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Immobilization of *Candida rugosa* Lipase on Different Ionic Supports to Improve the Enantioselectivity on Mandelic Acid Resolution

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Abstract: This work describes the improvement in enantioselectivity on the resolution of mandelic acid catalyzed by the *Candida rugosa* lipase (CRL) immobilized and stabilized on two different ionic supports. CRL was quickly immobilized on anionic diethylaminoethyl (DEAE) support and, due to the low content of positive charges on enzyme surface; the enzyme could only be successfully immobilized on cationic carboxymethyl support after chemical amination. Immobilized the enzyme presented higher thermal stability and higher stability to pH than the free enzyme. In relation to the free-enzyme, the DEAE derivative was 2-fold more stable in acid pH, while the carboxymethyl derivative was 2-fold more stable in alkaline pH. When incubated at pH 7.0 and 50 ºC, the carboxymethyl derivative was more stable retaining 80% of activity even after 7 h incubation, and the DEAE derivative presented half-life of 6.6 h. Due to this promising characteristics, both CRL derivatives were evaluated on the hydrolysis of (R,S)-mandelic acid ethyl ester under different pH. The CRL immobilized on DEAE support presented stereochemical preference for the R isomer in pH 5.0 and 7.0, while in pH 9.0 the hydrolysis of the S isomer hydrolysis was faster with a higher E-value of 21.2. On the other hand, the carboxymethyl derivative showed opposite results regarding stereochemical preference with higher E-value at pH 5.0 (E > 200) demonstrating excellent enantioselective transesterification towards the S-isomer of mandelic acid with a theoretical 50% conversion yield and a 99.9% enantiomeric excess.

Key words: *Candida rugosa* lipase, Immobilization, Stabilization, Enantioselectivity

Introduction

Lipases (glycerol ester hydrolases E.C. 3.1.1.3) are the most popular enzymes in biocatalysts because they couple a wide specificity for substrates, allowing their employment in many different reactions (Palomo et al., 2006).

One of the main applications of lipases is for resolution of racemates (Liu et al., 2004). Due to its complex mechanism of action, intense conformational changes in enzyme structure during catalysis should be taken into account (Torres et al., 2006). According to this mechanism, lipases present two different structural forms. The closed and inactive form possess an active site secluded from, in turn the reaction medium by an oligopeptide chain called “lid”. The open form has a displaced lid and the active center is exposed to the reaction medium allowing the enzyme becomes active (Torres et al., 2006; Palomo et al, 2002a). Lipase molecules are in equilibrium between the open-active and the closed-inactive structures of the immobilized lipases. However, upon exposure to a hydrophobic substrate such as a lipid droplet, an interfacial activity occurs, shifting the equilibrium towards the open form (Miled et al., 2001. It is very likely that if this equilibrium or the exact shape of the enzyme is altered in any way, the catalytic properties may be altered. This could be achieved via immobilization techniques involving different areas of the enzyme (Palomo et al, 2002b). This could reduce the freedom of the lid of the enzyme to move it, altering the shape of the final open form of the lipase leading to a

variety of results (Palomo et al, 2002c). Studies have shown that enzyme derivatives from lipases exhibit different catalytic properties (Oladepo et al., 1995).

The advent of new technologies such as genetic recombination, protein engineering and directed evolution have been providing to obtain enzymes with new characteristics (activity, chemo, regal and enantioselectivity) and more specific to certain industrial processes, thereby promoting an expansion market for enzymes, in particular for lipase (Machado et al., 2011).

Recently, the use of lipases in the enantioselective resolution of racemic mandelic acid for obtaining chiral enantiomers has received considerable attention (Cao et al., 2014; Yao et al., 2013).

Enantiomers of mandelic acid and their derivatives have been considered as important substances because they are utilized extensively for synthetic purposes as well as in stereochemical investigations. Mandelic acid enantiomers are employed in the resolution of racemic alcohols and amines (Zingg et al., 1988; Whitesell-Reynolds, 1983). (*R*)-mandelic acid is used as a versatile intermediate for preparation of semisynthetic cephalosporins, penicillins, anti-tumor agents and anti-obesity agents (Yadav-Zivakumar, 2004).

Ultimately, the modulation of the enzyme properties trying to alter the active center of enzymes via controlled immobilization and experimental conditions, called "conformational engineering", seems efficient to enzymes, as lipases, that suffer dramatic changes in their conformation during catalysis (Oladepo et al., 1995).

The fungus *Candida rugosa* demonstrated to be a valuable lipase producer in medium supplemented with soybean molasses (a byproduct generated from liquid evaporation during the drying of concentrated soy protein) at low pH, intermediate temperatures and within a short cultivation period (Morais Junior et al., 2016), encouraging new studies about the produced lipase. In this paper we have studied the improvement of the enantioselectivity on resolution of mandelic acid using *Candida rugosa* lipase immobilized on different supports by one-point covalent bonding, multipoint covalent boding and ionic bonding on anionic and cationic support.

Material and Methods

Materials

Diethylaminoethyl-Sepharose (DEAE) and carboxymethyl agarose supports were obtained from GE Heathcare. Bovine serum albumin (BSA), p-nitrophenylbutyrate (p-NPB), ethylenediamine (EDA), carbodiimide (EDAC), (R,S)-mandelic acid ethyl ester, (R)(-) mandelic acid and (S)(+) mandelic acid were obtained from Sigma-Aldrich (St.Louis, MO, USA). Other reagents were analytical or HPLC grade. *Candida rugosa* lipase (CRL) was produced under submerged fermentation in 250 mL flasks containing 50 mL of medium supplemented with 200 g/L soybean molasses pH 3.5. Cultivation was carried out in a rotary shaker at 130 rpm, 25 ºC for 12 h as described by Morais Junior et al. (2016).

Supports Preparation

DEAE and carboxymethyl supports were previously washed abundantly with distilled water and filtered under vacuum.

Standard Activity Determination

This assay was performed by measuring the increase of *p*-nitrophenolate at 348 nm, produced by hydrolysis of 50 mM *p*-nitrophenylbutyrate (*p*-NPB). The reaction was initiated with 20 µL of lipase dissolved in 2.5 mL 25 mM sodium phosphate pH 7 and 25 ºC. One international unit of activity (U) was defined as the amount of enzyme that hydrolyzed 1 µmol of *p*-NPB per minute under the conditions described previously. Supernatant of the suspensions containing supports were obtained using a pipette-tip-filter. Suspensions were assayed using cut-pipette-tips. Protein concentration was determined by Bradford's method at 595 nm using BSA as standard.

Different İmmobilization Techniques of the *Candida rugosa* lipase

Five grams of the support was added to 25 mL of the lipase solution diluted in 25 mL of 25 mM sodium phosphate pH 7.0 at 25 °C, and maintained under mild stirring. Immobilization of CRL was carried out on DEAE and carboxymethyl supports separately. Immobilization was followed by periodically taking supernatant and suspension samples and measuring the enzymatic activity. Supernatants were obtained using a pipette-tip-filter and suspensions samples were collected using cut-pipette-tips.

The preparations of all derivatives were then filtered, washed with distilled water and stored at 4 ºC.

Biochemical Characterization of İmmobilized Lipase

The effect of pH on lipase activity was studied at pH values 4, 5, 6, 7, 8, 9 and 10. Buffer systems were used at a concentration of 25 mM: sodium citrate buffer for pH 4 and 5, sodium phosphate buffer for pH 7 and 8, and sodium bicarbonate buffer for pH 9 and 10. A hundred milligram (0.1 g) of derivative was incubated with 1 mL of desired pH buffer at ambient temperature. The lipase activity was determined using the standard assay as described previously.

Thermal stability was studied by incubating the enzyme derivatives (1 g in 10 mL of 25 mM sodium phosphate pH 7) at 30, 40 and 50 °C for 7 hours and sampled per hour. The lipase activity was determined, using the standard assay as described earlier. The residual activities were calculated by lipase activity assay.

Enzymatic hydrolysis of (R,S)-mandelic acid ethyl ester

The activities of different CRL derivatives on the hydrolysis of (R,S)-mandelic acid ethyl ester were investigated by adding 0.1 g of each derivatie to 10 mL of 10 mM (R,S)-mandelic acid ethyl ester under different conditions of pH (25 mM sodium acetate pH 5, 25 mM sodium phosphate pH 7 and 25 mM sodium bicarbonate pH 9). The suspension was kept under mechanical stirring at 37 °C. At different times samples was taken and the degree of hydrolysis was confirmed by reverse-phase HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic UV1000) on a hydrophobic C18 (25 cm×0.4 cm) column. Products were eluted using a mobile phase 30% acetonitrile/70% ammonium phosphate buffer 10 mM (v/v) at pH 2.95 at a flow rate of 1.5 mL/min and 254 nm in the UV detector. Each assay was performed three times and the experimental error was never higher than 5%.

At different degrees of conversion, the enantiomeric excess (*ee*) of the produced acid was analyzed by elution of the samples on Chiral Reverse-Phase HPLC with chiral column Chiralcel OD-R. The mobile phase was composed of a 5% acetonitrile/95% ammonium phosphate buffer 10 mM, pH 2.1 and the analyses were performed at a flow rate of 0.5 mL/min by recording the absorbance at 225 nm. Retention times for the standard S-mandelic acid and R-mandelic acid were 9.5 and 10.5 min, respectively. The enantiomeric ratio (*E*) was calculated from the ratio between the R and S mandelic acid enantiomers.

Results and Discussion

Immobilization of CRL on İonic Supports

The results of immobilization yields and specific activity from each *Candida rugosa* lipase (CRL) derivative supports are shown in Table 1.

Table 1. Table of immobilization

Supports	% Immobilization	Specific Activity (U/g support)
DEAE	99.1 ± 0.5	55.1 ± 0.2
Carboxymethyl	98.4 ± 1.1	54.7 ± 0.3

Enzyme activity – final derivative activity after immobilization

The immobilization on DEAE occurred within 1 hour, reaching an immobilization yield of 99.1% resulting in the derivative with a specific activity of 55.1 U/g of support.

Initially, it was verified that even after long-term incubation of the CRL, the enzyme could not be immobilized on carboxymethyl. This fact may be attributed to the lack of reactive groups, such as amine groups (with positive charges), on the enzyme surface. In order to introduce more amino groups on CRL surface rendering higher reactivity to the enzyme, full chemical amination of the CRL was carried out to in solid-phase, i.e. the enzyme was treated after adsorption of on octyl-Sepharose. The enzyme activity of aminated CRL (CRL-A) immobilized preparations was only mildly affected by the treatment. The activity of CRL-A was 11.48 U/mL, corresponding to 89.8% of the initial activity.

After the chemical modification, the immobilization of CRL on carboxymethyl occurred within 1 hour, reaching an immobilization yield of 98.4% resulting in the derivative with a specific activity of 54.7 U/g of support. The results show that CRL structure has a higher affinity for ionic supports.

In nature when enzymes are applied as biocatalysts, processes may undergo inactivation becoming unstable and disturbing the reaction efficiency. Therefore, enzyme immobilization is an interesting process because it promotes a simple and efficient mechanism of the procedure in different kinds of supports activated with different groups reversible, covalent or multipoint procedures.

Stability of the immobilized CRL

The effect of pH on lipase activity was measured between pH 3.0 and 10.0 and results presented in Figure 1.

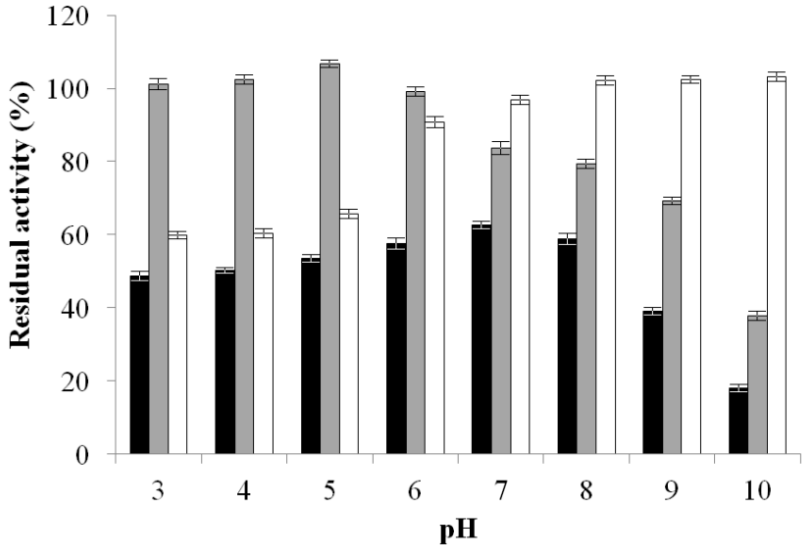

Fig 1. pH stability of different derivatives from CRL. (■) soluble CRL; (■) DEAE derivative; (□) carboxymethyl derivative

Figure 1 shows that the immobilized CRL on DEAE support kept residual activity higher than 100% in acid pH. The residual activity of the DEAE derivative was higher at acidic pH, demonstrating an inactivation of the enzyme above pH 7. For the carboxymethyl derivative, there is a difference. Figure 1 shows that the residual activity of CRL immobilized on carboxymethyl was higher in the alkaline pH, reaching 103.2% of this activity at pH 10.

Depending on the conditions applied, one can choose a different derivative to immobilize the enzyme, for example, in reactions where the favorable pH is acidic, one may utilize derivatives immobilized on DEAE and if the reaction occurs in medium with alkaline pHs, one may utilize a derivation immobilized on carboxymethyl.

The derivatives promoted some changes in the hyperactive effect. This phenomenon occurred because of the nature of lipases in general (a shared mechanism of interfacial adsorption that interacts with small and hydrophobic compounds that can easily accede to the active site of the enzyme) (Palomo et al., 2006).

The immobilization of lipases on ionic supports performed in this study improves the stability of this enzyme in

relation to pH.

Figure 2 shows the results obtained by thermal stability studies of CRL derivatives. The experiments were performed in a 25 mM sodium phosphate buffer of pH 7 by incubating samples at 30, 40 and 50 ºC.

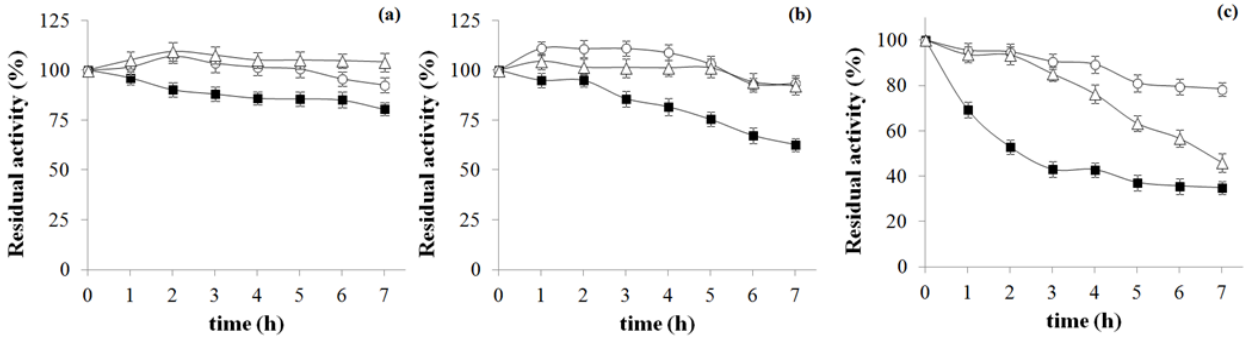

Fig 2. Thermal of inactivation of CRL immobilized on DEAE and carboxymethyl supports. Incubation was carried out on 25 mM sodium phosphate pH 7 at (a) 30 ºC; (b) 40 ºC; (c) 50 ºC. (■) free enzyme; (Δ) DEAE derivative; (○) carboxymethyl derivative

At 30 °C, CRL immobilized on DEAE and carboxymethyl were quite stable, retaining more than 90% of the initial activities after 7 h of incubation (Fig 2.a). However, the DEAE derivative was more stable, hyperactivating and reaching a residual activity more than 100% after 7 hours.

At 40 ºC (Fig 2.b), although both derivatives showed similar stability profiles not producing a significant inactivation, carboxymethyl derivative shows higher stability, hyperactivating and reaching a residual activity more than 100% at the end of the experiment.

The results obtained in the experiments performed at 50 °C (Fig 2.c) show that the greater stability obtained was CRL immobilized on carboxymethyl, whereby there was reduced residual activity of 21.5% at the end of 7 hours. On the other hand, the DEAE derivative was mildly inactivated in the first 2 hours of incubation at 50 °C, and the residual activity reduced by 54%.

When comparing the CRL immobilized on different ionic supports, it can be observed that the results were quite different, as seen in the carboxymethyl derivative at the three temperatures. In terms of explaining this result, this effect may have occurred because of the structure of the enzyme. The chemical modified CRL there is a structure with greater affinity with carboxymethyl support fostering more resistance to promote the interaction (enzyme-support) (Palomo et al., 2006).

Effect of Different Derivatives of CRL on Enzymatic Hydrolysis of (R,S)-Mandelic Acid Athyl Ester

Table 2 shows a high heterogeneity in the results of the enantioselectivity of the CRL immobilized derivatives towards the hydrolysis of (R,S)-mandelic acid ethyl ester at 37 ºC and different conditions of pH.

Table 2. Enantioselectivity hydrolysis of (R,S) – mandelic acid methyl ester catalyzed by CRL derivatives immobilized on different ionic supports

Derivative	pH	Yield (%)	*ee*[a]	*E*[b]	Stereochemical preference
DEAE	5.0	45.9	18.9	14.7	R
	7.0	46.3	33.3	19.9	R
	9.0	50.9	35.9	21.2	S
Carboxymethyl	5.0	43.6	> 99.0	> 200	S
	7.0	50.8	69.2	1.8	S
	9.0	48.4	12.4	12.8	R

[a] Enantiomeric excess; [b] Enantiomeric ratio (specificity)

The CRL derivatives showed an opposite behavior. The enantioselectivity of DEAE derivative increases the hydrolytic production of the R isomer between pH 5.0 and 7.0, obtaining a greater enantiomeric ratio of 19.9 at pH 7.0. At pH 9.0 the reaction becomes faster for the S isomer having a higher *E*-value (21.2) producing 35.5%

more than the R isomer. The derivatives immobilized on carboxymethyl increased its E-value by 111-fold more when the pH decreases from 7.0 to 5.0, producing more than 99% of the S isomer at pH 5.0. At pH 9.0 the reaction the R isomer was faster hydrolyzed, having an E-value by 12.8 producing 12.4% more than the S isomer. These changes hardly can be obtained so simply using another technique. In fact, the same lipase behaves as different lipases when immobilized on different supports (Palomo et al., 2002a).

Conclusions

From the results presented in this work, we concluded that the properties of a lipase may be modulated by directed immobilization and design of the experimental conditions. Thus, the immobilization on different supports can change the catalytic properties of the same lipase, determining a different E-value and different stereochemical preference. Changes of the reaction pH conditions can alter the lipase properties; e.g. CRL immobilized on DEAE support have a stereochemical preference to the R isomer at pH 5 and 7, while at pH 9 the S isomer hydrolysis is faster. On the other hand, the carboxymethyl derivative shows opposite results regarding the stereochemical preference. Ultimately, this mechanism of altering the properties via controlled immobilization and experimental conditions, called "conformational engineering", seems efficient to enzymes, as lipases, that suffer dramatic changes in their conformation during catalysis.

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