8	1.7	1.8
18:4*n*3	1.6	2.8	2.3	0.9	1.3	1.5
20:1*n*9	12.9	5.4	6.3	3.1	2.2	1.6
20:4*n*6	0.4	0.7	n.d.	2.1	2.1	2.1
20:4*n*3	0.7	0.5	n.d.	n.d.	n.d.	n.d.
20:5*n*3	9.5	10.8	9.0	7.5	7.5	6.8
22:1*n*9	9.5	4.8	5.3	1.9	1.1	n.d.
21:5*n*3	0.5	n.d.	n.d.	0.5	0.8	n.d.
22:5*n*3	1.3	2.1	2.3	1.4	2.1	2.3
22:6*n*3	11.1	23.4	30.1	22.1	27.1	35.9
Others	4.8	2.0	1.2	3.3	3.5	0.8