XXI SINAFERM
XII SHEB
Aracaju - Sergipe

XXI SIMPÓSIO NACIONAL DE BIOPROCESSOS
XII SIMPÓSIO DE HIDRÓLISE ENZIMÁTICA DE BIOMASSA

3 a 6 de setembro de 2017
Aracaju, Sergipe, Brasil

STUDY OF THE STABILITY AND CATALYTIC VERSATILITY OF THE LIPASE B FROM *Candida antarctica* IMMOBILIZED ON IMMOBEAD-350

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ABSTRACT

The strategy of enzyme immobilization is studied with the objective of producing more stable biocatalysts that can be used in reactions of high industrial interest. Lipase B from Candida antarctica (CALB) has been covalently immobilized on epoxy Immobead-350 (IB-350) and some properties have been compared to glyoxyl-agarose-CALB and Novozym 435. The thermal and solvent stability was higher than that of the glyoxyl-agarose-CALB, and the CALB-IB350 preparation was more stable at pH 5,0 and 70 °C ($t_{1/2}$= 68 min). The activity of the new preparation versus tributyrin and triacetin was about 4 and 17 fold higher than that of Novozym 435, respectively. Thus, the covalent attachment between enzyme and support produced a stable and active biocatalyst under different conditions and substrates.

1. INTRODUCTION

Lipases are enzymes that have a great biotechnological potential and are among the most commonly used in biocatalysis due to their wide specificity, high stability in different reaction conditions and broad spectrum of reactions that they are able to catalyze. However, the use of these biocatalysts has been limited by their unstable nature when submitted to stringent conditions of pH and temperature. The strategy of immobilization on solid supports have been used in order to overcome these limitations and to ensure optimal industrial outcomes (Sheldon & van Pelt 2013).

The Immobead-350 (IB-350) support was used to immobilize the lipase from *Candida antarctica* (CALB). The IB-350 beads matrix, a crosslinked co-polymer of methacrylate, allows the covalent immobilization of a variety of enzymes, once they are functionalized with epoxy binders, which react mainly with groups of amino acids present on the surface of the enzyme (Dhake et al. 2012).

In the present work, CALB was covalently immobilized on Immobead-350. Special focus will be placed on the use of this biocatalyst in the thermal and solvent stabilities and in the hydrolysis of tributirin and triacetin substrates, being compared with glyoxyl-agarose and Novozyme 435 preparations.

2. METHODOLOGY

2.1. Enzymatic immobilization

The enzyme was immobilized on support by covalent attachment. Standard immobilizations were performed using 50 mg of protein per g of glyoxyl and dried IB-350. The corresponding amount of CALB was added in 1 mL of 25 mM sodium bicarbonate buffer at pH 10, containing 0.01% (v/v) triton X-100. The mixture was placed under gentle stirring at 25°C.

2.2. Determination of enzyme activity and parameters of immobilization

The activity of the soluble and immobilized CALB was determined according to a methodology described by (Santos et al. 2017), with modifications. One activity unit (U) was defined as the amount of CALB capable of hydrolyzing 1 µmol of *p*NPB per min at pH 7,0 and at 25° C.

The yield of immobilization (*IY*), theoretical activity (At_t) and recovery activity (At_r) were the parameters of immobilization used to quantify the immobilization process. These parameters were calculated according to (dos Santos et al. 2017).

2.3. Thermal and solvent stabilities

To check the stability of enzyme derivatives, 1 g of biocatalyst (enzyme loading of 50 mg protein/g support) was suspended in 10 mL of 25 mM of citrate buffer at pH 5.0, sodium phosphate at pH 7.0 or sodium bicarbonate at pH 10, at 50°C, 60°C or 70°C, respectively. Periodically, samples were withdrawn and the activity was immediately measured using *p*NPB. The half-life was calculated from enzyme deactivation according to the (Henley & Sadana 1985).

2.4. Hydrolysis of tributyrin and triacetin

Hydrolyses of tributyrin and triacetin catalyzed by soluble and immobilized CALB (enzymatic loading: 50 mg/g) were carried out using a pHstat (Automatic titrator: Mettler Toledo – T50), at 37ºC, under stirring at 11000 rpm. 50 mM NaOH solution was utilized as titrating reagent. One unit of lipase activity (U) is the amount of enzyme capable to release 1 µmol of butyric acid or acetic acid under these assay conditions.

3. RESULTS

3.1. Thermal and solvent stabilities

CALB-IB-350 is the most stable preparation under all studied conditions, e.g., increased enzyme stability by a 13-fold-factor at 70 °C and pH 5. In the presence of both dioxane and acetonitrile, CALB-IB-350 preparation was also the more stable than CALB-GLX. This stabilization may be explained by enzyme rigidification due to multipoint covalent attachment (Garcia-Galan et al. 2011).

Table 1. Half-lives in different conditions (expressed in minutes) of CALB soluble, CALB-IB-350 and CALB-GLYOXYL under different inactivation conditions.

Biocatalyst	**Inactivation conditions**				
	pH 5 - 70°C	pH 7 - 60°C	pH 10 - 50°C	Dioxane 30% - 65°C	Acetonitrile 30% - 65°C
CALB-IB-350	68	57.5	29	42.3	27.8
Soluble CALB	5	12	6	3.8	6.3
CALB-GLYOXYL	16.94	6.44	2.71	2.04	10.92

3.2. Hydrolysis of tributyrin and triacetin

The results of the hydrolysis of tributyrin and triacetin (Table 2) showed that CALB-IB-350 was able to hydrolyze both substrates with an activity almost 4 times higher than the Novozyme and more than 7 fold higher than CALB-GLX using tributyrin substrate. The CALB-IB350 activity versus tributyrin was around 6 folds higher than that obtained with triacetin, while Novozyme was 27 folds higher using tributyrin than using triacetin as substrate. Thus, the results showed that the enzyme specificity can be strongly modulate for the immobilization, and that the commercial CALB-IB-350 is the most active immobilized preparation among the assayed ones versus these model substrates.

Table 2. Hydrolysis activities (U) of the substrates tributyrin and triacetin.

	Biocatalyst			
Substrate	CALB-IB-350	CALB-GLX	Soluble CALB	Novozym 435
Tributyrin	1.5 ± 0,07	0.195 ± 0.02	18.77 ± 0.17	0.41 ± 0.02
Triacetin	0.265 ± 0.02	0.005 ± 0.001	0,76 ± 0.04	0.015 ± 0.007

4. CONCLUSION

The results showed that CALB-IB-350 is more thermostable and stable in the presence of organic solvents than GLX-CALB and the activity of CALB-IB-350 was higher than other biocatalyst preparations in the hydrolysis of tributyrin and triacetin.

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