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Synthesis of structured triacylglycerols enriched in *n-3* fatty acids by immobilized microbial lipase

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ABSTRACT

The search for new biocatalysts has aroused great interest due to the variety of microorganisms and their role as enzyme producers. Native lipases from *Aspergillus niger* and *Rhizopus javanicus* were used to enrich the *n-3* long-chain polyunsaturated fatty acids content in the triacylglycerols of soybean oil by acidolysis with free fatty acids from sardine oil in solvent-free media. For the immobilization process, the best lipase/support ratios were 1:3 (w/w) for Aspergillus niger lipase and 1:5 (w/w) for Rhizopus javanicus lipase using Amberlite MB-1. Both lipases maintained constant activity for 6 months at 4 °C. Reaction time, sardine-free fatty acids:soybean oil mole ratio and initial water content of the lipase were investigated to determine their effects on *n-3* long-chain polyunsaturated fatty acids incorporation into soybean oil. Structured triacylglycerols with 11.7 and 7.2% of eicosapentaenoic acid + docosahexaenoic acid were obtained using Aspergillus niger lipase and Rhizopus javanicus lipase, decreasing the *n-6/n-3* fatty acids ratio of soybean oil (11:1 to 3.5:1 and 4.7:1, respectively). The best reaction conditions were: initial water content of lipase of 0.86% (w/w), sardine-free faty acids:soybean oil mole ratio of 3:1 and reaction time of 36 h, at 40 °C. The significant factors for the acidolysis reaction were the sardine-free fatty acids:soybean oil mole ratio and reaction time. The characterization of structured triacylglycerols was obtained using easy ambient sonic-spray ionization mass spectrometry. The enzymatic reaction led to the formation of many structured triacylglycerols containing eicosapentaenoic acid, docosahexaenoic acid or both polyunsaturated fatty acids.

Introduction

Lipases (acylglycerolacylhydrolases, EC 3.1.1.3) are biocatalysts with many industrial applications, naturally acting on carboxylic ester bonds to catalyze the hydrolysis of triacylglycerols (TAGs). In non-aqueous media, they can catalyze esterification, alcoholysis, acidolysis and transesterification reactions. The screening, kinetic characterization and immobilization of microbial lipases for use in biocatalysis has been

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an important focus of research in our laboratory. Previously we have described the selection by screening techniques on solid and liquid media of lipase-producing microorganisms and the characterization of the catalytic performance of fungal lipase preparations for hydrolysis and esterification reactions.[1–4] Among these strains, *Aspergillus niger*, *Rhizopus javanicus*, *Fusarium oxysporum* and *Penicillium solitum* are described as lipase-producers for the first time.[3,5] The chemo-, regio- and enantiospecific behavior of these enzymes are the great industrial interest. Most microbial lipases such as those from *A. niger*, *Rhizopus delemar*, *Rhizopus miehei*, *Mucor javanicus*, *R. javanicus* and *Yerrowia lipolytica* show 1,3-positional specificity releasing 2-monoacylglycerol and 1,2- and 2,3-diacylglycerol as products from the substrate.[2] Lipase from *A. niger* gave the best yields and enantioselectivities in the esterification of racemin ibuprofen.[1,6]

Lipases can be employed in the modification of the chemical structure of natural TAGs to improve their physicochemical properties and/or their health benefits. For instance, long chain polyunsaturated fatty acids such as the *n*-3 family (*n*-3 LCPUFAs) may be incorporated into TAGs from oils and fats *via* lipase-catalyzed reactions. These *n*-3 LCPUFAs, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), may promote specific effects related to metabolism and health and they show great promise for nutraceutical applications.

The concept of structured triacylglycerols (STAGs) implies modification of the fatty acids composition and/or fatty acids location on the glycerol backbone, and improvement of the physical and/or physiological properties of dietary lipids. Studies have also shown that enzymatically or chemically modified TAGs exhibit similar or, in some cases, greater benefits compared to blended oils with similar fatty acid compositions.[7–11]

Soybean oil (SO) is a cheap and abundant commodity in Brazil, representing an important source of fatty acids for regular consumption. SO presents an *n*-6/*n*-3 fatty acids ratio ranging between 7.1:1 and 11:1.[12,13] Based on experimental and clinical studies, the most favorable *n*-6/*n*-3 ratio for human nutrition is proposed to range between 2:1 and 4:1.[14]

In a previous study, we reported that TAGs from SO enriched in EPA and DHA can be obtained by acidolysis catalyzed by a commercial *R. miehei* lipase immobilized on a macroporous anion-exchange resin (Lipozyme RM IM®).[13] We also reported that some lipases, derived from native microorganisms, provide good results for obtaining PUFA concentrates as acylglycerols by hydrolysis of borage oil, salmon oil and sardine oil.[3,5,15] There are some studies reporting the production of TAGs enriched with *n*-3 PUFA by enzymatic acidolysis.[16] However, most of them have used commercially-available lipases in reaction media containing organic solvents and few studies exploring native lipases have been conducted. This fact highlights the importance of exploiting the capacities of novel microbial lipases to increase the content of *n*-3 LCPUFAs in vegetable oils.

Immobilization of the native lipases is an important part of the process insofar as the immobilized enzymes can be easily recovered and reused thereby making the process economically viable. On account of the relatively high surface hydrophobicity of lipases, the process of simple adsorption onto suitably hydrophobic supports has been the more used strategy for lipase immobilization. Therefore, this work has three main objectives: (i) to immobilize the native lipases from *A. niger* (AN) and *R. javanicus* (RJ) on Amberlite MB-1 using different lipase/support ratios; (ii) to determine the activity, stability and degree of incorporation of *n*-3 LCPUFAs by acidolysis of SO with a mixture of free fatty acids from sardine oil (sardine-FFA) in solvent-free media, by free and immobilized lipases; and (iii) to investigate some of the variables which may influence the incorporation of *n*-3 LCPUFA into SO by enzymatic acidolysis.

Materials and methods

Chemicals

Amberlite MB-1 and chemical and culture media reagents were obtained from Merck & Co., Inc. (Darmstadt, Hesse, Germany) and from Sigma–Aldrich Co. (St. Louis, MO, USA). Olive oil (Carbonell®) (Deoleo, Madrid, Spain), soybean oil (Liza®) (Cargill, Inc., São Paulo, São Paulo, Brazil) and sardine oil (Catalent Pharma Solutions, Inc., Sorocaba, São Paulo, Brazil) were purchased in the local market. The preparation of free fatty acids from sardine oil (sardine-FFA) was carried out using alkaline hydrolysis followed by acidification with acetic acid.[17] Its major component fatty acids were stearic (5.7%), myristic (7.4%), palmitoleic (8.1%), palmitic (16.5%) and oleic (15.3%) acids, EPA (19.8%) and DHA (11.4%).

Production and determination of lipases activities

This study was performed using AN and RJ lipases produced in our laboratory as previously described.[6] The production was carried out in a basal medium with an initial pH value of 6.0 that consisted of 2% peptone, 0.5% yeast extract, 0.1% $NaNO_3$, 0.1%, KH_2PO_4 0.05% $MgSO_4$. $7H_2O$ and 2% of olive oil. Cultures were carried out in Erlenmeyer flasks (500 mL) containing 120 mL of the growth medium. The cultures were inoculated with 1 mL of spores suspension (10^5–10^6 spores/mL) and the flasks were agitated on a rotary shaker (130 rpm) at 35 °C for 72 h. After that, the cultures were filtered and cleared filtrates were treated with ammonium sulphate (80% saturation). The precipitates were dialyzed overnight in sodium phosphate buffer, pH 7.0, and lyophilized for use as a crude lipase preparation in powder form. The residual water of the lyophilized lipases was 0.84% (w/w). Hydrolytic activity was determined by triolein, with olive oil as the substrate and employing a standard oleic acid curve. One activity unit (U) was defined as 1 μmol of oleic acid released per minute of reaction. The specific activity was expressed as U/mg of total protein. The amount of protein was determined by the method of Lowry et al.[18] AN lipase with 7.5 and RJ lipase with 6.8 U/mg were used, without further purification.

Immobilization by adsorption

The crude lipases were immobilized by adsorption onto Amberlite MB-1 support at different lipase/support ratios (w/w). The crude lipase preparation (0.07–0.17 g) was dissolved in 5 mL of sodium phosphate buffer (50 mM, pH 7.0), mixed