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Stabilization of lipase from *Thermomyces lanuginosus* by crosslinking in PEGylated polyurethane particles by polymerization: Application on fish oil ethanolysis

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ABSTRACT

The adsorption of *Thermomyces lanuginosus* lipase (TLL) on PEGylated polyurethane particles as support permitted the development of several strategies to improve the properties of this commercial low-cost enzyme. The supports were synthesized by miniemulsion technique using isophoronediisocyanate (IPDI) and poly(ε-caprolactone) diol (PCL530) as monomers. The aqueous phase was composed of distilled water, surfactant sodium dodecyl sulfate (SDS), and poly(ethylene glycol) with different molar mass (PEG 400, 4000 or 6000). Polyethyleneimine (PEI) and trehalose were used to coat the PU-PEG polyurethane particles in order to increase the stability. In general, the coating with PEI (20%) allowed a greater stability of the derivatives. (100% of relative activity at 50 °C during 8 h). TLL immobilized on PEGylated polyurethane particles was efficiently used in the production of ethyl esters from fish oil compared to the free TLL (data not shown). The values of ethyl esters production of EPA and DHA were dependent on the support used for immobilization, which proved to be a determining factor in the activity. The highest selectivity obtained value was 45.8 for the PU-PEG4000-PEI20 derivative.

1. Introduction

Miniemulsion are classically defined as aqueous dispersions of relatively stable oil droplets within size range of 50–500 nm prepared by shearing a system containing an organic phase, water, surfactant, and "co-surfactant" [1–6]. Nanoparticles obtained by miniemulsion are used as drug carrier, such as particles of biodegradable poly(urea-urethane) (PU). Recent studies disclose the use of this technique in the synthesis of supports for immobilization of enzymes [7,8]. The use of polymers such as polyurethane as a support for the immobilization of enzymes are well studied, but the use of PU synthesized from miniemulsion is still barely investigated for this purpose. The advantage of using this technique to obtain PU particles is the one-step synthesis, by interfacial polycondensation in miniemulsion.

Lipases immobilization has been widely studied, mainly due to the industrial importance of the catalysts obtained [6–9]. Although much discuss about immobilization, there is still a search for the "ideal support", that allows obtaining a biocatalyst efficient, low cost, simple synthesis, which results in higher production of the compound of interest. Previous results [13] verified that PU-PEGylated (PU-PEG) particles as support obtained by the miniemulsion are promising to *Thermomyces lanuginosus* lipase (TLL), showing satisfactory results in fish oil esterification. However, the stability of the catalyst can compromise its application, so soluble stabilizing additives (eg polyethyleneimine and trehalose) can be used after the immobilization process in order to provide greater stability to the derivative.

TLL is an important enzyme for several industrial applications, although initially oriented toward the food industry. This enzyme is also widely used, for example, in biodiesel and fine chemicals production [14]. An important application for this enzyme

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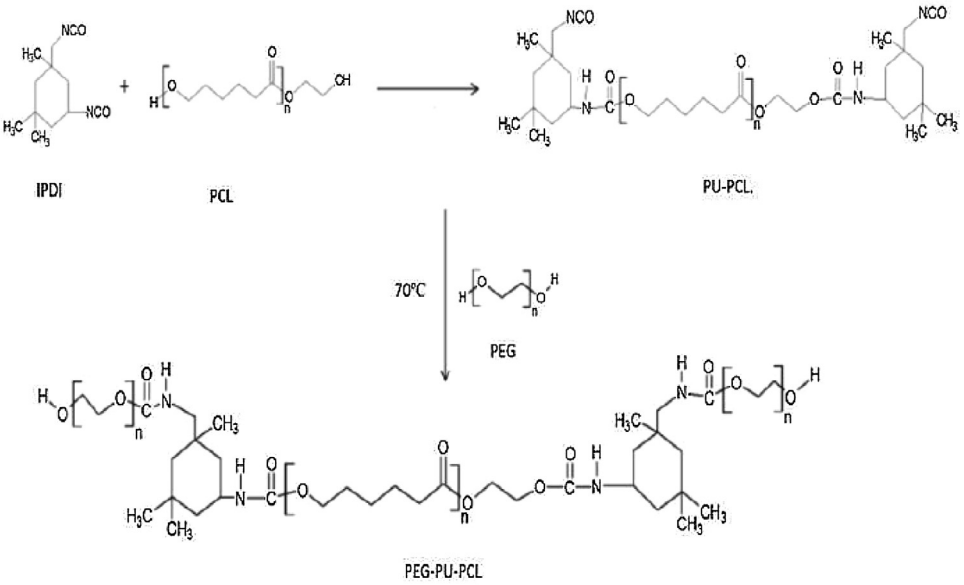

Fig. 1. Scheme of formation of PU-PEGylated particles.

is in the production process of long-chain polyunsaturated fatty acids (PUFAs), especially docosahexaenoic acid (DHA, C22:6n-3) and eicosapentaenoic acid (EPA, C20:5n-3) [11,12].

The use of fish oil, rich in Omega-3 fatty acids in reactions with enzymes is an attractive approach since enzymatic processes can be carried out under mild conditions without undesirable byproducts formation [13,14]. The ethanolysis reaction of fish oil allows the formation of ethyl esters rich in Omega-3 using mild temperatures, thereby protecting the polyunsaturated fatty acids from oxidation, and an alternative for obtaining specific structured lipids [19]. Moreover, the separation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from enzymatic catalysis (selective hydrolysis of methanolysis) is very interesting due to the similar molecules that are hard to separate by physicochemical protocols [17,20].

This report investigated the effects of polyethyleneimine and trehalose in the stability of immobilized derivatives from TLL lipase. PU-PEG particles synthesized by miniemulsion polymerization were used as enzyme support. The derivatives were used as a catalyst is the solvent-free transesterification of fish oil.

2. Experimental

2.1. Chemicals

Isophoronediisocyanate (IPDI, 98%, Mw 222 g/mol) and cyclohexane (97%) were purchased from Alfa Aesar (USA). Poly(ethylene glycol) diol with nominal molar mass of 400 (PEG400), 4000 (PEG4000) and 6000 Da (PEG6000), polycaprolactonediol with molar mass 530 Da (PCL530), and polyethyleneimine 25,000 Da (PEI 25,000) were purchased from Sigma-Aldrich (Germany). Surfactant sodium dodecyl sulfate (SDS) obtained from Aldrich Chemicals Ltd. D-Trehalose, 99% anhydrous was purchased from ACROS Organics. *p*-nitrophenyl butyrate (*p*-NPB), ethanol, cyclohexane were obtained from Sigma Chemical Co. (USA). Free *T. lanuginosus* (TLL) was generously donated by Novo Nordisk (Denmark). Sardine oil was obtained from Biotec BTSA (Spain). All others reagents and solvents used were of analytical or HPLC grade.

2.2. Enzymatic activity determination

The enzymatic activity was measured by hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB) used a spectrophotometer (JASCO V-630) with thermostatic cell and continuous magnetic stirring (500 rpm) for 2.5 min. The increase in absorbance at 348 nm produced by *p*-nitrophenol released during the hydrolysis of 0.4 mM of *p*-NPB in 25 mM sodium phosphate at pH 7 and 25 °C was measured. The value of activity was calculated using $\varepsilon = 5.150\ M^{-1}\ cm^{-1}$. One unit of enzyme activity (U) was defined as μmol of hydrolyzed *p*-NPB per minute per mg of enzyme under the described conditions [17]. The results were obtained in triplicate.

2.3. Determination of protein concentration

The protein concentration was determined spectrophotometrically according to Bradford [21]. Bovine serum albumin was used as standard protein for calibration curves.

2.4. Synthesis of PU-PEG nanoparticles by miniemulsion polymerization

PU-PEG nanoparticles were synthesized by miniemulsion polymerization based on the procedure previously described by Valério and coauthors [4]. The organic phase was composed by IPDI and PCL530 solution (2.5 NCO: OH molar ratio). The aqueous phase was prepared with 10 wt% of surfactant (SDS) and 10 wt% of different PEG were used (400, 4000 or 6000). All of the quantities were related to the organic phase. The IPDI and PCL were dissolved in 2 mL of cyclohexane under magnetic stirring for 10 min at room temperature (25 °C). The aqueous phase was added in the organic phase and kept for 2 min forming an unstable emulsion. The miniemulsion was prepared by sonication of the previous emulsion with an ultrasonic probe (Fisher-Scientific-Ultrasonic Dismembrator 500, 400 W) set to 70% of power intensity for 2 min. Polymerization was conducted at 70 °C during 3 h in a jacketed flask (50 mL) (Fig. 1). PU-PEG support was lyophilized for further use.

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