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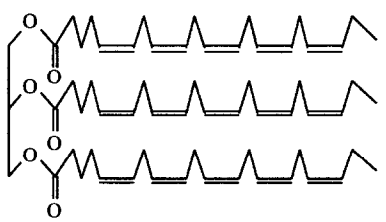
The Preparation of Homogeneous Triglycerides of Eicosapentaenoic Acid and Docosahexaenoic Acid by Lipase

Gudmundur G. Haraldsson*, Birgir Ö. Gudmundsson and Örn Almarsson

Science Institute, University of Iceland, Dunhaga 3, 107 Reykjavik, Iceland.

Abstract: The highly efficient generation of homogeneous triglycerides of either pure eicosapentaenoic acid, **1**, or docosahexaenoic acid, **2**, by an immobilized nonregiospecific yeast lipase from *Candida antarctica* is described.

The long-chain n-3 type polyunsaturated fatty acids characteristic of marine fat, such as *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA), have received considerable attention of scientists due to their beneficial biochemical and pharmacological effects on human health[1-3]. Fish oil is the most common source for the n-3 fatty acids in which they are bound into triglycerides usually within the range of 10 - 25 %[4]. Within the pharmaceutical industry there has been growing demand for these fatty acids in the natural triglyceride form[5,6]. The long-chain n-3 polyunsaturated fatty acids are highly labile and the traditional chemical modification processes that involve extremes of pH and high temperature will most certainly partially destroy their natural all-*cis* n-3 framework by oxidation, *cis-trans* isomerization or double-bond migrations[7].



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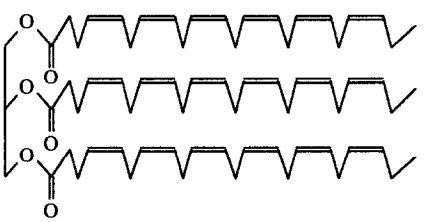

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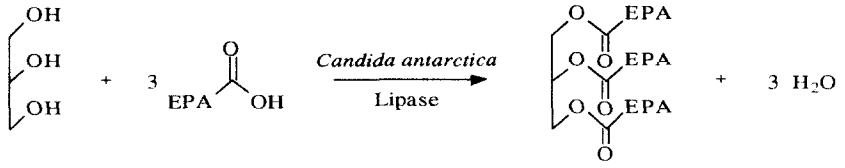
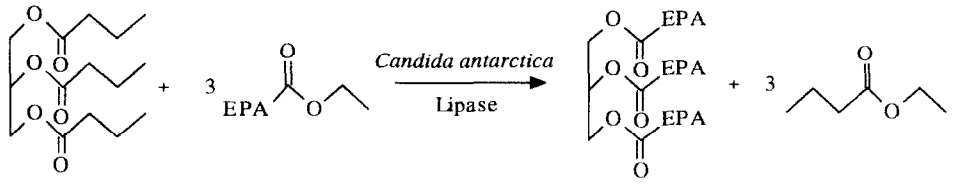
In the course of the last ten years the application of enzymes in organic synthesis has continued to grow enormously, and enzymes are now widely recognized as practical catalysts for asymmetric synthesis[8-10]. The most important feature of enzymes in organic synthesis is their selectivity, but also their high catalytic efficiency and the mildness under which enzymes exhibit their activity. The mild enzymatic modification processes involving fish oils and the highly labile n-3 polyunsaturated fatty acids could be expected to be especially favourable when taking into account the rather drastic conditions applied during the traditional chemical modification processes for triglyceride preparation[7,10].

A few years ago we reported on the preparation of triglycerides highly enriched with n-3 polyunsaturated fatty acids (higher than 70 %) in high purity and excellent yield. This was accomplished by the lipase-catalyzed transesterification of cod liver oil with concentrates of EPA and DHA as free fatty acids or ethyl esters, in the absence of a solvent, employing the catalytic effect of immobilized 1,3-regiospecific fungal lipase from *Mucor miehei*. At an equilibrium the extent of incorporation of the n-3 fatty acids into the triglycerides was limited to a weighted average of the fatty acid composition of the triglycerides and the concentrates. In spite of the regiospecificity of the lipase the fatty acids were randomly distributed among positions of the triglycerides as a consequence of acyl migrations[12]. Various lipases have also been utilized by several other groups to prepare concentrates of EPA and DHA by discrimination between those two by hydrolysis[13-15] and esterification[15-17] reactions.

In this communication we report a modification of our previously described procedure which has resulted in a highly efficient generation of homogeneous triglycerides of either pure EPA, **1**, or DHA, **2**. This was accomplished by an immobilized non-regiospecific yeast lipase from *Candida antarctica* provided by Novo-Nordisk A/S in Denmark. Two methods were employed: Direct esterification of glycerol and interesterification (ester-ester interchange) of tributyrin with stoichiometric amount of 99 % pure EPA or DHA as free fatty acids and ethyl esters, respectively, *in vacuo* without a solvent. The volatile co-products were condensed into a cooled trap during the progress of the reactions, thus shifting the equilibrium toward completion (Scheme 1, only shown for EPA). The resulting crude products were afforded in excellent yields (93 - 96 %) of very high purity (> 98 %).



Direct esterification



Interesterification

Scheme 1

In a typical procedure stoichiometric amounts of substrates were stirred at 65 °C *in vacuo* (0.01 - 0.1 Torr) with 10 % dosage of lipase based on the weight of substrates, without any solvent. The volatile co-products, water or ethyl butyrate, depending on type of reaction, were condensed into a liquid nitrogen cooled trap. They were regularly weighed during the reactions and their weights used to monitor the progress of the reactions. Moisture-free lipase, apart from the essential water for the lipase to maintain its integrity, was used, since under these conditions all additional water escaped into the cooled traps. In spite of this low water content the

enzyme retained sufficient activity very well, even in the interesterification reaction where no water was formed. In the latter reaction, however, it was found beneficial to use lipase containing 10 % water for speeding up the initial reaction rate.

Figure 1. demonstrates a graphical presentation for the progress of the direct esterification reaction of glycerol with pure (99 %) EPA and DHA as free fatty acids as monitored by 250 MHz ^{1}H NMR spectroscopy. The NMR technique became of great value when dealing with homogeneous substrates in terms of fatty acid composition. These results were in full consistence with the weight measurements mentioned earlier. It is evident that EPA is a considerably better substrate as compared to DHA. 97 % incorporation had been reached after 24 hours for both EPA and DHA, but after 72 hours both reactions had proceeded to completion and reached 100 % incorporation as based on mol equivalents. The reason for lower activity of the enzyme toward DHA as compared to EPA is believed to be the fact that the carbon-carbon double bond in the nearest proximity of the carboxyl group is located one bond closer to the carboxyl group in DHA than EPA[18]. This presumably affects its ability to fit properly into the active site of the enzyme.

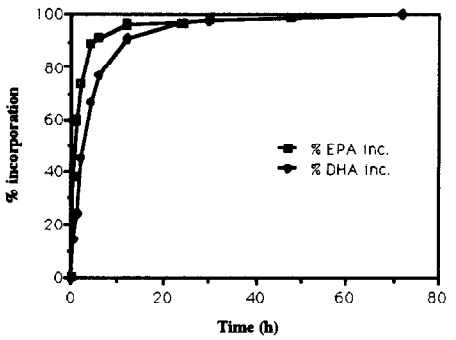


Figure 1. Incorporation of EPA and DHA during direct esterification of glycerol.

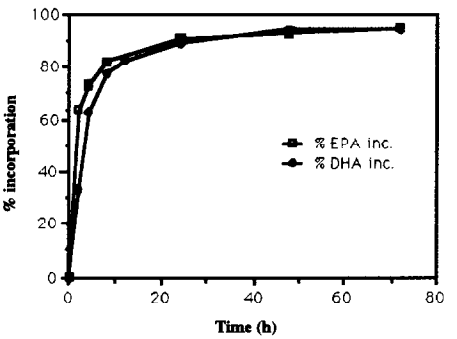


Figure 2. Incorporation of EPA and DHA during interesterification with tributyrin.

Figure 2. displays a graphical presentation for the progress of the incorporation of the corresponding pure EPA and DHA ethyl esters into tributyrin during the interesterification reaction as based on 250 MHz ^{1}H NMR spectroscopy and the weight measurements. This reaction was considerably slower than the direct esterification reaction. Also, as before, DHA incorporation was significantly slower as compared to EPA. After 72 hours 96 % incorporation had been reached for EPA and 95 % for DHA. 97 % incorporation was reached after 96 hours for both EPA and DHA. The extended time required for the incorporation of the very last few percentages is noteworthy and must be related to the purity of the substrates. This may be improved by using slight excess (1-2 %) of the fatty acid substrates. Under these conditions the extent of hydrolysis side-reaction was vanishingly low.

The triglycerides were fully characterized after preparative HPLC purification on silica gel, using 10 % ethyl ether in n-hexane as an eluant, by high-field ^{1}H and ^{13}C NMR spectroscopy, IR spectroscopy, accurate mass spectrometry and iatroscan analysis (TLC-FID). They were afforded as colourless oil and stored at -25 °C under argon. Under these conditions the EPA triglyceride compound was found remarkably stable, since after two years storage there was no indication of any disintegration as established by ^{1}H NMR. The corresponding DHA derivative was not as stable, and after a few months storage, decomposition was noticed.

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