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Properties of methoxy(polyethylene glycol)-lipase from *Candida rugosa* in organic solvents

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Abstract

Lipase from *Candida rugosa* was modified with methoxy(polyethylene glycol)–*p*-nitrophenyl carbonate (NPC–mPEG). A total of 34 out of 35 lysine chains were substituted, and the modified lipase retained 98% of the hydrolytic activity of the non-modified enzyme. mPEG-lipase is perfectly soluble in organic solvent and efficiently catalyzes the esterification of lauric acid, with nearly 100% of yield over 6 h in toluene. Moreover, mPEG-lipase exhibits a preference for the *S*-isomer over the *R*-isomer in the stereoselective resolution of racemic naproxen. © 1998 Elsevier Science S.A. All rights reserved.

Keywords: Lipase; Methoxy(polyethylene glycol); Protein modification; Esterification; Enantioselectivity; Enzymes in organic solvents

1. Introduction

Chemical modification of proteins with methoxy-(polyethylene glycol) (mPEG) has become a highly studied medical and biotechnologic application due to the unique properties of PEG and its general compatibility with biological systems [1]. Linking the enzyme to amphipathic polymers also increases its solubility in organic solvents, thus facilitating homogenous biocatalysis in organic syntheses (e.g. esterification or transesterification reactions or peptide syntheses). PEG binding can improve the solubility of proteins in organic solvents, in which some of the proteins retain their biological activity. In fact, the enzymes are essentially insoluble in organic solvents and, as a result, the suspended enzymes tend to coagulate, forming dense macroscopic aggregates in which only the outer surface is accessible to the substrate.

The basic strategy behind the preparation of mPEG–protein conjugates is to synthesize an 'activated' mPEG containing a reactive terminal group that can be readily coupled with a functional group on the protein, such as the ε-amino group of a lysine residue. Lipases are enzymes whose biological function is to catalyze the hydrolysis of triacylglycerols. They also act on a wide range of water-insoluble carboxylic esters.

This paper describes the purification of lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) from *Candida rugosa* and its modification with methoxy(polyethylene glycol)–*p*-nitrophenyl carbonate (NPC–mPEG) [2]. The modified, soluble lipase can be used as a catalyst in chemical reactions performed in organic solvents. The paper also deals with a kinetic study of the esterification of fatty acid, as well as a study of the stereoselective resolution of racemic naproxen in transparent organic solvents by methoxy(polyethylene glycol)-lipase.

2. Experimental

2.1. Materials

Lipase from *C. rugosa* was obtained from Amano Pharmaceutical Co., Ltd (Nagoya, Japan). 4-Dimethylaminopyridine and 4-nitrophenyl chloroformate were purchased from Fluka (Buchs, Switzerland); methoxy-(polyethylene glycol) 5000, *p*-nitrophenyl laurate, and 2,4,6-trinitrobenzensulfonic acid (TNBS) were obtained from Sigma Chemical Co. (St. Louis, USA); *o*-phthaldialdehyde and 2-dimethylaminoethylmercaptan were obtained from Aldrich (Steinheim, Germany); DEAE Sephacel was obtained from Pharmacia (Uppsala, Swe-

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den); BCA protein assay reagent was purchased from Pierce Chemicals (Rockford, IL, USA); and naproxen was donated by Alfa Chemicals (Milan, Italy). The other reagents were of analytical grade.

2.2. Lipase assay

Hydrolytic activity was evaluated spectrophotometrically on emulsified *p*-nitrophenyl laurate (pNPL) according to Carnielli et al. [3].

One volume of a 16 mM solution of pNPL in acetonitrile was mixed just before use with nine volumes of a 50 mM pH 7.5 phosphate buffer. Then, 1 ml of this mixture was pre-equilibrated at 25°C in the 1 ml cuvette of a UV–Vis spectrophotometer. The reaction was started by adding 10 μl of enzyme solution appropriately diluted with the 50 mM pH 7.5 phosphate buffer. The variation of the assay's absorbance at 410 nm against a blank solution without enzyme was monitored for 2 min. The reaction rate was calculated from the slope of the absorbance versus time curve by using an apparent molar extinction coefficient of 12.75×10^6 cm^2/mol for *p*-nitrophenol (pNP), which was determined from the absorbance values of standard solutions of pNP in the reaction mixture. One unit of activity is the amount of enzyme that produces 1 μmol of pNP per minute under the above conditions.

2.3. Spectroscopic measurements

Absorption spectra were obtained with a Lambda 5 Perkin–Elmer spectrophotometer.

2.4. Analytical methods

Protein concentrations were determined spectrophotometrically using the BCA protein assay reagent or the Bradford method [4]. The degree of modification of the lipase was determined by titration of the unmodified amino groups with 2,4,6-trinitrobenzensulfonic acid (TNBS) [5] or *o*-phthaldialdehyde (OPA) [6]. Automatic titrations were performed on a Radiometer pH-stat made up of a PHM 84 meter coupled with a TTT 80 titrator, an ABU autoburette, and a REC 80 servograph recorder.

GC analyses were performed with a Perkin–Elmer Sigma 3B Dual-FID gas chromatograph equipped with an SE 30 column, 100–200 mesh, $I = 15$ cm (Alltech, Deerfield, IL, USA); N_2 carrier gas, 25 ml/min, detector and injector temperature of 350°C (initial temperature 190°C, final temperature 230°C, rate/°C min 15, initial time 2 min, final time 3 min).

Esterification was monitored by HPLC Perkin–Elmer series 4, using a Chiracel OD (Daicel Chemical Industries, Ltd., Japan) chiral column capable of separating the *R*- and *S*-isomers of naproxen and their esters without derivation. The mobile phase was a v/v mixture of 96% *n*-hexane, 3% isopropanol and 1% acetic acid, at a flow rate of 0.4 ml/min. A variable-wavelength Perkin–Elmer model LC 75 UV detector at 270 nm was used for quantification at ambient temperature.

2.5. SDS-PAGE

Migrations were performed on slab gels using the mini Protean II cell (Bio-Rad Lab. Inc., USA). The resolving gels (pH 8.8; 12% acrylamide) were run at a constant voltage (200 V) and prepared according to the method originally described by Laemmli [7], which is based upon the discontinuous Ornstein–Davis buffer system with SDS present.

2.6. Lipase purification

C. rugosa powder (25 g) was suspended in 25 mM pH 7.5 phosphate buffer (200 ml) and centrifuged at $20\,000 \times g$ for 20 min at 4°C. The supernatant was treated with two volumes of ice cold ethanol and constantly stirred at 0°C during the addition of ethanol and for 1 h after addition. The precipitate (95% of the active lipase) was recovered after centrifugation, dissolved in 25 mM pH 7.5 phosphate buffer and extensively dialyzed against the same buffer. This step also removed most of the coloring material from the unpurified mixture.

The clear solution was loaded into a DEAE Sephacel column equilibrated with the same phosphate buffer and washed with the same buffer until no absorbance could be detected in the eluate at 280 nm. Elution of the lipase was achieved by a linear gradient of 0–0.5 M NaCl in 25 mM pH 7.5 phosphate buffer. The fractions showing lipase activity were pooled and concentrated by filtration with an Amicon Diaflo XM-50 membrane (50 000 cutoff).

The eluted peak appeared to be symmetrical for protein content and enzymatic activity. Analysis by PAGE revealed only one band with lipase activity.

2.7. Preparation of NPC–mPEG

A 0.2 mmol sample of mPEG, average molecular weight 5000, were dissolved in toluene (100 ml) and dried by azeotropic distillation. After cooling to 4°C, 1.0 mmol of 4-dimethylaminopyridine was added. Then, 1.0 mmol of 4-nitrophenyl chloroformate dissolved in anhydrous methylene chloride was added slowly under magnetic stirring. After standing 4 h, the reaction mixture containing a white precipitate of 4-dimethylaminopyridine hydrochloride was filtered through a fine sintered-glass funnel. The supernatant was concentrated under vacuum to approx. 10 ml and

added drop-by-drop to 100 ml of stirred diethyl ether at 0°C. The resulting precipitate of NPC–mPEG was collected by filtration, crystallized twice by ethyl acetate, and then stored dry and desiccated at 4°C. The degree of activation (which was evaluated spectrophotometrically at 400 nm on the basis of 4-nitrophenol in alkaline media for 15 min) was 98% (ε of *p*-nitrophenol at 400 nm = 17 000 M^{-1} cm^{-1}).

2.8. Covalent binding of lipase to activate NPC–mPEG

Solid NPC–mPEG was added slowly to a solution (5 mg/ml) of purified lipase in 0.2 M pH 8.2 borate buffer at 4°C. The reaction mixture, which was maintained at pH 8.2 by pH-stat, was stirred for 3 h. NPC–mPEG was added in a 5:1 molar ratio with respect to the ε-amino group on the lysine. During the modification process, the enzymatic activities of native and modified mPEG-lipase were assayed, and no decrease was detected. The degree of modification was determined by titrating the unreacted ε-amino group on the lysine with 2,4,6-trinitrobenzensulfonic acid (TNBS) [5] or with *o*-phthaldialdehyde (OPA) [6].

After the mixture was stirred overnight at room temperature, the unreacted mPEG was removed by filtration with an Amicon Diaflo XM 50 membrane and 0.05 M pH 7.2 phosphate buffer.

3. Results and discussion

3.1. Purification

The commercial lipase preparation from *C. rugosa* (Amano) contained 1% of protein, as determined by BCA protein assay or by the Bradford method [4]. The nature of the remaining 99% of the dry commercial powder is not known. It may consist of salts or other additives that were added to increase enzymatic stability.

The crude commercial powder (25 g) was treated with ice-cold ethanol to remove these impurities and most of the coloring material.

The proteins were separated from one another onto DEAE-Sephacel by ion-exchange chromatography. The protein elution profile showed one major peak for lipase activity, when eluted with approx. 0.05 M NaCl (data not shown). After dialysis against deionized water, the purified lipase solution was lyophilized and stored at 4°C.

The protein compositions of the crude and purified lipase were analyzed by SDS-PAGE. From this gel, the purity of the lipase preparation was estimated to exceed 95%.

A total quantity of 25 mg was collected.

3.2. Synthesis of mPEG-lipase

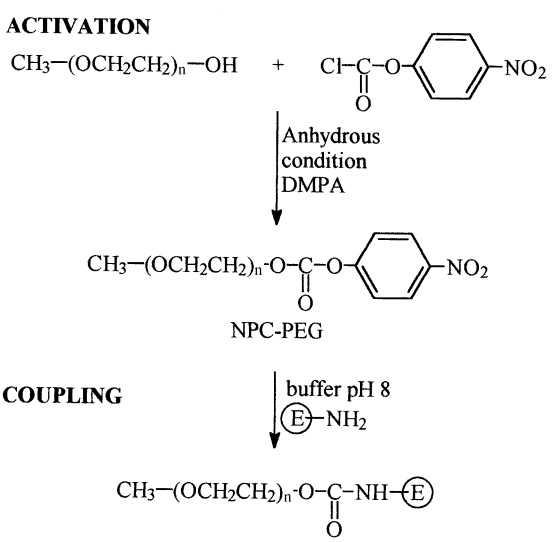
In most synthesis methods describing covalent attachment of mPEG to the lysil residues present in lipase, lipolytic activity is inversely proportional to percent modification [8–10], whereas the mPEG-lipase prepared in our laboratory retains 98% of its native lipolytic activity.

The relative data are as follows:

Modification	97%
Residual activity	98%
Lys mod/Lys native	34/35 [11]

Upon successful modification with mPEG, lipase acquires the property of forming clear solutions in some organic solvents. Since polyethylene glycol is amphipathic, it can modify enzymes in aqueous solution and it enables modified enzymes to function in a hydrophobic environment. After modification with NPC–mPEG (Scheme 1), lipase was soluble in various aromatic and chlorinated solvents such as benzene, toluene, chloroform and methylene chloride. Thus, this modified lipase can be used to study kinetics in homogeneous organic solutions rather than in emulsions. Derivatization also may lead to the development of catalysts with altered properties, such as substrate selectivity.

Khan et al. [12] hypothesized that PEG modified proteins are actually not soluble in organic solvents, but instead of formed microparticulate suspensions in organic solvents that look optically transparent. This distinction, however, is not important to our study, because we are only trying to use the ability of forming transparent solutions in organic solvents to distinguish the mPEG-modified enzyme from its native insoluble form.



Scheme 1.

Lipase has been widely used as a biocatalyst in the synthesis of esters and polyesters in organic solvent systems. For improved performance, transesterification can be used instead of esterification to prevent water from forming during the reaction, and an activated ester may be employed to increase reaction rate.

3.3. Rates of N-acylation with NPC–mPEG

A number of modifications were initially carried out on lipase at pH 8.2 and 25°C using different molar ratios of NPC–mPEG. Solid NPC–mPEG was added under stirring to a solution of purified lipase (5 mg/ml) in 0.2 M pH 8.2 borate buffer, and the pH of the resulting solution was maintained with pH-stat. The reaction was allowed to proceed until a plateau value was reached. The sample was then subjected to extensive dialysis and analyzed. The protein content was determined spectrophotometrically using the BCA protein assay reagent or the Bradford method [4], whereas the residual content of primary amino groups was determined by titration with the TNBS [5] or OPA [6] method.

The mPEG-lipase had been dried in vacuum over P_2O_5 from deionized water; in fact, we noted that mPEG-lipase undergoes a dramatic decrease in enzymatic activity after lyophilization. The native enzyme behaves similarly when lyophilized in the presence of PEG too. We suggested that the strong hygroscopic nature of the polymer enables water molecules to be stripped off the enzyme. These water molecules are essential to the existence of the enzyme's active structure.

The results shown in Fig. 1 suggest that lipase may be strongly acylated even when a low ratio of reagent to amino groups is used. Indeed, 34 mPEG chains (out of 35 possible chains) were linked in this experiment using 3 mol of NPC–mPEG/mol of NH_2.

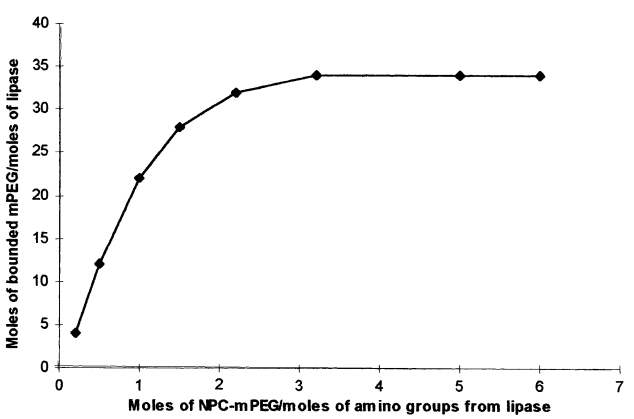


Fig. 1. Titration curve of *C. rugosa* lipase by NPC–mPEG. Each dot represents the results of a single experiment. Modifications with NPC–mPEG were carried out at pH 8.0 and 25°C. At the end of the modification, the mixture was dialyzed against water and analyzed (for protein content and residual amino functions).

3.4. Kinetic parameters

Hydrolysis of insoluble substrates by lipases takes place at the interface between the lipid and aqueous phases. Therefore, since the reaction rate is influenced by the substrate concentration in the bulk solution, it is not possible to determine kinetics parameters for a lipase acting on an insoluble substrate [13]. For this reason, we used a soluble substrate (pNPL) to determine the kinetics parameters of the free and modified lipases from *C. rugosa*. The rate of pNPL hydrolysis was measured at substrate concentrations from 1 to 8 mM. The kinetic constants were estimated by non-linear regression.

As is shown in Fig. 2, a plot of the rate of pNPL hydrolysis versus substrate concentration for free and for mPEG-lipase respectively is hyperbolic, which indicates Michaelis–Menten kinetics. The increase of reaction ratio as a function of substrate concentration was confirmed by the linearity of the Lineweaver–Burk plot (Fig. 2(a)). The (1.5-fold) increase in the K_m value for the modified lipase can be owed to a steric hindrance of methoxy(polyethylene glycol) chains, which renders the enzyme less accessible to the substrate.

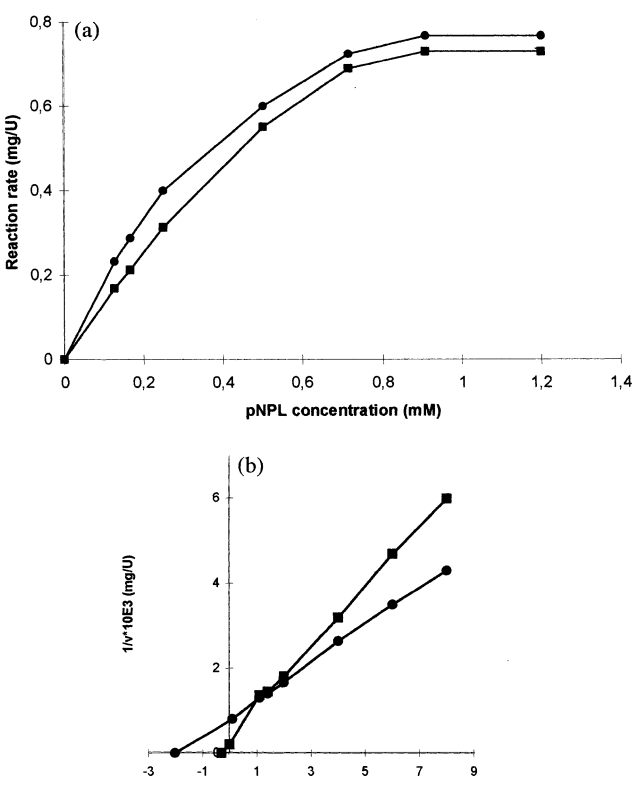


Fig. 2. Effect of pNPL concentration on initial reaction rate. The activities of the free (●) and modified (■) lipases from *C. rugosa* were measured at different concentrations of pNPL. The results are the average of three measurements of activity, with S.D. $< 10\%$.

3.5. Effect of organic solvents on enzymatic activity

The nature of organic solvents is crucial for maintaining the water content necessary to ensure catalytic activity. More hydrophilic solvents tend to strip the essential hydration water from the enzyme, thus distorting its catalytic conformation. Generally, however, it is observed that only a small amount of water is needed to employ enzymes successfully in organic solvents [14,15]. On the other hand, more hydrophobic solvents retain their catalytic activity, thus leaving a water layer that adheres to the enzyme surface and acts as a protective sheath [16].

As a result of these considerations, we carried out a series of experiments to examine the effect of the hydrophobicity of organic solvents on the activity of soluble mPEG-lipase. Our objective was to study the stability of modified lipase by measuring its residual enzymatic activity under fixed conditions after incubation for appropriate periods. Residual enzymatic activity was expressed as percentage of zero-time activity.

As is shown in Fig. 3, the best non-aqueous media for enzymatic reactions are hydrophobic solvents, since they do not strip essential water from the enzymes. These data suggest that the effect of an organic solvent on an enzyme is primarily due to interaction with the essential layer of water bound to the enzyme rather than with the enzyme itself. Indeed, these reactions are thought to occur in the aqueous microenvironment that surrounds the enzyme, with reactants and products partitioning the two fluid phases.

A study on the stripping of water from enzymes by organic solvents has indicated that there are at least two fractions of enzyme-bound water [17]. The first fraction is mobile water, which is stripped by polar solvents relatively easily. The second fraction, on the other hand, is structural in nature and its partitioning into an organic solvent would be thermodynamically unfavorable. As a result, the extent to which enzyme catalysis is possible in polar solvents depends upon the relative quantities of mobile and structurally bound water.

For this reason, we investigated the esterification of lipase-catalyzed fatty acid in toluene, which was dried with 3 Å molecular sieves.

Modified lipase was dissolved in a solution of toluene and lauric acid contained in small tubes with screw caps, and benzyl alcohol was added to start the reaction. In these experiments, both the lauric acid and the benzyl alcohol were dissolved at a temperature of 25°C in anhydrous toluene in equimolar amounts in the 0–2 M range. The tube was sealed and agitated on a rotary shaker. At the end of each specified time period, a sample was withdrawn from the tube and analyzed by gas chromatography using the method described in Section 2.4. As shown in Fig. 4, the synthesis of the ester and the accompanying disappearance of the fatty acid occurred fairly rapidly. More than 90% conversion was observed after 6 h.

Recently, lipase from *C. rugosa* [18], suspended in organic solvents, was found to esterify preferentially the *S*-isomer of racemic naproxen. Moreover, mPEG-lipase (in which 97% of all ε-amino groups in the molecule are coupled with polyethylene glycol) is soluble in organic solvents and retains 98% of the hydrolytic activity of the native enzyme. As a result, we tried to evaluate whether mPEG-lipase presents high enantioselectivity for the *S*-form of racemate naproxen under these conditions as well.

Esterification of chiral acid (*R*,*S*)-naproxen by mPEG-lipase with trimethylsilylmethanol as an acyl acceptor was tested in a mixture of 80% isooctane and 20% toluene by volume. The reaction mixture was stirred at 37°C, and a sample was injected into the above-mentioned HPLC system for different periods of time using the procedure described in Section 2.4.

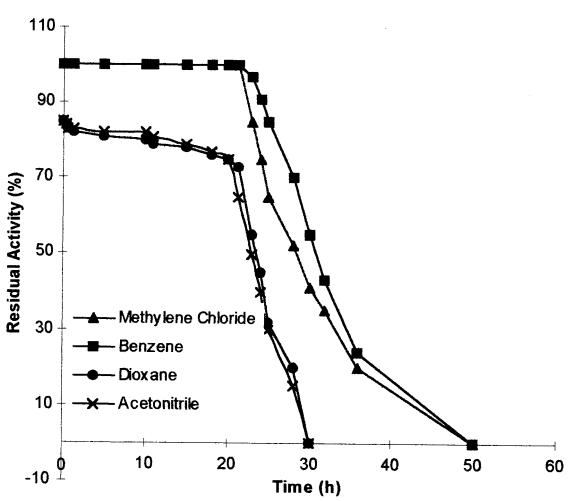


Fig. 3. Stability of soluble mPEG-lipase in organic solvents. The modified enzyme (0.1 mg/ml) was dissolved in dry organic solvent, samples were withdrawn according to a predetermined time schedule, and the samples were then assayed.

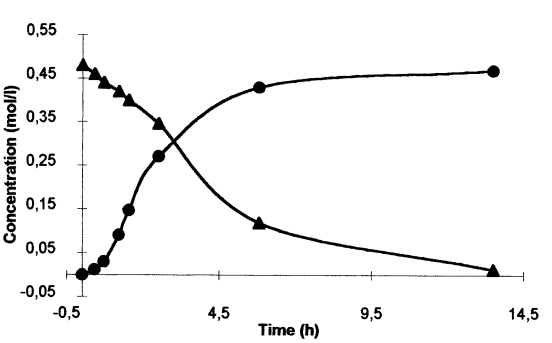


Fig. 4. Benzyl laurate (●) synthesis and lauric acid (▲) consumption with time. The substrates are in equimolar amounts at 0.5 mol/l. $T = 25$°C.

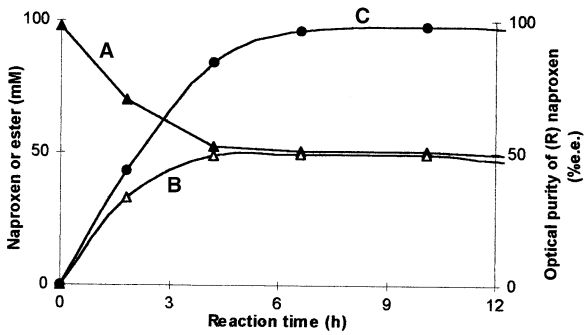


Fig. 5. Time line of optical resolution of (*R*,*S*)-naproxen. Esterification of (*R*,*S*)-2-(6-methoxy-2-naphthyl)propionic acid with trimethylsilylmethanol by mPEG-lipase in toluene. ▲, amount of (*R*,*S*)-naproxen and △, (*S*)-naproxen ester, respectively. ●, optical purity of unreacted (*R*)-naproxen.

As shown in Fig. 5, the amount of (*R*,*S*)-2-(6-methoxy-2-naphthyl)propionic acid decreases over time to approach a constant level of 50% (curve A).

Curve B shows the course over time of the amount of (*S*)-trimethylsilylmethoxy-2-(6-methoxy-2-naphthyl)-propionate produced by esterification. Product yield remains at a constant level of 50% during more than 5 h of incubation. The optical purity of the non-reacted acid, (*R*)-naproxen, reaches 99% e.e. in 3 h, as shown by curve C. The conversion of the racemic naproxen, the enantiomeric excesses for the substrate e.e. (s) and the product, e.e. (p), as well as the enantiomeric ratio, *E*, were calculated from the previous definition [19].

4. Conclusions

From the results obtained above, we conclude that mPEG-lipase from *C. rugosa* catalyzes esterification in organic solvents in a highly stereoselective manner. We also conclude that in organic solvents, it catalyzes not only ester synthesis but also the stereoselective resolution of racemic naproxen.

As a result, such modified lipase could be extremely useful in many practical applications.

Acknowledgements

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