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Selective enrichment of polyunsaturated fatty acids by hydrolysis of fish oil using immobilized and stabilized *Rhizomucor miehei* lipase preparations

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A B S T R A C T

Immobilization of *Rhizomucor miehei* lipase (RML) by two different methods is reported. In the first approach RML was directly immobilized on epoxy functionalized silica particles in a mild condition. In the other approach and to perform oriented immobilization, the epoxy functionalized support was partially modified by introducing iminodiacetic acid groups fallowed by addition of Cu^{2+}. In this type of immobilization the covalent linkage takes place after initial adsorption of the enzyme on the support via metal chelate affinity interaction. The results showed higher thermal stability for the derivative obtained by this method. Co-solvent stability of the derivatives and free RML was also studied in presence of 10 and 20% of six polar organic solvents (DMSO, THF, acetonitrile, 1-propanol, 2-propanol and dioxane). The results showed improved stabilities for both derivatives, silica-epoxy-IDA-RML in particular. The ability of the immobilized preparations and the free enzyme to catalyze hydrolysis of fish oil was determined at different conditions. The results revealed that all the derivatives discriminate between *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) in favor of EPA. The content of EPA and DHA increased with time and the amount of biocatalyst during the hydrolysis process. Reusability of the enzyme greatly improved after immobilization.

Keywords: *Rhizomucor miehei* lipase; Oriented immobilization; Silica; Selective hydrolysis; Fish oil; Polyunsaturated fatty acids

1. Introduction

The production of fatty acids by the hydrolysis of oils is a known way in the economic use of these renewable important substrates (Park et al., 2000). Oils are part of a group of materials known as fatty acid esters and their hydrolysis by water produces valuable free fatty acids. Among them, fish oil consists virtually of pure triacylglycerols comprising more than fifty different fatty acids with chain lengths range from C14 to C24 of different degree of instauration, from fully saturated to polyunsaturated (Barlow and Stansby, 1982). *n*−3 polyunsaturated fatty acids (PUFA) are characteristic of marine fats. *cis*-5,8,11,15,17-Eicosapentaenoic acid (EPA) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) are the main bioactive omega-3 fatty acids. The beneficial health effects of marine oils are now well documented and attributed to *n* − 3 PUFA, EPA and DHA in particular (de Deckere, 2001). EPA and DHA are ingredients used in dietary supplements, healthy foods, and pharmaceutical products (Kamal-Eldin and Yanishlieva, 2002). The American Heart Association has recommended that all individuals should eat fish; twice a week and those with documented coronary heart disease should

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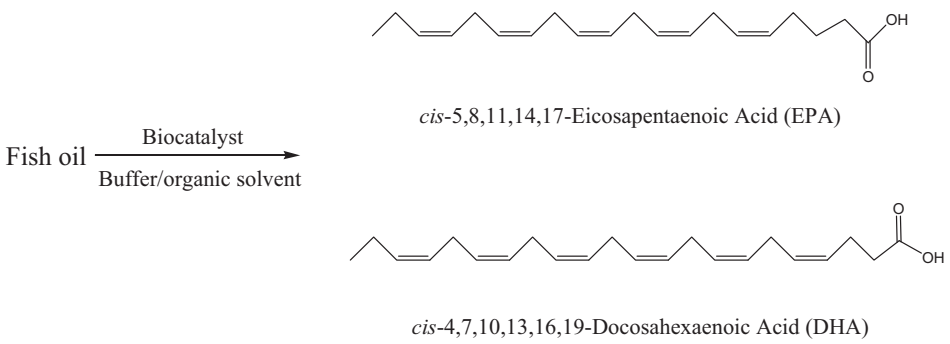

Fig. 1 – Selective hydrolysis of fish oil using free and immobilized RML.

take fish oil supplements that supply 1 g/day of EPA and DHA. Consumption of EPA and DHA reduces the risk of coronary heart disease by lowering triglyceride levels and blood pressure (Kris-Etherton et al., 2003). Studies have shown that those who incorporate fish into their diets had a lower risk of mortality from coronary heart disease (Kromhout et al., 1995). The long chain fatty acids EPA and DHA have also been reported to have ability to the reduction of certain types of diabetes, cancers and mental health disorders (Nettleton and Katz, 2005).

Due to the importance of omega-3 polyunsaturated fatty acids, various techniques have been used for enrichment of these compounds. One of the most promising techniques is the use of lipase-catalyzed enzymatic hydrolysis of fish oil.

Lipases have found applicability in oil chemistry due to their fatty acid selectivity which can be utilized in concentrating groups of fatty acids from oils by kinetic resolution. There are numerous reports in the literature showing the use of commercially available free lipases to concentrate EPA and DHA from marine oils (Faber, 2011). They reveal that most of lipases discriminate against $n-3$ PUFA and those lipases that display any significant activity toward $n-3$ fatty acids usually prefer EPA to DHA (Fernández-Lorente et al., 2012).

The use of soluble enzymes in chemical reactions has many disadvantageous (high cost, low stability in reaction media, recovery problems). In order to overcome these limitations, it is necessary to use immobilized form of enzymes. Therefore, improvements in enzyme immobilization are current focus of research in fat and oil industries (Fernández-Lorente et al., 2012; Pizarro et al., 2012). Lipases can be immobilized by several methods such as adsorption, cross-linking, multipoint covalent attachment and physical entrapment (Habibi et al., 2013; Villeneuve et al., 2000). Selection of a suitable support and the use of proper immobilization technique would be a powerful way to improve the enzyme properties (Mateo et al., 2007; Pedroche et al., 2007). Generally, most of enzyme immobilization techniques take place via a non-specific adsorption of enzyme on a solid support or by the reaction of several residues on the surface of proteins (carboxylic acids, amines, hydroxyls, thiols) with reactive groups on the support. In both cases, the corresponding proteins are attached to the surface in random orientation which may cause to some drawbacks like decrease in enzyme activity and reproducibility problem. Covalent immobilization of enzymes by the orientation of a protein via a particular amino acid on the other hand, allows proteins to be arranged in a more controlled style. In general, functional properties of an immobilized enzyme (e.g., activity toward different substrates, selectivity, stability, etc.) depend on the way that the protein is immobilized on support.

In this paper, two different techniques have been used to prepare immobilized derivatives of RML. In the first approach epoxy functionalized silica particles (silica-epoxy) were prepared as a conventional support for random immobilization of the lipase. In the other approach, partial modification on silica-epoxy was made using iminodiacetic acid in order to have a heterofunctional support with ability to coordinate with histidine groups of the protein (silica-epoxy-IDA). In this way enzyme immobilization was directed via histidine group and then further incubation led to covalent binding of the enzyme to the support via the reaction between remaining epoxy groups on the support and nucleophilic groups on the enzyme surface. The influence of immobilization technique on catalytic behavior of the derivatives was also evaluated in selective hydrolysis of poly unsaturated fatty acids (EPA/DHA) from fish oil (Fig. 1).

2. Materials and methods

2.1. Materials

The lipase from *Rhizomucor miehei*, fish oil from menhaden, *cis*-4,7,10,13,16,19-docosahexaenoic acid, ethylenediaminetetraacetic acid (EDTA), *p*-nitrophenyl butyrate (*p*-NPB), iminodiacetic acid disodium salt monohydrate (IDA) and glycidoxypropyltrimethoxysilane (GPTMS) were from Sigma. Silica gel (70–230 mesh), 1,4-dioxane, 1-propanol, 2-propanol, dimethyl sulfoxide (DMSO), tetrahydrofuran (THF) and acetonitrile were purchased from Merck. *cis*-5,8,11,14,17-Eicosapentaenoic acid was from Cayman company. Other reagents and solvents were of analytical or HPLC grade. Fourier transform infrared spectra (FT-IR) were recorded on a Bomen FT-IR-MB-series instrument with a KBr pellet technique. Thermogravimetry (TGA) and differential thermal analysis (DTA) were carried out from 10 °C to 800 °C at a heating rate of 20 °C/min in air atmosphere using a STA 503 M system from Bähr GmbH, Germany.

2.2. Methods

2.2.1. Preparation of silica-epoxy support

The dry silica gel particles (1 g) were dispersed in 50 mL of dry toluene then 1 mL of GPTMS and 0.15 mL Et_3N were added. The resulting mixture was refluxed under nitrogen atmosphere and vigorous stirring for 4 h. The modified silica gel was collected by filtration and washed thoroughly with THF. Finally the modified particles were dried at 120 °C for 8 h. The presence of epoxy groups on the support was confirmed by IR spectroscopy and TGA–DTA analysis.

2.2.1.1. Determination of epoxy groups on the support. Determination of the epoxy groups on the support was carried