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Preparation of n-3 PUFAs ethyl esters by an efficient biocatalyzed solvent-free process

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ABSTRACT

alpha-Linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been subjected to esterification with ethanol in presence of lipase B from *Candida antarctica* (Novozym 435®), in solvent free condition. The use of alcohol donors triethyl orthoformate (TEOF) or diethyl carbonate (DEC) instead of free ethanol, allowed working in irreversible esterification conditions and ALA-, EPA- and DHA-ethyl esters were obtained in quantitative yields.

1. Introduction

Today the design of new environmentally friendly and economically valuable syntheses is one of the main challenges for companies operating in the chemistry sector. The "green approach" seeks to respond to this requirement by developing procedures with high or quantitative yields, safety of reagents and products, possibility to recycle the catalyst(s), and when feasible by performing the reactions in water or solvent free conditions. Biocatalysis, exploiting the action of natural catalysts such as enzymes that are able to work in mild and eco-friendly conditions, can often accomplish these "green" requirements. In chemical transformations of sensitive compounds these conditions are mandatory. This is the case of n-3 polyunsaturated fatty acids (n-3 PUFAs) esterification.

The interest towards n-3 polyunsaturated fatty acids dates back to about thirty years ago when some studies suggested that the elevated concentrations of n-3 PUFA in the diet of Greenland Eskimos were responsible for the low mortality for cardiovascular disease in this population [1]. The beneficial effects of n-3 PUFAs on human health are now well documented [2–4] and the main macro-consequences have been their much higher consumption and a consequent increase in the market demand.

Since they are easily oxidised, the free n-3 PUFAs are usually preserved during storage in the form of triglycerides or ethyl esters [5]. In general, having these acids in the form of esters also facilitates their manipulation, such as in the multistep preparation of valuable epoxy fatty acids [6,7].

While an extensive series of data is available for the transformation of PUFA into triglycerides, little is reported about the preparation of PUFA ethyl ester by direct esterification. Recently Silva et al. [8] published an $H_3PW_{12}O_{40}$ catalyzed esterification of different fatty acids, including unsaturated acids. Although the process gives ethyl esters in satisfactory yield, the large excess of alcohol and the use of tungsten-catalyst makes the process unsuitable for a green application. Considering that PUFA triglycerides represent the natural substrates for lipases, these enzymes are optimal candidates to act as mild catalysts in the preparation of their corresponding ethyl esters. But so far, although this approach has been reported for fatty acids [9], few investigation have been made for the sensitive PUFAs and, to our knowledge, the only example is the use of *Phycomices nitens* lipase in the esterification of EPA with ethanol [10]. In the light of this, we decided to try extending our experience in the esterification of aryl acids to PUFAs, and here we report the irreversible esterification, in presence of lipase B from *Candida antarctica*, of three different n-3 PUFAs: α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Fig. 1) in order to prepare the corresponding ethyl esters in high yield.

2. Materials and methods

2.1. Materials

ALA (9,12,15-octadecatrienoic acid), EPA (5,8,11,14,17-eicosapentaenoic acid), DHA (4,7,10,13,16,19-docosahexaenoic acid), stearic acid (SA), ethanol absolute and lipase B from *C. antarctica* (Novozym 435®) were obtained from Sigma–Aldrich; diethyl carbonate (DEC) and triethyl orthoformate (TEOF) were

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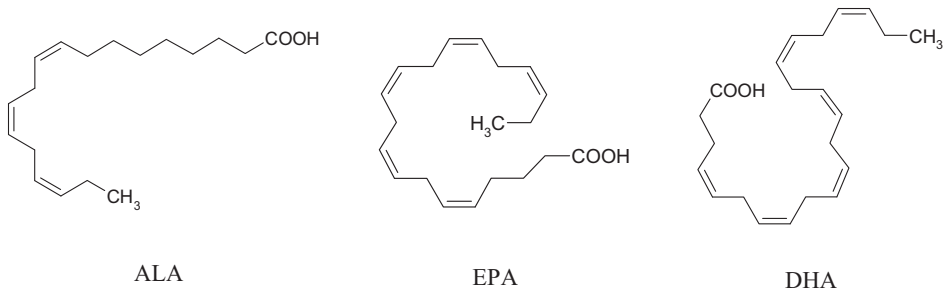


Fig. 1. n-3 polyunsaturated fatty acids.

purchased from Fluka. All analytical biotransformations were performed in triplicate.

2.2. Esterification of SA (equilibrium reaction)

The different experiments were performed by adding 1 g of SA, ethanol (1, 2, 3 or 6 equiv.) and 50 mg of Novozym 435 in a 12 ml vial. The mixture was kept in the shaker at 60 °C and 300 rpm. The course of reaction was monitored by GC at defined times.

2.3. Esterification of SA by irreversible procedure

To a 1 g of SA (3.52 mmol), in a 12 ml vial, was added an alcohol donor (for DEC: 0.5, 1 or 2 molar ratio were used; for TEOF 1 molar ratio). Then 50 mg of Novozym 435 and 5 μl of ethanol were added to start the reaction. The mixture was kept in shaker at 60 °C and 300 rpm. The course of reaction was monitored by GC at defined times.

2.4. Esterification of n-3 PUFAs (equilibrium reaction)

To a 1 g of chosen n-3 PUFA, in a 12 ml vial, were added to 2 equiv. of ethanol and 50 mg of Novozym 435. The mixture was kept in shaker at 60 °C and 300 rpm. The course of reaction was monitored by GC at defined times.

2.5. Esterification of n-3 PUFAs by irreversible procedure

To a 1 g chosen n-3 PUFA, in a 12 ml vial, was added to 1 equimolar amount of alcohol donor, DEC and TEOF respectively. Then 50 mg of Novozym 435 and 5 μl of ethanol were added to start the reaction. The mixture was kept in the shaker at 60 °C and 300 rpm. The course of reaction was monitored by GC at defined times.

2.6. Esterification of ALA in preparative scale

TEOF 12 ml (0.072 mol) and 1 g of Novozym 435 were added to ALA 20 g (0.072 mol). The mixture added of 10 μl of ethanol and incubated at 60 °C under shaking at 300 rpm. After 4 h the GC analysis evidenced the complete conversion of the substrate, and the reaction was stopped by filtering off the enzyme. The obtained oil, kept under vacuum (2 mbar) for 1 h at rt, furnished 22 g of ALA ethyl ester (in >99% yield), which nature was confirmed by comparison in GC analysis with an authentic sample.

2.7. GC analysis

The trend of the esterification reactions was analyzed by GC methodology. Aliquots of the reaction mixture were injected on a Shimadzu GC-17A equipped with a fused-silica capillary column from Phenomenex (ZB-FFAP 30 m, 0.33 mm, 0.50 μm); nitrogen

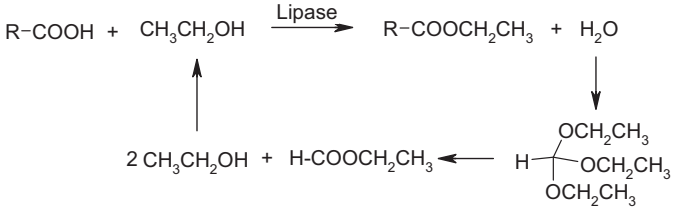


Fig. 2. Scheme of irreversible esterification with TEOF as alcohol donor.

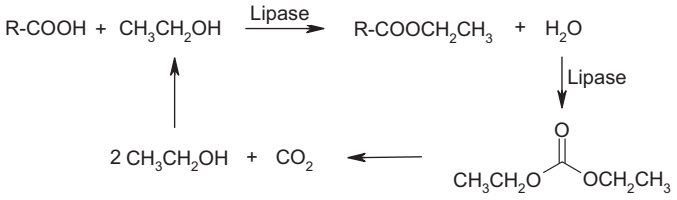


Fig. 3. Scheme of irreversible esterification with DEC as alcohol donor.

was the carrier gas. Reaction mixture was analyzed using temp. prog.: 100 °C for 1 min, 100–250 °C at 5 °C/min, 250 °C for 10 min. The injector ad detector temperatures were 250 and 280 °C respectively. Conversion values were calculated from the areas of the product and the residual acid peaks, using response factors determined by calibration with standards containing equimolar mixture of free fatty acid and the corresponding ethyl ester.

3. Results and discussion

Preliminary esterification experiments were performed on stearic acid, chosen as an inexpensive model substrate. All reactions were catalyzed by Novozym 435, already in use in our laboratory for biodiesel production studies. To enhance the green nature of the procedure, we worked in solvent free conditions, and in order to melt the solid stearic acid a 60 °C temperature condition was adopted.

Since formation of ester by direct reaction of alcohol and acid is a reversible process, the removal of water produced as by-product, allowed a complete conversion of reagents into the desired ester. To achieve this condition we have chosen TEOF and DEC as alcohol donors, already successfully used in the past in our laboratory [11,12]. As reported in Fig. 2, TEOF reacting with the water, produced in the first step of the reaction, gave ethyl formate and two molecules of ethanol for progression of reaction.

In the case of DEC, the mechanism is slightly different since the hydrolysis of carbonate is lipase-catalyzed reaction producing CO_2, which spontaneously is subtracted from the reaction medium, and two molecules of ethanol used in the progression of reaction (Fig. 3).

The performance of these reactions was compared with that obtained using free ethanol. The esterification of SA with ethanol

Table 1
Biocatalyzed esterification of SA with ethanol.

Time (h)	Conversion (%)			
	SA/EtOH 1:1 (molar ratio)	SA/EtOH 1:2 (molar ratio)	SA/EtOH 1:3 (molar ratio)	SA/EtOH 1:6 (molar ratio)
0	0	0	0	0
1	90	91	91	29
6	89	91	92	30
24	89	92	92	31

Experimental conditions: SA: 1 g (3.52 mmol); EtOH: 205.2 μl for 1 equiv., 410.4 μl for 2 equiv., 615.6 μl for 3 equiv., 1.23 ml for 6 equiv.; Novozym: 50 mg; temp. shaker: 60 °C; 300 rpm. The conversion values were determined by GC.

Table 2
Biocatalyzed esterification of SA with DEC and TEOF.

Time (h)	Conversion (%)			
	SA/DEC 1:0.5 (molar ratio)	SA/DEC 1:1 (molar ratio)	SA/DEC 1:2 (molar ratio)	SA/TEOF 1:1 (molar ratio)
0	0	0	0	0
0.5	29	62	68	60
1	45	76	84	85
2	74	87	93	96
4	90	95	96	99
8	96	98	99	100
12	97	100	100	100

Experimental conditions: SA: 1 g (3.52 mmol); DEC: 213.1 μl for SA/DEC 1:0.5, 426.0 μl for SA/DEC 1:1, 852 μl for SA/DEC 1:2; TEOF: 584.7 μl (3.52 mmol); Novozym: 50 mg; temp. shaker: 60 °C; 300 rpm. The conversion values were determined by GC.

(Table 1) reached the equilibrium value around 90% conversion; the use of an excess of alcohol (6 equiv.) did not favour the shift of the reaction towards a total consumption of the acid, rather a dropping of the conversion was observed, probably due to the inhibition of lipase [13].

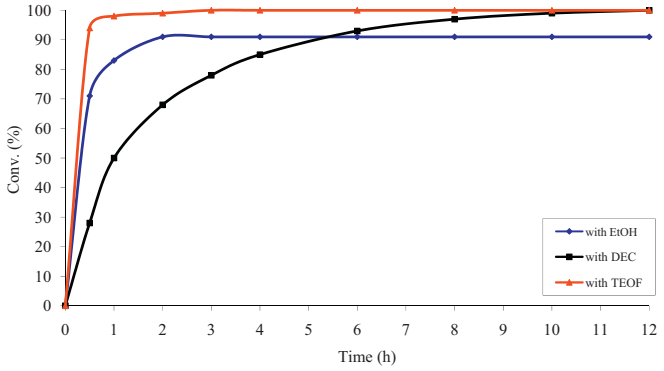
Conversely, the total conversion of SA in ethyl ester was realized when TEOF and DEC were used as alcohol donors (Table 2).

In particular, the esterification in presence of DEC, followed a linear progression depending closely on the amount of utilized carbonate: when a molar ratio 1:0.5 was used (it should noted each DEC molecule releases two ethanol molecules), the acid reached a 97% conversion value in 12 h, and when the molar ratio acid/alcohol donor was increased (1:1 or 1:2), the total transformation of stearic acid was observed. Further experiments were performed in presence of TEOF (Table 2), and the use of this alcohol donor gave better results in terms of reaction rate: indeed a molar ratio 1:1 SA/TEOF was enough to obtain the complete conversion of stearic acid into the corresponding ethyl ester in 8 h.

With these preliminary results we undertook the esterification of three major n-3 PUFAs: ALA, EPA and DHA.

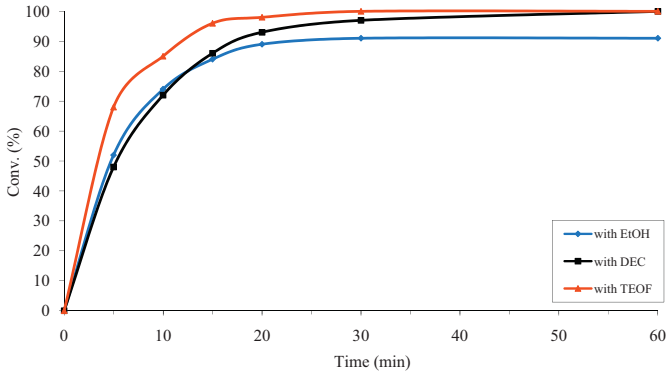
In presence of Novozym 435, two esterification reactions of ALA were performed with equimolar amounts of DEC and TEOF respectively. Both the alcohol donors gave a progressive esterification of the acid but, as expected, TEOF allowed a total conversion in a shorter time (3 h) while with DEC the same result was obtained after 12 h. In the same reaction conditions, the use of two equivalents of ethanol as free alcohol, gave a maximum conversion value of 90% at the equilibrium point (Fig. 4).

The esterification of EPA, carried out in the same conditions adopted above, showed a similar trend with an higher enzymatic efficiency, perhaps due to the higher unsaturation level of the substrate: the reactions, compared both to the first preliminary experiments with SA and to the reactions with ALA were faster, and TEOF and DEC permitted, respectively in 30 and 60 min, the complete transformation of EPA into the desired ethyl ester (Fig. 5).



Experimental conditions: ALA: 1 g (3.59 mmol); DEC: 435.3 μl (3.59 mmol); TEOF: 596.3 μl (3.59 mmol); EtOH: 418.6 μl (7.18 mmol); Novozym: 50 mg; temp. shaker: 60 °C; 300 rpm. The conversion values were determined by GC.

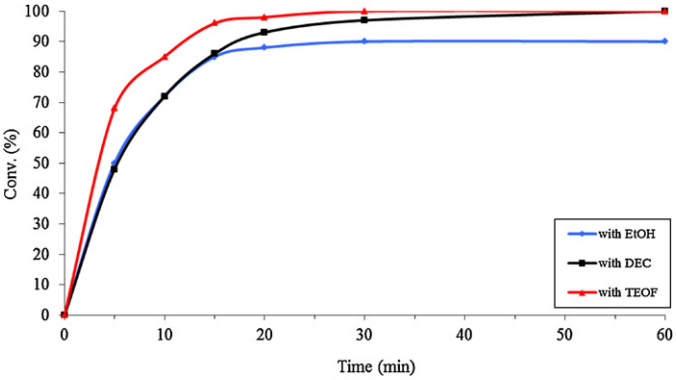
Fig. 4. Trend of biocatalyzed esterification reactions of ALA.



Experimental conditions: EPA: 1 g (3.31 mmol), DEC: 400.7 μl (3.31 mmol);TEOF: 549.8 μl (3.31 mmol); EtOH: 385.9 μl (6.62 mmol) Novozym: 50 mg; temp. shaker: 60 °C; 300 rpm. The conversion values were determined by GC.

Fig. 5. Trend of biocatalyzed esterification reactions of EPA.

When DHA was used as substrate, in the same working conditions, the esterification catalyzed by Novozym 435 occurred with similar trends to those observed for EPA: in a time range of 30–60 min, the total conversion of the acid into ester was observed (Fig. 6). Again the higher unsaturation level of the substrate seems to enhance the efficiency of the biocatalyst. These experimental results show that Novozym 435 is a very efficient biocatalyst in promoting the synthesis of PUFA esters in a free solvent system.



Experimental conditions: DHA: 1 g (3.05 mmol), DEC: 368.9 μl (3.05 mmol); TEOF: 506.6 μl (3.05 mmol); EtOH: 355.6 μl (6.1 mmol) Novozym: 50 mg; temp. shaker: 60 °C; 300 rpm. The conversion values were determined by GC.

Fig. 6. Trend of biocatalyzed esterification reactions of DHA.

The need to have gram-scale amounts of ALA ethyl ester for further parallel investigations in our laboratory, gave us the opportunity to verify the practical exploitation of the process. To this end, a preparative reaction of ALA (20 g) with TEOF as alcohol donor, in a molar ratio 1:1, was carried out: after 4 h the reaction was quenched by filtering off the biocatalyst and the mixture was subjected to vacuum for 1 h in order to remove both the ethanol excess and the ethyl formate obtained as side product. GC analysis of the residual oil confirmed the presence of the ALA ethyl ester as sole final product and the absence of unwanted side-reaction products, such as epoxide derivatives.

4. Conclusions

In the present work we have developed a procedure to transform sensitive polyunsaturated fat acids, such as EPA, DHA and ALA, into ethyl esters. The irreversible conditions which is it possible to realize using TEOF and DEC as ethanol donors, allowed to performing reactions with the total conversion of the acid into the desired ethyl esters. The use of solvent-free conditions enhances the green nature of this proposed process in the transformation of PUFAs.

Acknowledgements

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