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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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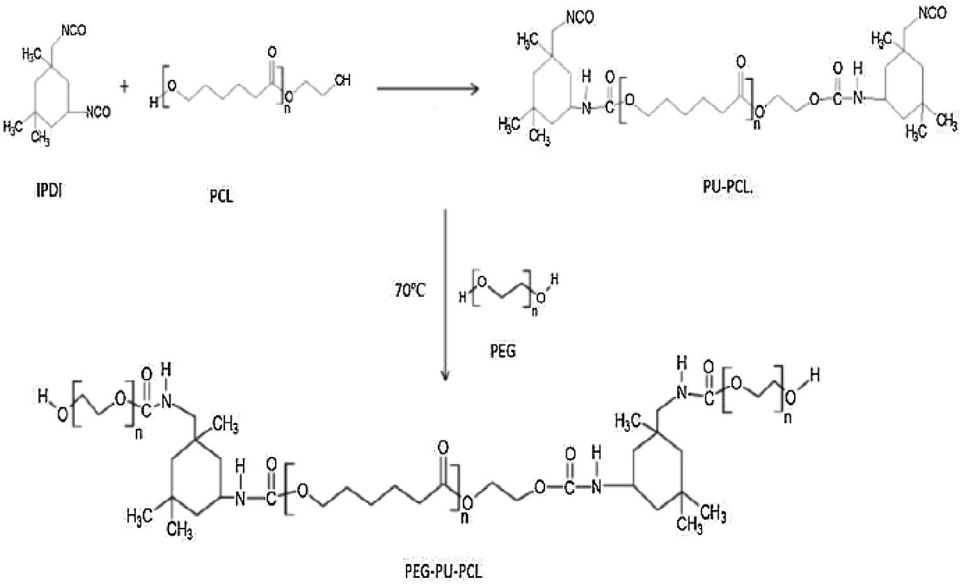

Fig. 1. Scheme of formation of PU-PEGylated particles.

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.

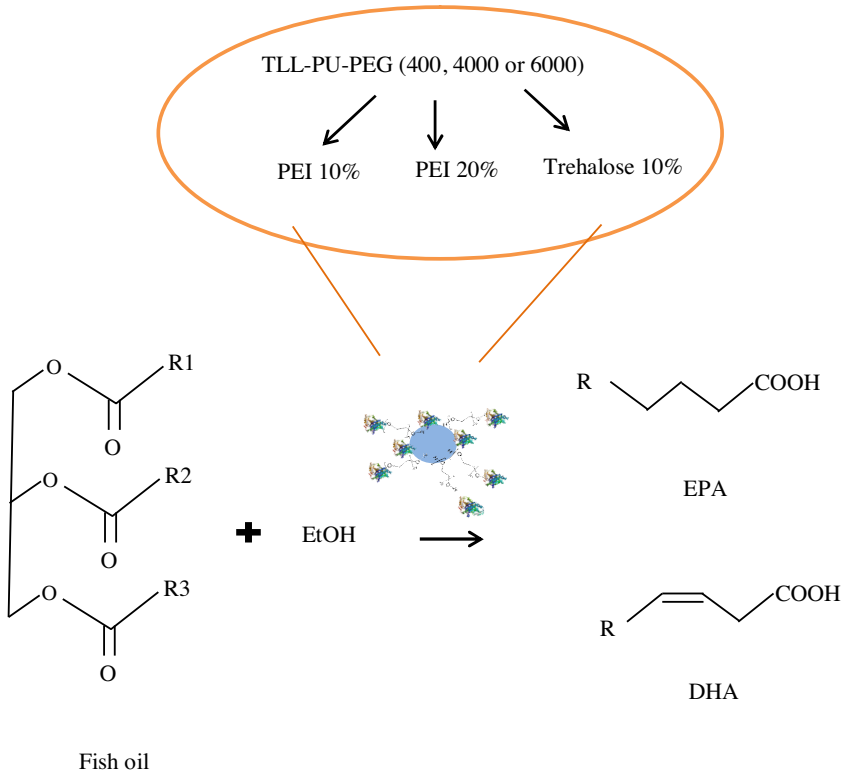

Fig. 2. Scheme of EPA and DHA production from TLL immobilized in PEGylated PU particles covered with PEI or trehalose.

2.5. *Immobilization of* T. lanuginosus *lipase (TLL) in PU-PEG particles*

The lipase (previously dialyzed on distilled water) was diluted 1:10 (m/v) using 5 mM phosphate buffer pH 7.0. PU-PEG particles (1 g) were used in the immobilization process. The immobilization mixture was stirred at room temperature (25 °C) until achieving the maximum immobilization percentage. Samples were taken periodically for enzymatic activity measurement according to item 2.2. The adsorption was monitored by the enzymatic activity in the suspension and in the supernatant [22]. The yield of immobilization (I) and recovered activity (RA) was calculated by Eq. (1) and (2), respectively

$$I = \frac{A - B}{A} \quad (1)$$

$$AR = \frac{C \times 100}{A \times I} \quad (2)$$

where I is the percentage of immobilization, A is the activity offered support for immobilization, B is the enzyme activity of the supernatant at the end of the immobilization process and C is the derivative of the activity at the end of the immobilization process [23].

2.6. *Preparation of coated supports- PU-PEG-PEI and PU-PEG-Trehalose*

After the end of immobilization process, the derivatives were coated with polyethyleneimine (PEI) (10 or 20%wt/v) or trehalose (10%wt/v). A solution of PEI or trehalose was prepared in sodium phosphate buffer pH 7.0 and 0.05 M. The derivatives (PEGylated PU-PEG400, 4000 and 6000 Da) were kept at stirring overnight at 25 °C. Finally, the suspensions were filtered and stored at 4 °C.

2.7. *Determination of kinetic parameters of free and immobilized* T. lanuginosus *lipase (TLL)*

The kinetic constants were determined using *p*-NPB as substrate in different concentrations (12.5, 25, 50, 75, 100, and 150 mM). The apparent K_m and V_{max} values for immobilized lipase in PU-PEG particles were calculated from Lineweaver–Burk plots, using the mass of protein. The results shown were obtained from the resulting equation of the means of the values plotted in the graph.

2.8. *Free and immobilized enzyme TLL in coated PU-PEG particles: thermal stability and pH effect*

Free and immobilized enzyme TLL in PU-PEG particles were kept at room temperature (25 °C) for 2 h in buffer sodium citrate 50 mM (pH 5), buffer phosphate 50 mM (pH 7), and buffer sodium bicarbonate 50 mM (pH 9). TLL enzyme activity was measured according to the methodology described previously (Section 2.2). For thermal stability free and immobilized TLL in PU-PEG particles (0.1 g) were diluted in 1 mL of buffer phosphate 25 mM, pH 7, then incubated at 50 °C for different times. The enzyme activity was evaluated according to the Section 2.2. The results were obtained in triplicate.

2.9. *Production of ethyl esters of Omega-3 fatty acids*

The immobilized TLL was used for the production of ethyl esters of Omega-3. The immobilized TLL in PU-PEG particles (0.1 g) was added to the substrate solution with 0.701 mmol of sardine oil and 5.77 mmol of ethanol, without organic solvents, and 0.2 g of molecular sieve (3 Å) (Fig. 2). The experiments were carried out at 37 °C for 72 h under magnetic stirring. The production of eicosapentaenoic acid ethyl ester (EE-EPA) and docosahexaenoic acid ethyl ester (EE-DHA) was determinate by HPLC [17]. The results were obtained in triplicate.

2.10. Monitoring of esters production

The content of esters was determined by RP-HPLC (Spectra Physic SP 100 coupled with UV detector Spectra Physic SP 8450) using a reversed-phase column (Ultrabase-C8, 150 × 4.6 mm, 5 μm). The flow rate was 1.5 mL/min with acetonitrile/water/acetic acid (80:20:0.1, v:v), and pH 3. The UV detection was performed at 215 nm. The synthetic yields were calculated according peak corresponding at the pure compounds, EPA (retention time of 10 min) and DHA (retention time of 13 min).

3. Results and discussion

3.1. Effect of coating on the T. lanuginosus lipase (TLL) activity immobilized in PU-PEG particles

Table 1 shows the specific activity and recovered activity for the TLL immobilized in PU-PEG particles. PEI is a polybasic aliphatic amine, used in immobilization techniques as a minor constituent, giving a hydrophilic character and greater mechanical strength to the preparations of immobilized enzymes [24]. PEI treatment can result in better retention activity, thermal stability, and support coated with amine could allow more effective proteins adsorption [11]. The PEI coating derivatives with 20% showed the highest activity values. In previous experiments we found that the enzyme activities of derivatives without PEI coatings were 67, 180 and 177 U/g support for PU-PEG400, PU-PEG4000 and PU-PEG6000, respectively. The addition of PEI had a positive influence on the interaction with the enzyme (Table 1), increasing considerably the activity by hyperactivation.

The effect of coating with trehalose, as a possible protective agent, was not observed. On the contrary, it appears to have destabilized enzyme-support structure, resulting in a decrease on the specific activity compared to the results of the work previously cited. Only the support with greater polymeric chain showed a satisfactory activity. According to Table 1, it can be considered the PU-PEG6000 derivative as the more rigid, not requiring further treatment such as crosslinking, to ensure its high enzyme activity. For other derivatives, treatment with polyethyleneimine (20%) was considered most appropriate to ensure higher activity values.

3.2. Study of pH in activity of immobilized TLL coated PU-PEG particles

The derivatives were incubated at pH 5.0, 7.0 and 9.0 for 2 h, and the relative activity was calculated (Fig. 3). The enzymatic activity was expressed as relative activity, the calculation was conducted by the derivative of the difference at the starting time of the reaction (after the addition of the buffer, this value was considered as 100%) and the end of 2 h, the percentage was calculated. The coatings applied to the PU-PEG400 resulted in stable derivatives across the range studied. On the other hand, PU-PEG4000 and PU-PEG6000 showed different behavior. This should be because of agglomeration, first the size of PEI and PEG as well as its interaction with some important amino acids in catalytic center. Clearly, was possible to see this behavior in the study of effect of pH and in the enzymatic activity. In both cases, the increase in the size of the polymer (PEG) derivatives tends to destabilize it more due to the effects of diffusion and solvation of amino acids involved in the catalytic center of the derivative with the molecules and loads of PEI.

In our previous study showed that the interaction between enzyme and support, as expected, is performed by adsorption. [13]. The same study showed, where the use of the supports without coating was tested, showed high stability, with slight reduction in the pH, except for the PU-PEG4000 at pH 9.0 and 10.0 that showed

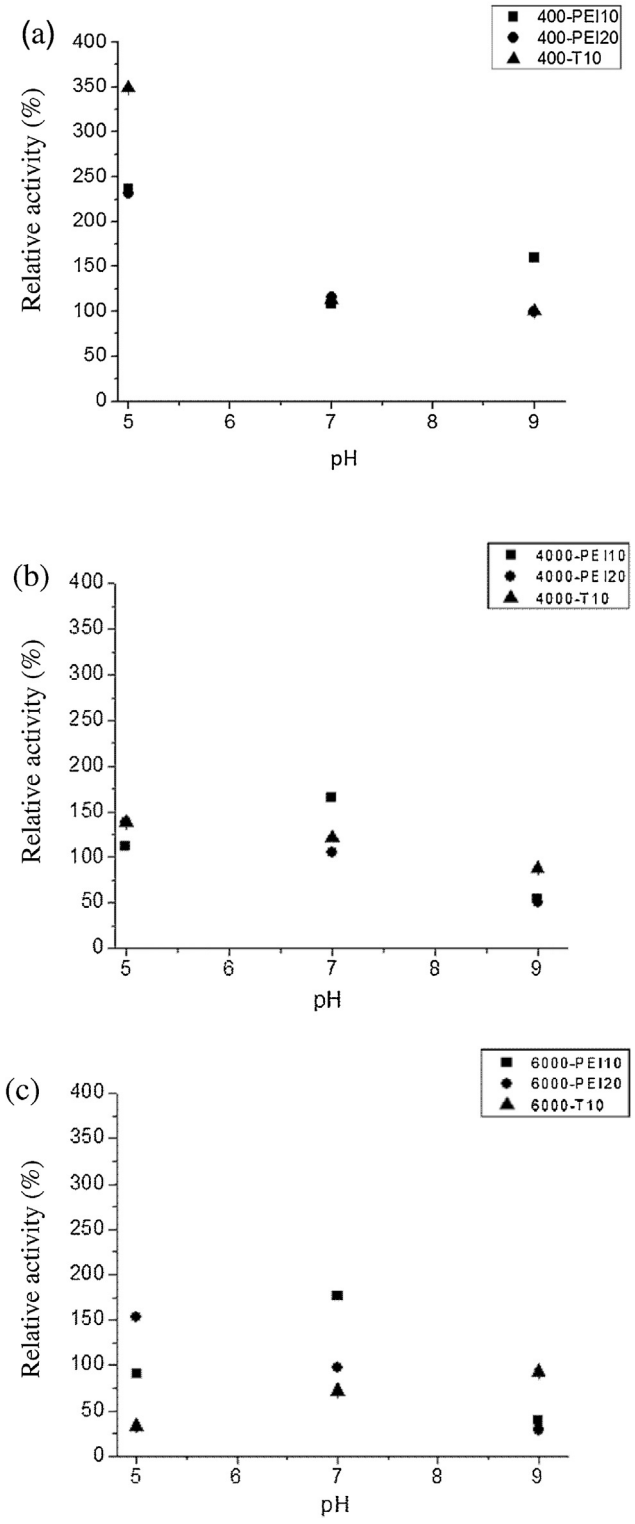

Fig. 3. Effect of pH in the enzyme activity of TLL immobilized in PU-PEG400 (a), PU-PEG4000 (b), and PU-PEG6000 (c) coated with PEI10 (■), PEI20 (●), and T10 (▲).

50% of relative activity. In general, the coatings were effective at pH 5.0 and 7.0 (Fig. 3). The PU-PEG 6000 support with trehalose 10% (T10) covering showed opposite behavior, with 34% of relative activity at pH 5.0. The trehalose, in this case, could be playing a role deactivator at lower pH values. Due to the fact that this enzyme presents isoelectric point below 7, and in these conditions predominate positive charges on the surface of the enzyme, it may be

Table 1
Effect of coating on the TLL enzyme activity using different PU-PEG particles as support.

Enzyme	Specific activity (*p*-NPB) [U/g support][a]	Recovered activity (%)
Free TLL	175.0 ± 0.1[b]	–
PU-PEG400-PEI10	46.7 ± 0.2	54.0
PU-PEG400-PEI20	104.2 ± 0.5	119.0
PU-PEG400-T10	26.1 ± 0.3	29.8
PU-PEG4000-PEI10	132.3 ± 1.0	75.6
PU-PEG4000-PEI20	229.2 ± 1.3	130.0
PU-PEG4000-T10	69.1 ± 0.6	39.4
PU-PEG6000-PEI10	168.8 ± 2.0	96.6
PU-PEG6000-PEI20	193.7 ± 0.1	110.7
PU-PEG6000-T10	146.1 ± 0.5	83.5

[a] Means ± SD (n = 3).
[b] Offered activity.

Table 2
Kinetic parameters of immobilized lipase in PU-PEG particles.

Enzyme	K_m (mM)	V_{max} (μmol/min/$mg_{ofderivative}$)	V_{max}/K_m
Free TLL	0.212	292.9	1381.37
PU-PEG400-PEI10	0.539	15.9	29.50
PU-PEG400-PEI20	1.127	9.4	8.34
PU-PEG400-T10	1.426	4.0	2.81
PU-PEG4000-PEI10	1.912	17.4	9.10
PU-PEG4000-PEI20	2.239	20.0	8.93
PU-PEG4000-T10	5.908	4.7	0.80
PU-PEG6000-PEI10	5.247	13.5	2.57
PU-PEG6000-PEI20	3.210	12.0	3.74
PU-PEG6000-T10	0.921	13.2	14.33

playing an opposite role (destabilizing) with aminoacid structures in protein.

3.3. Thermal stability of immobilized TLL in coated PU-PEG particles

The thermal stability study is very important for further applications. Fig. 4 shows the relative activity of PU-PEG particles evaluated at 50 °C for 8 h. The support coated with 20% of PEI (PEI 20) showed higher stability and 100% of relative activity in all studied range. The coating with PEI20 influenced positively all supports studied, PU-PEG400, PU-PEG4000 and PU-PEG6000. The results suggested that TLL lipase adsorption in the PEGylated PU particles coated with PEI20 increased the thermal stability of the biocatalyst. This is due to the presence of numerous charges of the amino groups in the PEI, promoting many intense interactions between enzyme and the support with PEI, increasing considerably stability.

There is much in the literature about the thermal stability of the immobilized enzymes, and this is among the most important objectives of developing immobilization techniques, the enzyme to become more stable and resistant to severe conditions, like wide ranges of pH and temperature, and may also preserve enzyme activity through several cycles [25]. This paper presents a novel attempt to improve the synthesis method of the support previously proposed, which is the miniemulsion technique [13], widely used in the production of drug carrier [26]. The authors believe that other ways of improving the support stability and the use of other crosslinking agents can also have application on supports with respect to thermal stability.

3.4. Kinetic properties of immobilized lipases

The kinetic constants were determined using *p*-NPB as substrate. The activities of free and immobilized TLL enzyme at different concentrations of substrate (12.5, 25, 50, 75, 100 and 150 mM) were calculated (Table 2). The concentration of enzyme was the same in all experiments. K_m is a measure of enzyme-substrate affinity. Free TLL showed a value of 0.212 mM, and the enzyme derivative that showed approximate value to free TLL enzyme was the PU-PEG400-PEI10 with 0.539 mM. The increase in the K_m values may be just due to slight distortions of the enzyme structure associated to the immobilization [27]. The V_{max} value for free TLL enzyme was determined as 292.9 μmol/min/mg. As expected, the V_{max} declined with the immobilization process and the highest results for the enzyme derivative were obtained when PU-PEG4000-PEI20 was used (20.0 μmol/min/mg). There may also be enzyme microenvironment effect, where loads of crosslinking agents influencing the immobilization process. Diffusional effects which hinder the input substrate or product output of immobilized system can also be caused the increased in the K_m value. Another possible explanation for the increased K_m is the modifications in the zeta potential, PEGylated PU particles can change the zeta potential of substrate, thus leading to change of electric attraction between enzyme and substrate.

The enzyme affinity for the substrate was significantly decreased, as in the use of PU-PEG4000-T10, that showed K_m of 5.908 mM The catalytic efficiency, given by V_{max}/K_m, was also calculated, and in this work we can highlight the value found for PU-PEG400-PEI10 (29.50 μmol/min/mg) as support.

3.5. Ethanolysis of fish oil in solvent free system catalyzed by immobilized TLL enzyme in coated PU-PEG particles

To enhance the PUFA levels in food, EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) can be stabilized as ethyl esters. Although free fatty acids and triglycerides may be metabolized more rapidly and completely than the ethyl esters, the fatty acids are easily oxidized. Currently, most commercial concentrates are derived from ethyl esters of Omega-3 [17,28,29]. Thus, this work contributes to the study of immobilization process, with the proposed use of a polymer synthesized by a route not explored in immobilized biocatalysts, the process of miniemulsion, such as application in catalysis reaction with rich triglycerides in polyunsaturated fatty acids.

TLL immobilized in coated PU-PEG particles was used as catalyst for enzymatic ethanolysis of sardine oil in solvent free system. All derivatives were used for this purpose. Fig. 5 shows the kinetic of ethyl esters production. The results are promising in terms of production of ethyl esters of Omega-3 and at a minimum 4 times higher than the values obtained in previous work using PU-PEG particles without coatings [13]. The derivatives coated with PEI showed the best results. This cationic polymer is widely used in literature and it's appointed as used in at differents pHs [30]. The same was not observed when using coating trehalose; this sugar probably caused a destabilization in the derivative during the reaction of production of esters, hindering the formation of the desired product. The selectivity is an important factor for study the production of ethyl ester. As seen in Table 3, the selectivity obtained in production

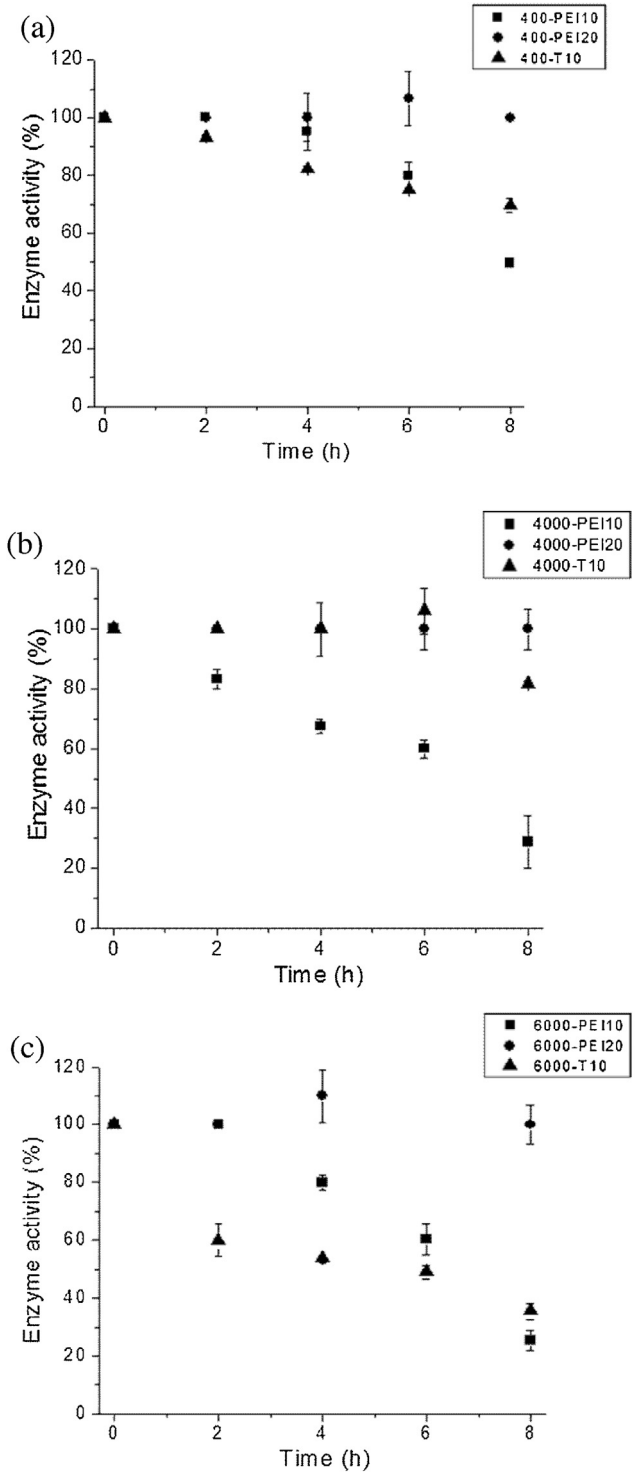

Fig. 4. Thermal stability of TLL immobilized in PU-PEG400 (a), PU-PEG4000 (b), and PU-PEG6000 (c),coated with PEI 10 (■), PEI 20 (●), and T10 (▲) at 50 °C.

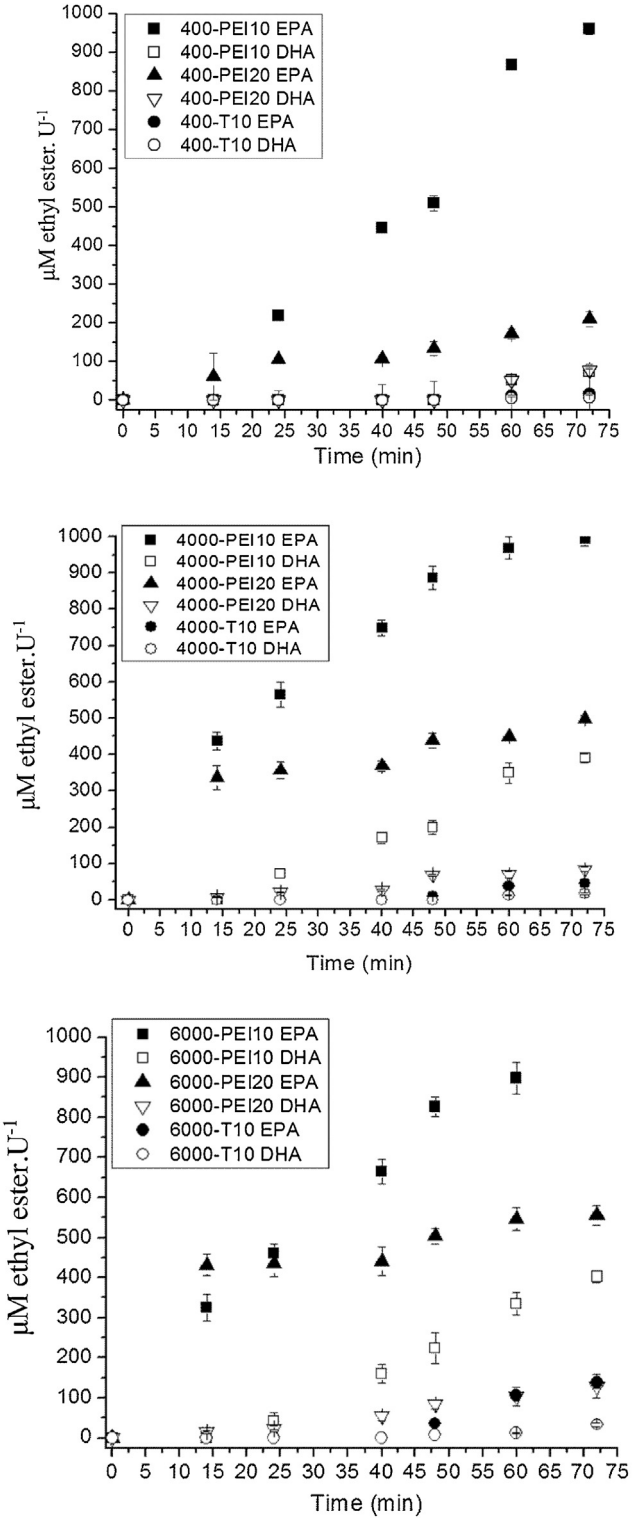

Fig. 5. Ethanolysis of Omega-3 fatty acids in solvent free system catalyzed by TLL immobilized in polymeric particles: PU-PEG400 (a), PU-PEG4000 (b), and PU-PEG6000 (c), coated with PEI10 EPA (■), PEI10 DHA (□), PEI20 EPA (▲), PEI20 DHA (Δ),T10 EPA (●), and T10 DHA (○).

reactions of ethyl esters was directly influenced by the media used in the immobilization process. It is likely that the derived coated with trehalose may have suffered diffusional effects, impairing the enzyme's interaction with the substrate. The selectivity EPA/DHA of TLL varied from 0.9 to 45.8. In all cases the derivative coated with 20% PEI (PEI20) plays a very important role in both production and selectivity.

4. Conclusions

Whereas the more stable the complex enzyme-support is, more interesting for large-scale application. This study showed that the polymeric derivative covered with trehalose or polyethyleneimine can be interesting for improving the enzyme stability and

Table 3
Selectivity of immobilized TLL in PEGylated PU nanoparticles.

Enzyme	Selectivity[a]
Free TLL	17.4
PU-PEG400-PEI10	4.2
PU-PEG400-PEI20	21.8
PU-PEG400-T10	0.9
PU-PEG4000-PEI10	22.5
PU-PEG4000-PEI20	45.8
PU-PEG4000-T10	2.0
PU-PEG6000-PEI10	23.4
PU-PEG6000-PEI20	29.4
PU-PEG6000-T10	2.2

[a] Measured at 15% of conversion total ethyl esters produced (EPA + DHA). Selectivity expressed as molar ratio between synthesized EPA and DHA.

activity. The coating of the polymeric derivatives from TLL lipase with polyethyleneimine and trehalose has a positive effect in the stability. The study shows that derivatives of PU-PEG were efficient in the solvent-free transesterification of fish oil. Based on the results obtained in this study, the proposed support, synthesized by PU-PEG particles coated with polyethyleneimine and trehalose, exhibited promising results, high percentages of immobilization and recovered activities. The best recovered activities were obtained when using PEI (20%) as coating: 119.0, 13.00 and 110.7 for PU-PEG400-PEI20, PU-PEG4000-PEI20 and PU-PEG6000-PEI20, respectively.

The derivatives were effective in the production of ethyl esters, having a production at least 4 times higher compared to PU particles without coating. The highest selectivity obtained value was 45.8 for the PU-PEG4000-PEI20 derivative.

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