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Enzymatic synthesis of trieicosapentaenoylglycerol in a solvent-free medium

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Abstract

The enzymatic synthesis of trieicosapentaenoylglycerol from glycerol and eicosapentaenoic ethyl ester in a solvent-free medium is studied here. Novozym SP 435 (immobilized lipase from *Candida antarctica*) has appeared as a very efficient biocatalyst for this transesterification. A nitrogen bubbling has allowed a good mixing and also the shifting of the reaction toward synthesis by eliminating the ethanol formed. The effect of temperature and of the quantity of lipase has been studied. In the optimal conditions ($T = 80$°C, 5% (w/w) of lipase, 1 mol glycerol for 3 mol ethyl ester), pure triglyceride has been obtained after 10 h.

Keywords: Trieicosapentaenoylglycerol; Lipase; *Candida antarctica*; Transesterification; Solvent-free medium

1. Introduction

For more than a decade, consumers have been told to increase their intake of long-chain $n-3$ polyunsaturated fatty acids (PUFAs) by eating more fish. This advice first came from the observation that populations incorporating seafood as a major part of their diet had lower rates of cardiovascular diseases.

Since then, much research has proved the positive effects of $n-3$ PUFA present in marine fats (eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA) on human health, especially on cardiovascular diseases, arthritis, inflammation, diabetes, kidney and skin disorders, cancer, vision and brain development. Even if some effects are still to be proved, these components are now increasingly treated as 'food for the prevention of disease'. As people are more and more concerned by good eating habits, this has created a great demand for PUFA lipids. Most parts of these components can be found in fish oils. But the maximum quantity of triglycerides of $n-3$ PUFA in such products is generally 18% that is quite a low value. In order to enrich the food in PUFA lipids, EPA and DHA from fish oils are purified and stabilized as ethyl esters. It has also been shown that free fatty acids and triglycerides were metabolized more rapidly and completely than ethyl esters [1]. However, free fatty acids are oxidized most easily.

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Therefore, triglycerides can be considered to be the most desirable form of food.

For all these reasons, the synthesis of triglycerides of PUFA appears to be of great interest nowadays.

As the traditional chemical way presents some obvious drawbacks — the necessity to use high temperatures can destroy the long chain of PUFA and cost a lot — an enzymatic synthesis has been chosen with a lipase as biocatalyst. A synthesis in a solvent-free medium is often preferred as it has the great advantage of avoiding the use of toxic organic solvents. It also allows the recovery of the product without further complex purification steps.

Some examples of enzymatic esterifications in solvent-free medium are proposed. Reactions between glycerol and a free fatty acid (caprilic acid [2], oleic acid [3,4], mixing of fatty acids [5], cereal oil [6,7]) have been extensively studied.

Haraldsson et al. [8] have studied the direct esterification between glycerol and EPA fatty acid and also the interesterification between tributyrin and EPA ethyl ester. The reactions, catalyzed by Novozym SP 435, were conducted in the absence of any solvent, at 65°C, under vacuum. The equilibrium was shifted toward completion by the elimination of the coproducts. Better results were obtained in the case of the direct esterification. Ninety-seven percent of incorporation was reported after 24 h and 100% after 72 h.

In this paper, the enzymatic synthesis of homogeneous triglycerides of eicosapentaenoic acid in a medium only composed of glycerol, ethyl esters of $n-3$ PUFA and an immobilized lipase is studied. Our purpose is to find the optimal reaction parameters for this transesterification.

2. Materials and methods

2.1. Materials

The substrates used are glycerol, purchased from Sigma (purity $>99\%$), and eicosapentaenoic ethyl ester (purity 80% by gas chromatography, GC).

All the solvents are analytical grade. 2-Methyl-2-butanol was purchased from Merck, ethanol from Carlo Erba and isopropanol from Prolabo.

Two lipases (triacylglycerol acylhydrolase EC 3.1.1.3.) have been tested. Novozym SP 435 is a non-specific lipase from *Candida antarctica* immobilized on a macroporous acrylic resin. Lipozyme IM 60 is a 1,3-specific lipase from *Rhizomucor miehei* immobilized on a macroporous anionic resin. Both lipases were kindly provided by Novo Industri.

The thermostated bath used for the synthesis was a BIOBLOCK Polytest 20 (85 507).

2.2. Triglyceride synthesis

Various experimental apparatus are described for enzymatic esterifications in solvent-free medium, the important point being always to obtain a good mixing to put the two viscous liquid phases in contact without any mechanical destruction of the enzyme. Mostly, glass vials are placed in a thermostated bath [5]. The mixing is often obtained, thanks to a magnetic stirrer sometimes suspended in the medium [2,4] or by the agitation of the whole vial. Working under nitrogen atmosphere or under vacuum can be an efficient way to avoid the oxidation of the PUFA [5].

In a typical experiment, glycerol ($0.60\ \text{g} = 6.52 \times 10^{-3}$ mol), EPA ethyl ester ($6.5\ \text{g} = 1.97 \times 10^{-2}$ mol) and the enzyme are placed in 25 ml glass vials fixed in a shaking thermostated bath equipped with a plate with spring clamps. The glasses are shaken lengthwise at approximately 200 strokes/min and a stroke length of about 1.5 cm. This system of mixing avoids the mechanical destruction of the catalyst.

The esterification is performed under nitrogen atmosphere in order to avoid the oxidation of the PUFA. This is obtained, thanks to a bubbling (385 ml/min, $P = 1$ atm, $T = 20$°C) that also enhances the mixing of the medium and allows the evaporation of the ethanol formed during the reaction that shifts the reaction toward the synthesis.

2.3. Analytical procedures

Concerning the analytical aspect, the main problem is in fact to obtain a representative sample especially during the first part of the synthesis. The number of phases present in the medium is indeed not constant. Theoretically, at the beginning of the

reaction, two separate liquid phases — a hydrophilic one with the glycerol and a lipophilic one with the ethyl ester — and a solid one — the lipase immobilized —are in the reactor. Then mono- and diglycerides that have amphiphilic properties are synthesized and the medium becomes biphasic. At the beginning of the synthesis, samples cannot theoretically give any information on the composition of the whole medium. As the major part of the volume is composed of the lipophilic phase when working in stoichiometric proportions, i.e. 1 mol glycerol/3 mol PUFA, we can assume that the first samples are representative of this phase. According to Selmi et al. [2], who studied the reaction between the glycerol and the caprilic acid catalyzed by a lipase from *R. miehei* at 60°C, the medium is biphasic after only 15 min.

In order to obtain the time course of the reaction, the medium is regularly analysed. Fifty microliters of samples are taken, diluted in 5 ml 2-methyl-2-butanol and analysed by chromatographic techniques.

The decrease of the EPA ethyl ester is determined by GC. A CHROMPACK CP 9002 gas chromatograph equipped with a CP Wax 52 CB CHROMPACK capillary column (25 m × 0.25 mm), a split–splitless injector and a FID detector are used. Helium is used as a carrier gas at a column head pressure of 70 kPa. A split of 60 ml/min is taken. Both detector and injector are heated at 250°C. A temperature of 150°C is used at the beginning during 5 min, rising 10°C/min to a final temperature of 250°C, which is maintained for 5 min. One microliter is injected. In such conditions, the retention time of EPA ethyl ester is 15 min. The calibration curve has been obtained with solutions of EPA ethyl esters in 2-methyl-2 butanol from 3 to 10 g/l.

The glycerides (and also the EPA ethyl ester) are assayed by high-performance liquid chromatography (HPLC). A Merck Lichrospher 100, RP18, 10 μm HPLC column, a Merck L-6200 Intelligent Pump and a Eurosep evaporative light scattering detector DDL 21 were used. A solvent gradient permits to enhance the separation. The initial mobile phase is water/ethanol (75/25); from the beginning to 5 min, the mobile phase changes from water/ethanol (75/25) to pure ethanol and after 5 min until 25 min, the mobile phase is ethanol. From 25 to 30 min, it changes from pure ethanol to ethanol/isopropanol (50/50) and finally from 30 min to the end of the analysis, the mobile phase is ethanol/isopropanol (50/50). The flow rate equals 1 ml/min. One-hundred-microliter samples are injected. The conditions of the detector are 450 mV and 65°C. In such conditions, the retention times are: 700 s (monoglyceride of EPA), 810 s (EPA ethyl ester), 915 s (diglyceride of EPA) and 1300 s (triglyceride of EPA).

The ethyl ester calibration curve has been obtained with solutions of EPA ethyl esters in 2-methyl-2 butanol from 0.8 to 4 g/l. The glycerides of EPA are not commercially available. According to Ref. [9], the response of a light scattering detector is the same for different monoglycerides and for different diglycerides. In our case, solutions of commercial monolinoleine (between 0.4 and 4 g/l) and dilinoleine (between 0.4 and 4 g/l) in 2-methyl-2-butanol have been used for the calibration of the detector. The triglyceride calibration curve has been obtained with solutions of the reaction medium at the end of the synthesis (pure triglyceride of EPA) in 2-methyl-2-butanol (between 0.3 and 5 g/l).

2.4. Product characteristics

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of the triglyceride of EPA obtained at the end of the synthesis were recorded on a Bruker AC-200 P spectrometer in deuterated chloroform as a solvent.

3. Results and discussion

3.1. Comparison of the catalytic activities of Novozym SP 435 and Lipozyme IM 60 for the synthesis of triglyceride of EPA

The catalytic activity of two immobilized lipases (Novozym SP 435 and Lipozyme IM 60) have been compared by following by GC the decrease of EPA ethyl ester during the reaction. The optimal temperature for Novozym is 80°C while the one for Lypozyme is 60°C. That is why we have chosen to test these two temperatures. The results are presented in Fig. 1. When working with Lipozyme, at 80°C, it appears that there is no synthesis of triglyceride after

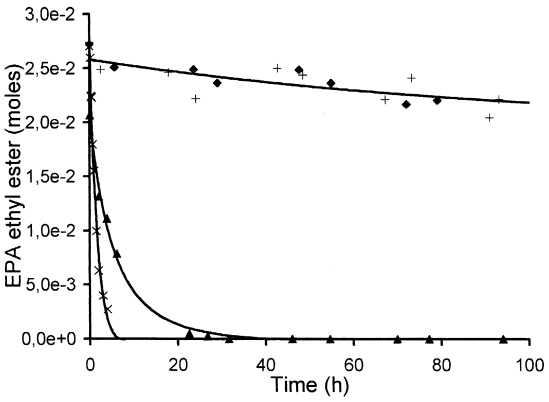

Fig. 1. Comparison of the catalytic activity of Novozym SP 435 and Lipozyme IM 60 for the synthesis of triglyceride of EPA by following the EPA ethyl ester quantity (by GC) vs. time. Lipase quantity = 5% (w/w). (♦) Lipozyme, 60°C; (+) Lipozyme, 80°C; (▲) Novozym, 60°C ; (×) Novozym, 80°C.

100 h. As 80°C is a quite high temperature, we can assume that there is thermal deactivation of the biocatalyst. But the results obtained with Lipozyme at 60°C are not better. For both temperatures tested, Novozym SP 435 clearly appears as an efficient catalyst for the transesterification studied. At 60°C, after 15 h, there is only 10% of the initial ethyl ester still in the medium. When working at 80°C, the same quantity is obtained after only 4 h. Similar results have been obtained [8] when comparing the activity of these two lipases during different esterifications. Thus, the next synthesis will be done with Novozym SP 435.

At the end of the synthesis catalyzed by Novozym at 80°C, the medium has been analyzed by HPLC. The chromatogram obtained shows that these media are composed of a pure product.

3.2. Characterisation of the product

This pure product has been characterized by ^{1}H and ^{13}C NMR. ^{1}H NMR: $\delta = 5.37$, 31 H, =C−*H*, −CH_2−C*H*−CH_2; $\delta = 4.29$–4.15, 4 H, −C*H_2*−CH−C*H_2*; $\delta = 2.84$, 24 H, =C−C*H_2*−C=; $\delta = 2.32$, 6 H, −C*H_2*−COO−; $\delta = 2.07$, 12 H, =CH−C*H_2*−CH_2−, CH_3−C*H_2*−CH=; $\delta = 1.69$, 6 H, −C*H_2*−CH_2−COO−; $\delta = 0.97$–0.88, 9 H, =CH−CH_2−C*H_3*. ^{13}C NMR: $\delta = 173.14$–172.78, 3 C, *C*=O; $\delta = 132.24$–127.33, 30 C, *C*−H; $\delta = 69.29$, 1 C, −*C*H−O; $\delta = 62.44$, 2 C, *C*H_2−O; $\delta = 33.81$–20.87, 24 C, *C*H_2; $\delta = 14.62$–14.41, 3 C, −*C*H_3. These results are characteristics of a homogeneous triglyceride of EPA. The spectra correspond to those reported by Haraldsson et al. [8].

3.3. Influence of the water quantity

An important factor in the study of enzymatic synthesis is the water quantity (water in the medium and water bound to the enzyme). In our case, no water is produced during the synthesis. Moreover, the bubbling and high temperatures used would lead to the elimination of the water molecules that could be present in the medium. So there should not be any accumulation of water. This result has been confirmed by Karl–Fisher measurements of the synthesis medium. During the reaction, no water is detected. This absence of water avoids the hydrolysis of the ethyl ester. Indeed, no free fatty acids have been detected by HPLC; that means there is less than 2×10^{-3} mol of free EPA in the medium. As there is no hydrolysis of the EPA ethyl ester, we can consider that the only reaction responsible for the decrease of the ethyl ester is the transesterification. So it is accurate to study only the EPA ethyl ester decrease.

3.4. Determination of the optimum quantity of enzyme

The first transesterifications have been conducted with a quantity of lipase equal to 10% (w/w) of the total mass of the substrates. This value is commonly used [2–8]. Other reactions have been conducted with less enzyme (from 0.5% to 5 %) in order to determine the minimum quantity needed to reach the equilibrium in a reasonable time.

Concerning the equilibrium, as could be expected, for all the quantities of enzyme tested, pure triglyceride of EPA is obtained at the end of the synthesis.

A way to determine the optimal quantity of enzyme is to study the initial velocity vs. the quantity of enzyme. We have chosen to study the quantity of EPA ethyl ester still in the medium after 1 and 2 h of transesterification vs. the lipase quantity (Fig. 2) instead of the initial velocity. This latter value is

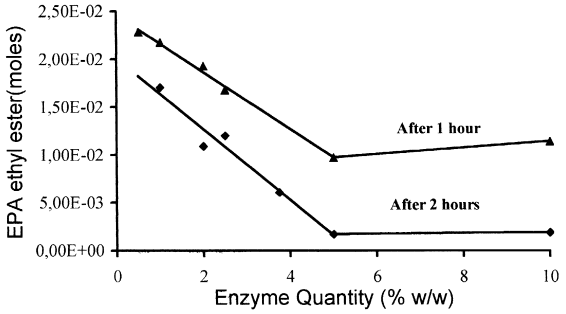

Fig. 2. Influence of the lipase quantity (Novozym) on the EPA ethyl ester quantity (by HPLC) after 1 h (▲) and 2 h (◆) of synthesis. Temperature = 90°C.

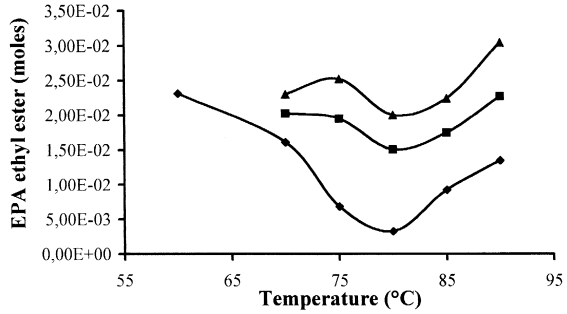

Fig. 3. Influence of the temperature on the EPA ethyl ester quantity (by HPLC) after 0.5 h (▲), 1 h (+) and 2 h (◆) of synthesis. Novozym quantity = 5% (w/w).

indeed quite tricky to calculate because of the inhomogeneity of the medium at the beginning. As can be seen, from 0.5% to 5%, there is a linear decrease of the quantity of EPA ethyl ester vs. the percentage of enzyme. When working with more than 5%, whatever the quantity of enzyme, the results are the same. So it is of no use working with 10% (w/w) of biocatalyst; half of this quantity is enough to obtain the same results. The next synthesis will be done with a quantity of biocatalyst equal to 5% (w/w) of the total mass of the substrates.

3.5. Influence of the temperature

Different temperatures from 60°C to 90°C have been tested.

Concerning the final state, for all the temperatures tested, no more EPA ethyl ester is detected and pure triglyceride of EPA is observed by HPLC. This result is logical as, thanks to the bubbling, all the ethanol formed are eliminated and thus, the reaction is totally shifted toward synthesis.

Concerning the kinetic aspect, as could be expected, a modification of the process temperature has an influence on the initial velocity of the synthesis. We have chosen to study the quantity of EPA ethyl ester still in the medium after 0.5, 1 and 2 h of transesterification vs. temperature (Fig. 3) instead of the initial velocity for the same reasons as before. As can be seen, at 80°C, there is a minimum in the three curves. So 80°C appears as the optimal temperature for the transesterification catalyzed by Novozym. This is coherent with the optimal temperature of Novozym predicted by Novo Industri which is 80°C. For temperatures greater than 80°C, there is some thermal deactivation of the biocatalyst.

3.6. Example of a synthesis with the optimal reaction parameters

Fig. 4 shows the time course of a transesterification. The quantities are followed by HPLC. The conditions for this synthesis, catalyzed by Novozym SP 435, are: $T = 80$°C, initial molar ratio = 1 mol glycerol/3 mol EPA ethyl ester — in order to obtain only triglycerides—, quantity of lipase = 5 % (w/w) of the total mass of the substrates, nitrogen bubbling = 385 ml/min. The time course experimentally obtained corresponds to the reactive scheme proposed

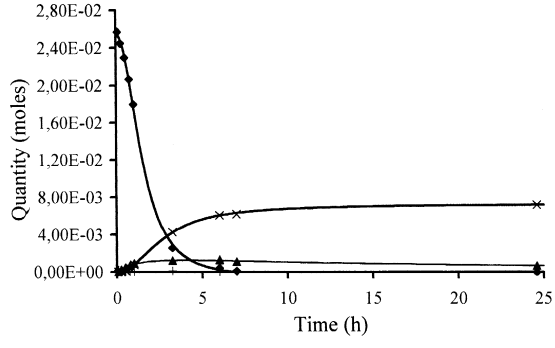

Fig. 4. Time course of the synthesis of triglyceride of EPA in the optimal conditions. $T = 80$°C, Novozym quantity = 5% (w/w). (◆) EPA ethyl ester by HPLC; (+) monoglyceride of EPA by HPLC; (▲) diglyceride of EPA by HPLC; (×) triglyceride of EPA by HPLC.

in the literature for the esterification between glycerol and free EPA [8]. Indeed, as can be seen on this figure, in the first part of the reaction, monoglycerides of EPA are synthesized, then diglycerides and lastly, triglycerides of EPA.

After 10 h of transesterification, no more EPA ethyl ester can be detected neither by GC nor by HPLC. This means that there is less than 2×10^{-3} mol of EPA ethyl ester in the medium (compared to 2.3×10^{-2} mol initially). The HPLC, after 10 h of synthesis, only shows one product that is triglyceride of EPA (see Section 3.2). As pure triglyceride of EPA (purity determined by HPLC) is obtained, we can conclude that the conversion of EPA ethyl ester in triglyceride of EPA is 100 % after 10 h of synthesis. Moreover, the mass balance is checked as 7×10^{-3} mol of tri EPA are formed while 2.2×10^{-3} mol of EPA ethyl ester ($\approx 3–7 \times 10^{-3}$) were in the medium at the beginning.

4. Conclusion

The enzymatic synthesis of trieicosapentaenoylglycerol from glycerol and eicosapentaenoic ethyl ester has been successfully conducted in a solvent-free medium, at 80°C with 5% (w/w) of Novozym SP 435 (immobilized lipase from *C. antarctica*). The transesterification is totally shifted toward synthesis, thanks to a nitrogen bubbling that eliminates the ethanol formed. After 10 h, pure triglyceride of eicosapentaenoic acid, characterized by ^{1}H NMR and ^{13}C NMR, has been obtained.

These first results are very encouraging. The aim is now to complete this study by scaling-up the results obtained in the laboratory in order to develop a process that could produce larger quantities of triglyceride.

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