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# Preparation of sn-2 long-chain polyunsaturated monoacylglycerols from fish oil by hydrolysis with a stereo-specific lipase from *mucor miehei*

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**RESUMEN**

**Preparación de sn-2 monoacilgliceroles poliinsaturados de cadena larga a partir de aceites de pescado mediante hidrólisis con una lipasa estereoespecífica de *mucor miehei.***

Se describe la preparación de sn-2 eicosapentaenoil glicerol y sn-2 docosahexaenoil glicerol mediante la hidrólisis de aceite de pescado por lipasa inmovilizada sn-1, sn-3 estereoespecífica (Lipozime IM-20) de *mucor miehei*. Los monoacilgliceroles obtenidos después de la hidrólisis enzimática se separaron por cromatografía en columna de ácido silícico impregnado de nitrato de plata. Ambos monoacilgliceroles pueden ser individualmente separados en forma casi pura por elución de la columna con una mezcla de solvente. La preparación de sn-2 monoacilgliceroles sustituidos de origen marino permite su utilización como sustratos para la síntesis de triacilgliceroles que contienen ácidos grasos poliinsaturados de cadena larga en posiciones específicas.

*PALABRAS-CLAVE: Aceite de pescado - Hidrólisis enzimática - Mucor miehei - sn-2 monoacilgliceroles (preparación).*

**SUMMARY**

**Preparation of sn-2 long-chain polyunsaturated monoacylglycerols from fish oil by hydrolysis with a stereospecific lipase from *mucor miehei.***

The preparation of sn-2 eicosapentaenoyl glycerol and sn-2 docosahexaenoyl glycerol by the hydrolysis of fish oil by the sn-1, sn-3 stereo-specific immobilised lipase (Lipozyme IM-20) from *mucor miehei* is described. Monoacylglycerols obtained after the enzymatic hydrolysis were separated by silver nitrate-coated silicic acid column chromatography. Both monoacylglycerols can be individually separated in almost pure form by elution from the column with a solvent mixture. The preparation of sn-2 substituted monoacylglycerols from marine origin allows their utilization as substrates for the synthesis of structured long-chain polyunsaturated fatty acid-containing triacylglycerols at specific positions.

*KEY-WORDS: Enzymatic hydrolysis - Fish oil - Mucor miehei - sn-2 monoacylglycerols (preparation).*

## 1. INTRODUCTION

In recent years, the use of lipases as biocatalysts to produce useful oleochemicals has been actively pursued (Marangoni & Rousseau, 1995). Lipase-catalyzed reactions offer several benefits over chemically catalyzed reactions, such as milder operating conditions, cleaner products and reduced waste production (Valenzuela & Nieto, 1994). The attractive feature of lipases is the specificity of the enzyme with respect to acylglyceride positions and fatty acid types, which could seldom by achieved with chemical catalysts. sn-1,3 Position specificity has been exploided in a number of applications to obtain high-value specialty fats (Mukherjee, 1990; Haraldsson, 1992).

Structured triacylglycerols (TGs) are synthetic compounds made up of both medium and long-chain fatty acids bound on the same glycerol molecule in a predetermined proportion to form a triacylglyceride (Heird *et al.,* 1986). Preparation of these structured TGs needs specific fatty acids (i.e. medium-chain fatty acids) to be reacted with a specific sn-2 long-chain monoacylglycerol to obtain a structured TG having medium-chain fatty acids at the sn-1 and sn-3 positions and a long-chain fatty acid at the sn-2 position. These type of structured TGs may have interesting nutritional and pharmacological applications especially when the fatty acid occupying the sn-2 position is a long-chain polyunsaturated fatty acid, such as eicosapentaenoic acid (EPA, C20:5, n-3) or docosahexaenoic acid (DHA, C22:6, n-3). Fish oils are good sources to obtain these long-chain polyunsaturated fatty acids due the high concentration of EPA and DHA of these oils (20% to 28% EPA+DHA) (Valenzuela *et al.,* 1993) and also because almost the total amount of these long-chain polyunsaturated fatty acids is at the sn-2 position of the triacylglycerols forming fish oils (Brockerhoff, *et al.,* 1963). Therefore, stereo-specific sn-1, sn-3 lipase-hydrolyzed fish oils may provide high concentrations of sn-2 EPA or sn-2 DHA monoacylglycerols suitable as substrates for interesterification with medium-chain fatty acids to obtain especially defined structured TGs. In this work we describe the preparation of almost pure sn-2 EPA- and sn-2 DHA-containing monoacylglycerols by the enzymatic hydrolysis of sardine oil with the sn-1, sn-3 stereo-specific immobilized lipase Lipozyme IM-20, obtained from *Mucor miehei.*

## 2. MATERIALS AND METHODS

Materials: Lipozyme IM-20 (activity 10,000 LU/g, water 7.2% w/w) a microbial sn-1, sn-3 specific lipase obtained from *Mucor miehei* and immobilized on a macroporous anion exchange resin, was kindly provided by Novo Nordisk A/S (Bagsveard, Denmark). Recently refined sardine oil (28% EPA+DHA) was obtained from CORPESCA SA (Mejillones, 2° Región, Chile). Kieselgel 60 F254 thin layer chromatography (TLC) plates were purchased from Merck (Darmstadt, Germany). All solvents and salts were purchased from J. T. Backer. Silicic acid (Sil A-200) and standards of fatty acids and monoacylglycerols for TLC or gas-chromatography were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Enzymatic hydrolysis of sardine oil: 50 mL of sardine oil previously subjected to high vacuum distillation to eliminate peroxides, other volatile compounds and cholesterol, as previously described (Dinamarca *et al.,* 1990) were mixed with 3 g of Lipozyme IM-20 and 125 mL of 0.1M sodium phosphate buffer pH 7.5. The mixture was vigorously stirred during 2 min. until an oil-in-water emulsion was formed, and after this the emulsion was transferred to a 150 mL water-jacketed (45° C) glass reactor. The hydrolysis was carried out under mechanical stirring (300-400 rpm) during 40 hours. Every 10 hours, a sample of 0.01 mL was taken for qualitative analyses of monoacylglycerols, diacylglycerols and triacylglycerols by TLC. Hexane/diethyl ether/ acetic acid (80:20:1) was used as solvent on Kieselgel 60 F254 plates with one hour develop time. Slightly dried plates were sprayed with 0.1% 2', 7'-dichlorofluorescein in 99% ethanol for detecting the spots at 254 nm (for monoacylglycerols), 290 nm (for diacylglycerols) and at 360 nm (for triacylglycerols) according Hoque *et al.,* (1973). After 40 hours of hydrolysis the emulsion was centrifuged at 2000 x g to recover the enzyme, and the free fatty acids and the remaining triacylglycerols were extracted with hexane (4 vol of hexane/vol. of hydrolysis product). The aqueous phase containing mainly sn-2 EPA monoacylglycerol and sn-2 DHA monoacylglycerol was applied to a 45 cm x 3.5 cm (i.d.) glass column containing silver nitrate (20%)-coated silicic acid (Hoque *et al.,* 1973). 50 mL of the hydrolyzed aqueous phase were applied to the column in each separation procedure, and after the elution of the non-adsorbed molecules (free fatty acids, triacylglycerols, and small amounts of free EPA and DHA) with 150 mL of 0.1M sodium phosphate buffer pH 7.5, the column was washed first with hexane:ethanol (1:4 v/v) to elute the sn-2 EPA monoacylglycerol, and after with hexane: ethanol (1:2 v/v) to release the sn-2 DHA monoacylglycerol component. The elution profile was spectrophotometrically recorded at 210 nm. Fractions containing each acylglycerol were collected, rotary evaporated under vacuum until 10% original volume, and kept under $N_2$ until use. The identification of sn-2 EPA- and sn-2 DHA monoacylglycerols and of the other minor components of each eluted fraction was carried out by TLC against the respective standard, and the concentration of each monoacylglycerol was determined by gas-chromatography.

Methyl esters of fatty acids constituting acylglycerols were prepared according to Totani *et al.,* (1991). Gas-chromatography was carried out in a Hewlett Packard 5890 series II chromatograph equipped with split injector and a flame ionization detector using a SP-2560 (Supelco, Inc, Bellefonte, PA, USA) capillary column (100 m x 0.25 mm i.d ). The column temperature was raised from 150° C to 210° C at 5° C/min. The injector and the detector temperatures were both 250° C. Hydrogen was used as carrier gas.

## 3. RESULTS AND DISCUSSION

Figure 1 shows the kinetic of hydrolysis of sardine oil after the treatment with Lipozyme IM-20. It can be observed that rapid reduction of the triacylglycerol concentration and the corresponding increase of the monoacylglycerols as product of the sn-1, sn-3 stereo-specific enzymatic hydrolysis occurred. From the beginning of the hydrolysis a small amount of sn-1 monoacylglycerols can also be detected as product of the sn-2 to sn-1 acylmigration that occurs during the enzymatic hydrolysis (Marangoni & Rousseau, 1995). A high proportion of sn-2 monoacylglycerols is formed after 40 hours of hydrolysis, however if the process is further prolonged, increase in the formation of sn-1 monoacylglycerol products is observed accompanied by reduction of the sn-2 monoacylglycerol product formation (data not shown). After the recovery of the enzyme and after the extraction of the free fatty acids and the remaining non-hydrolyzed triacylglycerols, the sn-2 monoacylglycerols were fractionated by silver-coated silicic acid column chromatography.

Figure 2 shows the elution profile of the hydrolysis products after the column chromatographic separation. The first peak corresponds to free fatty acids, diacylglycerols and triacylglycerols, which were remnants from the solvent extraction, that are not adsorbed to the column. When the hexane:ethanol (1:4 v/v) mixture is applied to the column a highly defined fraction is eluted (**Fm1**), which is almost exclusively composed by sn-2 EPA acylglycerol. The second solvent mixture (hexane: ethanol, 1:2 v/v)

was applied to the column after the absorbance of the eluate was reduced to less than 0.2 optical units. The elution allows the separation of another fraction (**Fm2**) which is mainly composed by sn-2 DHA acylglycerol accompanied by a minor proportion of sn-2 docosapentaenoic acid (C22:5, DPA) monoacylglycerol and sn-2 docosatrienoic acid (C22:3, DTA) monoacylglycerol. Each fraction (**Fm1** and **Fm2**) was analyzed by TLC and by gas- chromatography as described in methods. Table I shows the relative composition of the **Fm1** and **Fm2** fractions.

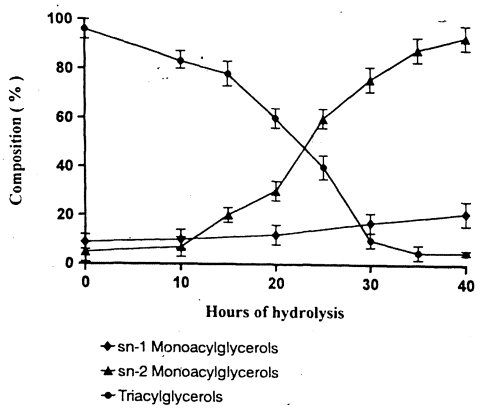


Figure 1

Kinetics of the enzymatic hydrolysis of fish oil by Lipozyme IM-20. Each point represents the average of six assays ± S. D. Other experimental conditions are in the text

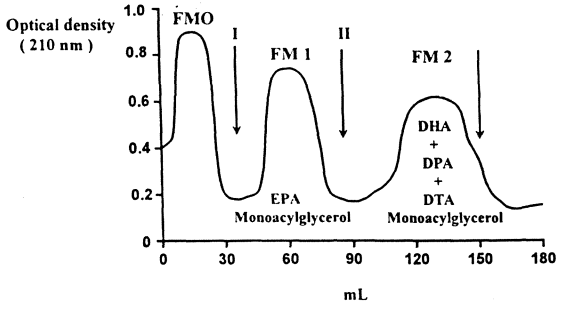


Figure 2

Spectrophotometric profile obtained after silver-coated silicic acid column chromatography of the products of the enzymatic hydrolysis of fish oil by Lipozyme MI-20. **FM0**: non adsorved products; **FM1**: fraction eluted after hexane:ethanol (1:4 v/v); **FM2**: fraction eluted after hexane:ethanol (1:2 v/v). The profile represents a typical fractionation assay. Oher experimental conditions are in the text

The procedure described allows the preparation of almost pure sn-2 EPA monoacylglycerol (98%) and sn-2 DHA monoacylglycerol (94%), the last product being mixed with relatively small amounts of sn-2 DPA monoacylglycerol (less than 4%) and sn-2 DTA monoacylglycerol (less than 3%). Both, sn-2 EPA and sn-2 DHA monoacylglycerols can be easily obtained in laboratory-scale with relatively high yield. These products are suitable as substrates for interesterification with medium- or long-chain fatty acids to obtain structured TGs with specific positional composition.

Table I
**Fatty acid* composition of FM1 and FM2 fractions**

| Fatty acid | FM1 | FM2 |
|---|---|---|
| C 20:5 (EPA) | 98.2 | 0.2 |
| C 22:6 (DHA) | — | 94.3 |
| C 22:5 (DPA) | — | 3.6 |
| C 22:3 (DTA) | — | 2.8 |
| N.I.** | 1.8 | — |

* Expresed as methyl ester derivatives
** Not identified

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