Lipase Catalyzed Ethanolysis of Fish Oil in Supercritical Carbon Dioxide

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ABSTRACT

Ethanolysis of a mixture of tuna and sardine oil catalysed by lipase Lipozyme 435 (*Candida antarctica* lipase B) has been studied in supercritical carbon dioxide as reaction medium. The reaction products were analysed by Normal Phase-High Performance Liquid Chromatography (NP-HPLC) and High Temperature Gas Chromatography (HTGC). The reaction has been performed in a stirred tank reactor operating in discontinuous mode at 323.15 K and 110 bar. The effect of initial molar reactant ratio ethanol:fish oil has been varied in the range 7:1 to 76:1. It can be observed that an increase in the amount of ethanol caused enzyme inhibition which decreased the reaction rate of ethanolysis. A maximum in the monoacyglyceride (MAG) production has been observed for some kinetics. At this point the different reaction products were separated by HPLC and collected to determine the fatty acid (FA) profile of each reaction product by capillary Gas Chromatography (GC). Almost 60% wt. of the FA collected in the MAG fraction was found to be docosahexaenoic acid, DHA.

INTRODUCTION

An adequate intake of omega 3 polyunsatturated fatty acids (n-3 PUFAs) such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) is recommended in a healthy diet. n-3 PUFAs are essential for normal growth and development of the brain and the nervous system and their health benefits are well established [1]. Therefore, the production of n-3 PUFA concentrates from different sources, such as fish oil, has drawn the attention of the pharmaceutical and food industry in the last decades.

Since PUFAs are easily oxidized to become hydroperoxydes, they should be preserved in the forms of acylglycerides or esters [2]. To obtain these fish oil derivatives, different methods are used including: alcoholysis, interesterification and acidolysis [3]. In this work, ethanolysis of fish oil has been performed to convert triacylglycerols (TAG) into fatty acid ethyl ester (FAEE) and di or monoacylglycerides (DAG or MAG). Among these forms, MAG has been shown as the most desirable because of its higher bioavailability and stability [4], [5].

Conventional chemical methods of lipid modification have low selectivity and undesirable side reactions may occur. As an alternative, lipases have been used as biocatalysts [6–8]. Since n-3 PUFAs are usually bound at the *sn*-2 position, the use of 1,3-regiospecific lipases as biocatalyst can result in monoglycerides containing high amounts of n-3 PUFAs. The same may occur with certain lipases that present steric impediments to reach the ester bond between glycerol and long unsaturated fatty acids, such as EPA and DHA [9].

To improve the mutual solubility of the reactants and help to create a homogeneous reaction medium, different organic solvents have been used as reaction media [10]. However organic solvents are not ecologically friendly and additional purification steps are needed to ensure the safety of the final products. The use of SC-CO_2 as reaction medium provides many advantages over conventional organic solvents, such as higher diffusivity and lower viscosity.

Moreover, the solvating power of SCFs is strongly density-dependent and it can be easily controlled by variation of temperature and pressure, allowing the fractionation and separation of reaction products. Several studies have taken advantage of these properties to conduct enzymatic catalyzed reactions such as oxidation, hydrolysis, trans-esterification, esterification and enantioselective synthesis in SC-CO_2 medium [11].
In this study, the immobilized lipase Lipozyme 435 from *Candida antarctica* was used to catalyse the ethanolysis of a mixture of tuna and sardine refined oil under SC-CO_2. The effect of operating parameters on the reaction, such as reaction time and initial molar reactant ratio, ethanol:fish oil, has been analysed. For some kinetics, a maximum in the MAG production has been observed. At this reaction time, analysis of the fatty acid (FA) profile for the different reaction products (TAG, EE, MAG and DAG) has been performed by capillary GC to determine the FA distribution.

MATERIALS AND METHODS

Materials

Commercial immobilized lipase, Lipozyme 435 (*Candida antarctica* lipase B immobilized on a macroporous resin) was kindly donated by Novozymes (Bagsvaerd, Denmark). Refined fish oil was kindly provided by AFAMSA S.A. (Pontevedra, Spain) being a mixture of tuna (*Thunnus* sp.) and sardine (*Sardina pilchardus*) oil. Absolute ethanol (99.9 %) was purchased from Merck KGaA. Carbon dioxide (99.9%) was supplied by Carburos Metálicos S.A. (Spain). All other chemicals used in different analyses were of analytical or HPLC grade.

Ethanolysis of fish oil in SC-CO_2

A schematic diagram of the experimental apparatus used for ethanolysis of fish oil by immobilized lipase under SC-CO_2 was shown in Figure 1. The reactor was made by stainless steel (SS-316), having an internal volume of 500 mL. The reactor temperature was controlled by an electric resistance heater and a thermocouple. Ethanol was added into the reactor together with the enzyme and oil. The reactor was then closed and CO_2 was pressurized into the reactor by means of a high pressure pump up to the desired pressure (110 bar), which was maintained by digital pressure controller. The reaction medium was stirred by means of a magnetic stirrer at 700 rpm. The reaction was performed for 24 h. A system of valves allows taking samples of the reaction medium at periodic intervals. The sampling line was washed with hexane and air-dried after every sampling. Samples have been analysed by HPLC and HTGC.

Analysis of fish oil

The fish oil used in this work was analysed by gas chromatography to determine the fatty acid profile by the AOAC method [12]. The fatty acid methyl esters were firstly prepared and then analysed in a Hewlett Packard gas chromatograph (6890N Network GC System) equipped with an auto-sampler (7683 B series) and a flame ionization detector (FID). A fused silica capillary column (OmegawaxTM-320, 30 m × 0.32 mm i.d.) was used. Calibration curves were made for several pairs formed by the internal standard (methyl tricosanoate) and representative chromatographic standards in order to find the corresponding response factors. Regiospecific analysis of fatty acids in the fish oil used in the work has been conducted by

ethanolysis of the oil catalysed by Lipozyme 435 and by using a great excess of ethanol. Although this lipase is nonregiospecific, it behaves as 1,3-regiospecific with a great excess of ethanol. The exactly procedure to carry out the regiospecific analysis has been previously described [13]. Table 1 shows the FA analyses of the mixture of the mixture of tuna and sardine oil by GC-FID. This Table also shows the FA distribution at the *sn*-2 position. It can be observed that more than 50 % of DHA is found at *sn*-2 position.

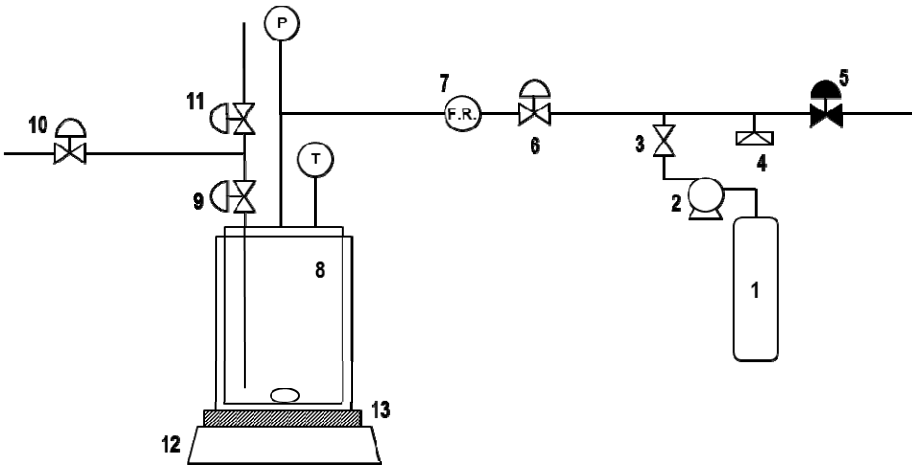

Figure 1 : Experimental apparatus for ethanolysis of fish oil by immobilized lipase under SC-CO_2.
1 : CO_2 cylinder ; 2 : Pump ; 3 : Back Pressure regulator ; 4 : Rupture disk ; 5 : Vent valve ; 6 : Inlet valve ; 7 : Mass flow meter ; 8 : High pressure reactor ; 9,10 : Sampling valves ; 11 : Washing valve ; 12 : Stirrer ; 13 : Electric heater ; F.R. : Coriolis mass flow meter ; P : Pressure indicator ; T : Thermocouple.

Analysis of reaction products

Quantification of the reaction products (monoacylgycerols, diacylglycerols, triacylglycerols and ethyl esters) has been carried out by normal phase HPLC. Separation was carried out at room temperature in a Lichrospher Diol column (5 mm, 4mm·250 mm) and detection was performed in an ELSD detector (Agilent Technologies 1200 Series Model) at 0.35 MPa and 35ºC. The mobile phase consisted of (A) isooctane and (B) methyl tert-butyl ether + acetic acid (0.1 %) at a constant flow rate of 1 mL min^{-1}. NP-HPLC method and calibration procedure have been previously described in detail [14].
Chromatographic analysis of the ethanol and glycerol content of the reaction samples was performed using High-Temperature Gas Chromatography (HTGC) with a Hewlett Packard (HP 6890 Series GC System) gas chromatograph equipped with a flame ionization detector (FID). A fused silica capillary column of 30 m × 0.25 mm i.d., coated with a 0.25 mm film thickness of 65 % Phenyl-methylpolysiloxane (65HT) as a stationary phase and an Agilent Technologies 7683B Series automatic injector were used. The GC method and calibration procedure have been previously described in detail [10].
On the other hand, at a certain reaction time, fractions corresponding to the different reaction products separated by HPLC were collected in special flasks to evaporate the solvent under vacuum. Separations were repeated at least 8 times to collect enough sample for the fatty acid profile analysis by GC.

RESULTS

In this work, the effect of the initial reactant molar ratio, ethanol:fish oil, on the kinetic reaction has been studied. The initial molar ratio has been varied in the range 7:1 to 76:1. Enzymatic ethanolysis in SC-CO_2 system was carried out at 110 bar and constant temperature of 323.15 K. Pressure and temperature were fixed based on previous studies taking into account the enzyme activity and stability of the biocatalyst. Enzyme loading was established at 5% wt. (based on total weight of substrates).

The time courses for the enzymatic ethanolysis of refined fish oil in SC-CO_2 system are shown in Figures 2 and 3. The TAG conversion at initial molar ratio of 7:1 was higher than at 76:1. The lipase catalyst could be irreversibly inactivated by contact with the higher amounts of alcohol (dead-end inhibition). This fact has been also described in literature for the ethanolysis of menhaden oil by Lipozyme TL-IM in SC-CO_2 [15]. Although in this work, the effect of other operating variables has not been studied, in literature it has been found that the higher the operating pressure the faster the ethanolysis reaction due to an improvement in the solubility of oil and ethanol.

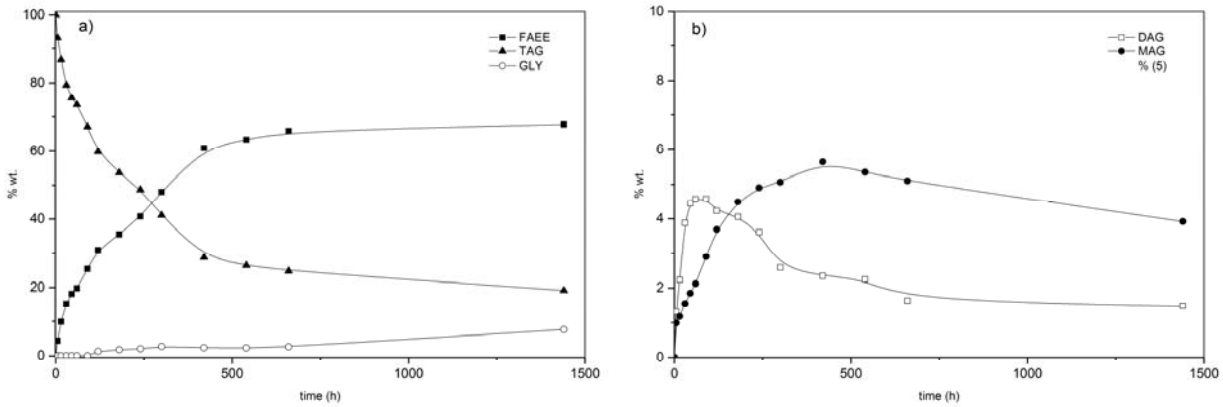

Figure 1 : Time course of the ethanolysis of fish oil by Lipozyme 435 in SC-CO_2. a) FAEE, TAG and glycerol (GLY) contents. b) DAG and MAG contents. (110 bar ; 323.15 K; enzyme loading = 5 % wt.; molar ratio 7:1)

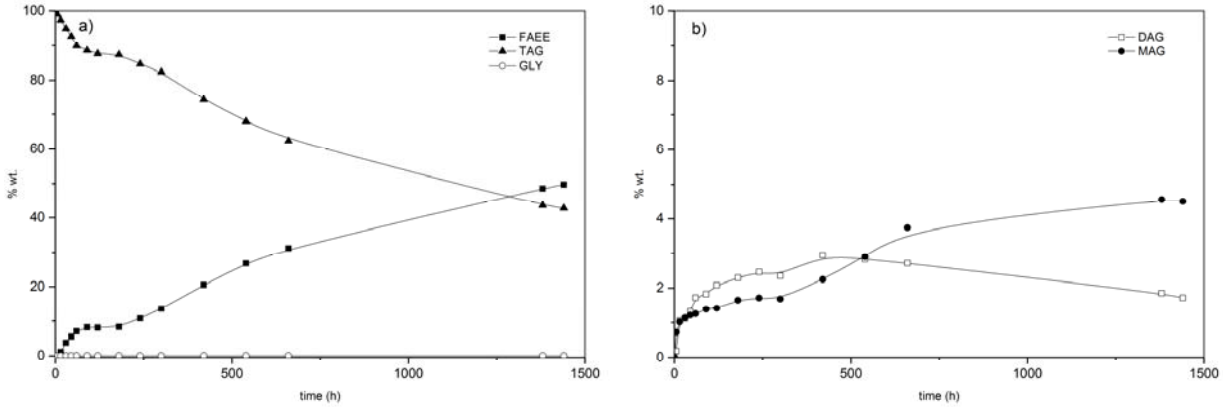

Figure 2 : Time course of the ethanolysis of fish oil by Lipozyme 435 in SC-CO_2. a) FAEE, TAG and glycerol (GLY) contents. b) DAG and MAG contents. (110 bar ; 323.15 K; enzyme loading = 5 % wt.; molar ratio 76:1).

MAG production reached a maximum at 7 h for an initial molar ration of 7:1 (Figure 2b). At this reaction time, the different fractions observed in the NP-HPLC chromatogram (Figure 4) were collected separately and the FA profile was determined for each type of lipid (FAEE, TAG, DAG and MAG) as it has been described in previous section to determine the extent of the n-3 PUFA enrichment. Results obtained are showed in Table 1.

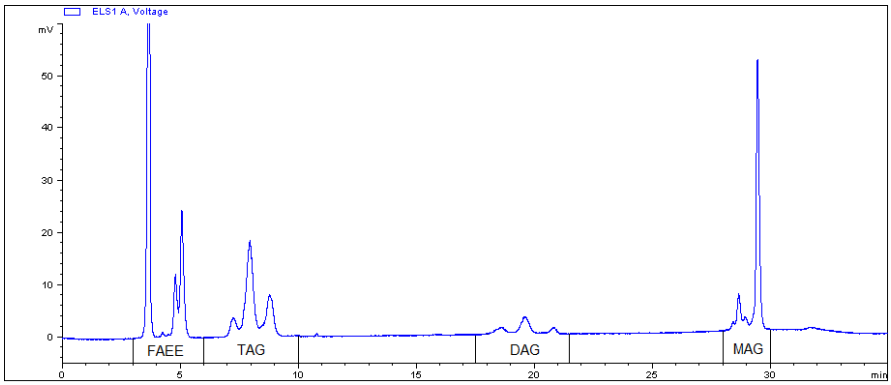

Figure 3 : Fractions observed in the NP-HPLC analysis of the reaction products at 7 h ; molar ratio 7:1.

From Table 1 it can be observed that DHA content in MAG and DAG fractions was considerably higher than in the EE and TAG fraction. In contrast, saturated fatty acids such as palmitic acid and monounsaturated fatty acids like oleic acid appeared mainly in the FAEE fraction formed during the reaction or remained in the unreacted TAG. This is supported by the fact that Lipozyme 435 is selective for substrates of small volume (i.e. short chain FA) due to the limited space of its active site [9].

Table 1 Fatty acid composition of fish oil (% mol) and in *sn*-2[a] of TAG in oils studied in this work.

		Tuna-sardine oil		FA distribution at 7 h reaction time (7:1)			
Fatty acid		TAG	*sn*-2	FAEE	TAG	DAG	MAG
Myristic (M)	14:0	4.7	41.4	4.1	3.3	3.0	3.3
Palmitic (P)	16:0	23.2	27.9	22.4	18.9	13.8	13.6
Palmitoleic (Po)	16:1n-7	6.9	32.3	6.6	5.6	5.5	5.3
Stearic (S)	18:0	6.1	8.4	5.7	3.9	2.6	1.8
Oleic (O)	18:1n-9	18.4	19.4	19.5	18.1	13.6	10.3
Vaccenic (V)	18:1n-7	3.1	16.2	3.1	1.9	1.5	1.2
Linoleic (Lo)	18:2n-6	2.4	33.8	4.7	7.5	3.2	2.4
Linolenic (Ln)	18:3n-3	0.7	36.5	0.8	1.0	0.8	0.7
Steriadonic (St)	18:4n-3	1.6	30.8	0.9	0.7	1.1	1.0
Eicosenoic (G)	20:1n-9	1.9	12.9	1.6	0.9	0.7	0.6
Eicosatrienoic (Et)	20:3n-3	1.8	35.0	1.8	1.5	1.4	1.3
Eicosapentaenoic (Ep)	20:5n-3	6.5	30.2	6.4	5.5	5.7	4.3
Docosapentaenoic (Dp)	22:5n-3	1.6	44.4	1.4	1.6	2.0	2.13
Docosahexaenoic (Dh)	22:6n-3	21.4	61.0	20.9	29.7	45.1	51.9
	NP-HPLC %area of each fraction			71.4	12.6	3.3	12.7

(a) % *sn*-2 = [mol % *sn*-2 fatty acid / mol % fatty acid in TAG*3] * 100

CONCLUSIONS

In this work, ethanolysis of fish oil catalysed by Lipozyme 435 has been conducted under SC-CO_2 reaction medium. Lipozyme 435 was efficient for biocatalysis in SC-CO_2. Results obtained shows that DHA was enriched in the MAG fraction. Initial substrate ratio should be more widely investigated because of greater excess amounts of ethanol inhibited the enzymatic reaction. Results obtained in this work could help to carry on with the purpose of selecting the appropriate reaction conditions in order to concentrate the n-3 PUFAs of fish oils in the most bioavailable form of MAG, which could be easily separated from the mixtures by SC-CO_2 fractionation.

ACKNOWLEDGEMENTS

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