Biochemical Engineering Journal 46 (2009) 257–264

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Production of structured triacylglycerols by acidolysis catalyzed by lipases immobilized in a packed bed reactor

Estrella Hita, Alfonso Robles*, Belén Camacho, Pedro A. González, Luis Esteban, María J. Jiménez, María M. Muñío, Emilio Molina

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

ARTICLE INFO

Article history:
Received 10 March 2009
Received in revised form 21 May 2009
Accepted 24 May 2009

Keywords:
Structured lipids
Structured triacylglycerols
Acidolysis
Docosohexaenoic acid (DHA)
Caprylic acid
Immobilized lipase
Packed bed reactor
Tuna oil
Lipase from *Rhizopus delemar*
Palatase 20000L

ABSTRACT

The aim of this work was to produce structured triacylglycerols (STAGs), with caprylic acid located at positions 1 and 3 of the glycerol backbone and docosohexaenoic acid (DHA) at position 2, by acidolysis of tuna oil and caprylic acid (CA) catalyzed by lipases Rd, from *Rhizopus delemar*, and Palatase 20000L from *Mucor miehei* immobilized on Accurel MP1000 in a packed bed reactor (PBR), working in continuous and recirculation modes. First, different lipase/support ratios were tested for the immobilization of lipases and the best results were obtained with ratios of 0.67 (w/w) for lipase Rd and 6.67 (w/w) for Palatase. Both lipases were stable for at least 4 days in the operational conditions. In the storage conditions (5 °C) lipases Rd and Palatase maintained constant activity for 5 months and 1 month, respectively.

These catalysts have been used to obtain STAGs by acidolysis of tuna oil and CA in a PBR operating with recirculation of the reaction mixture through the lipase bed. Thus, STAGs with 52–53% CA and 14–15% DHA were obtained. These results were the basis for establishing the operational conditions to obtain STAGs operating in continuous mode. These new conditions were established maintaining constant intensity of treatment (IOT, lipase amount × reaction time/oil amount). In this way STAGs with 44–50% CA and 17–24% DHA were obtained operating in continuous mode. Although the compositions of STAGs obtained with both lipases were similar, Palatase required an IOT about four times higher than lipase Rd.

To separate the acidolysis products (free fatty acids, FFAs, and STAGs) an extraction method of FFAs by water–ethanol solutions was tested. The following variables were optimized: water/ethanol ratio (the best results were attained with a water/ethanol ratio of 30:70, w/w), the solvent/FFA–STAG mixture ratio (3:1, w/w) and the number of extraction steps (3–5). In these conditions highly pure STAGs (93–96%) were obtained with a yield of 85%. The residual FFAs can be eliminated by neutralization with a hydroethanolic KOH solution to obtain pure STAGs. The positional analysis of these STAGs, carried out by alcoholysis catalyzed by lipase Novozym 435, has shown that CA represents 55% of fatty acids located at positions 1 and 3 and DHA represents 42% of fatty acids at position 2.

1. Introduction

The production of structured triacylglycerols (STAGs) for clinical [1,2] and nutritional purposes [3] is a subject of interest. Most attention has focused on triacylglycerols (TAGs) with medium-chain fatty acids (M) located in positions 1 and 3 of the glycerol backbone and a functional long-chain polyunsaturated fatty acid (PUFA) in position 2 (MLM structure). This composition and structure is interesting because absorption of the fatty acids depends on their type and on their position in the TAG molecule. Thus, pancreatic lipase, which is 1,3-specific, hydrolyzes the TAG of the diet giving free fatty acids and 2-monoacylglycerol (2-MAG) [4]; in addition this lipase shows higher activity toward medium-chain fatty acids than toward long-chain ones, especially PUFAs [5,6]. The liberated medium-chain free fatty acids are directly absorbed into the portal vein and used to provide energy [7,8], while the 2-MAG (with the essential long-chain fatty acid) are well absorbed via the lymphatic route [4] and are often used in biosynthesis [7,9]. In this way STAGs may provide a balanced nutrition.

The aim of this work is to obtain STAGs rich in caprylic acid (CA), located in positions 1 and 3 of the glycerol backbone, and docosohexaenoic acid (DHA) in position 2. This fatty acid is being used as an additive to the milk formula for premature children, due to its importance in the development of the central nervous system [1,10].

The simplest and most direct route for the synthesis of STAGs of MLM type is the acidolysis between long-chain TAGs (with a high content of a functional fatty acid in position 2, L), and a medium-

* Corresponding author. Tel.: +34 950015065; fax: +34 950015484.
E-mail address: arobles@ual.es (A. Robles).

1369-703X/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bej.2009.05.015

chain free fatty acid (M), catalyzed with a 1,3-specific lipase [11–18]. Lipases offer high catalytic efficiency, specificity and selectivity by incorporation of the required acyl group into a specific position of native TAGs. In addition, the process takes place in very mild conditions that maintain the structure of the functional fatty acid unaltered.

The production of STAGs by acidolysis has been studied in our laboratory [17–19]. The first catalyst used [17,18] was Lipozyme IM (Novo Nordisk A/S, Denmark), which is a 1,3-specific lipase, immobilized on an ion exchange resin. However, this resin may catalyze the acyl-migration of the long-chain acyl groups in the DAGs (which are intermediates in the acidolysis reaction) from position 2 to position 1 or 3 [20,21]. This undesired reaction eliminates the long-chain acyl group from position 2, decreasing the yield in STAGs of MLM type. In a posterior work [19] lipases Rd, from *Rhizopus delemar*, D, from *Rhizopus oryzae*, and AK, from *Pseudomonas fluorescens* were used immobilized on Accurel MP1000; this support is microporous polypropylene, which lacks superficial charges and as a result no acyl-migration was observed. With these catalysts STAGs were obtained with over 50% CA content at positions 1 and 3 and over 13% DHA content at position 2. These results were obtained immobilizing the lipase in a packed bed reactor (PBR) through which the reaction mixture was recirculated (operation in discontinuous mode).

The separation of the reaction products is an important aspect, which is often afforded less importance. The major products of an acidolysis reaction are FFAs and TAGs. The separation of these products is usually carried out by neutralization of FFAs with a hydroalcoholic solution of KOH or NaOH (forming soaps soluble in the hydroalcoholic phase) and extraction of TAGs by hexane [19]. However, this method can give low TAG recovery yields high amounts of FFAs lead to the formation of many soaps, in which a considerable proportion of TAGs remains occluded. In this work the separation of FFAs and TAGs has been tested by extraction of the former with water–ethanol mixtures. In this method the most important variable to optimize is the water/ethanol ratio, which depends on the type of FFAs and TAGs [22,23].

This work, therefore, has four main objectives: (i) to immobilize the lipases Rd, from *R. delemar*, and Palatase 20000L, from *Mucor miehei*, on Accurel MP1000, (ii) to determine the activity and stability of these immobilized lipases, (iii) to obtain STAGs of MLM type by acidolysis of tuna oil (highly rich in DHA) and CA, catalyzed by these lipases immobilized in a PBR operating in discontinuous and continuous modes, and (iv) to test the purification of STAGs by extraction of FFAs by water–ethanol mixtures.

2. Materials and methods

2.1. Lipases and chemicals

The lipases used were: Palatase 20000L from *M. miehei* (kindly donated by Novozymes A/S, Bagsvaerd, Denmark) and lipase Rd from *R. delemar* (Europa Bioproducts Ltd, Cambridge, UK). These lipases are 1,3-specific. The support used to immobilize these lipases was Accurel MP1000 (Membrana GmbH, Obernburg, Germany), which is a microporous support of polypropylene with a particle size ranging between 0.4 and 1 mm.

For the acidolysis reactions the following chemicals were used: caprylic acid (CA, 8:0, over 98% pure and a molecular weight of 144.2 g/mol, Sigma–Aldrich, St. Louis, MO, USA), cod liver oil (Acofarma, Terrassa, Barcelona, Spain), tuna oil (with a DHA (22:6n3) content of 20%, kindly donated by Brudy Technology S.L., Barcelona, Spain) and analytical grade hexane (95% purity, Panreac S.A., Barcelona, Spain). Table 1 shows the fatty acid composition of the cod liver and tuna oils, neither of which contained partial acylglycerols, as verified by thin layer chromatography.

Table 1
Fatty acid composition of cod liver and tuna oils (mol%).

Fatty acid	Cod liver oil	Tuna oil
14:0	4.7	4.8
16:0	13.1	20.8
16:1n7	9.4	7.0
16:2n4	1.5	1.3
16:4n1	–	–
18:0	2.8	5.7
18:1n9	20.8	15.9
18:1n7	6.2	3.0
18:2n6	1.3	1.9
18:3n3	0.2	0.7
18:4n3	1.5	1.1
20:1n9	11.4	3.0
20:2n6	–	0.4
20:3n6	–	0.3
20:4n6	–	2.1
20:4n3	–	0.6
20:5n3 (EPA)	8.6	7.3
22:1n9	8.2	2.0
21:5n3	–	0.4
22:5n3	1.2	1.7
22:6n3 (DHA)	9.2	20.1
Average molecular weight (Da)	910.0	994.4

2.2. Immobilization of lipases

The lipases were immobilized on Accurel MP1000 at different lipase/support ratios, following the procedure described by Soumanou et al. [24]: different amounts of lipase (between 1 and 10 g, Table 2) were dissolved in 25 ml phosphate buffer (20 mM, pH 6). The solution was added to 1.5 g Accurel MP1000 previously mixed with 5 ml ethanol. This mixture was stirred at 150 rpm at room temperature for 8 h, after which 5 ml chilled acetone (−15 °C) was added. The immobilized lipase was collected by filtration, washed three times with phosphate buffer (20 mM, pH 6.0), dried at room temperature under vacuum for 48 h, and stored at 5 °C until use. The lipase activity was measured by acidolysis reactions in the batch reactor as described below.

2.3. Acidolysis in the batch reactor

The reaction mixture consisted of cod liver oil, 150 mg; CA, 150 mg; hexane, 5 ml and immobilized lipase, 125 mg. These amounts determined a CA/cod liver oil molar ratio of $m_0 = 6$. This reaction mixture was placed in 50-ml Erlenmeyer flasks with silicone-capped stoppers. The mixture was incubated at 40 °C and agitated in an orbital shaking air-bath at 400 rpm for 24 h (Inkubator 1000, Unimax 1010 Heidolph, Klein, Germany). The reaction was stopped by separation of lipase by filtration and the reac-

Table 2
Influence of the lipase and the lipase/support ratio on the molar fraction of CA incorporated to the TAG of cod liver oil (F_M).

Lipase	Lipase/support ratio (w/w)	F_M (mol%)
Rd from *Rhizopus delemar*	0.25	6.3
	0.67	47.9
PALATASE 20000L from *Mucor miehei*	0.67[a]	14.0
	1.33[a]	34.1
	2.67[a]	37.4
	4.00[a]	42.2
	5.33[a]	45.7
	6.67[a]	48.6

[a] Weight of protein solution supplied by the manufacturer.