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REUTILIZATION OF THE MOST STABLE COIMMOBILIZED ENZYME TO BUILD A NEW COMBIENZYME WHEN THE LESS STABLE ENZYME HAS BEEN INACTIVATED USING GLUTARALDEHYDE CHEMISTRY

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ABSTRACT

In this study glutaraldehyde was used as a strategy to co-immobilize the lipase Eversa® Transform 2.0 and the ß-galactosidase from *Aspergillus oryzae*. The lipase Eversa® Transform 2.0 retained about 90% of the initial activity when immobilized onto aminated supports and after treated with glutaraldehyde and incubated at 55°C. However, the ß-galactosidase immobilized using a similar protocol, lost a significant value of its activity under the same conditions. To prevent the necessity of discarding all the combibiocatalyst, the lipase was immobilized following this protocol (covalently), reduced to eliminate chemical reactivity, and the ß-galactosidase was then coimmobilized via ion exchange. This way, the ß-galactosidase could be released of the support by incubation at 300 mM NaCl. The immobilized Eversa® Transform 2.0 was reused in three inactivation/release/reload cycles to build a new combienzyme with a margoinal loss of activity. To this purpose, the inactivated β-galactosidase was released from the support and replaced by a new enzyme bath in each cycle, while the immobilized lipase maintained its activity throughout all protocols. Therefore, this strategy was effective to reuse the covalently immobilized Eversa® Transform 2.0 after the reversible immobilized ß-galactosidase inactivation.

Keywords: Enzyme co-immobilization. Dissimilar enzymes stabilities. Reuse of stable co-immobilized enzymes. Enzyme release from combibiocatalyst.

1 INTRODUCTION

Co-immobilizing two enzymes that present, after immobilization, very different stability levels have some disadvantages. In this immobilization protocols, all biocatalysts will discard even if some of enzymes them are still fully active.[1,2] Some strategies have been reported to overcome this matter.[3,4] In this work, we propose a new strategy using aminated supports and the glutaraldehyde to co-immobilize enzymes.[5]

Glutaraldehyde (GLU) is a very versatile immobilization reagent and widely used.[6] One of these strategies consists of the glutaraldehyde treatment of the enzymes previously ionically immobilized on aminated supports. For this case, there must be only one glutaraldehyde molecule between the enzyme and the support.[5] In our previous research, we found possible to release immobilized enzymes onto reduced amino-glutaraldehyde supports.[7]

In this study, we immobilized the most stable enzyme on an aminated support, and then we treat the biocatalyst with one molecule of glutaraldehyde to get the enzyme covalent immobilization. Next, we reduce the biocatalyst with borohydride to eliminate all chemical reactivity, and we adsorb the least stable enzyme on the support. After the inactivation of the least stable enzyme, we release it just by incubation at high ion strength.

2 MATERIAL & METHODS

Lipase activity was quantified by determining the increase in absorbance at 348 nm produced by the release of p-nitrophenol.[8] The ß-galactosidase activity was determined by measuring the increase in absorbance at 380 nm produced by the release of o-nitrophenol.[9] Amino-glyoxyl–agarose (MANAE–agarose) support was prepared as described by Fernández-Lafuente et al.[10] Eversa® Transform 2.0 immobilization was carried using 3 mg of protein per g of support in a 5 mM Tris-HCl solution at pH 7.

MANAE-Eversa® biocatalyst was suspended in 50 mM sodium phosphate at pH 7.0 containing 1 % (v/v) GLU, and then reduced using 1 mg of sodium borohydride per mL under gentle stirring.[6] The ß-galactosidase was immobilized using 3 mg of protein per g of support in a 5 mM Tris-HCl solution at pH 7. The biocatalyst resulted is MANAE-EVR-GLU-ß-galactosidase.

To releasing inactivated ß-galactosidase from the support, the biocatalyst was incubated in 5 mM Tris-HCl containing 300 mM or 600 mM of NaCl at pH 7.0 for 2h at 25°C. The SDS-PAGE experiments were carried out following the Laemmli[11] protocol with some modifications. For MANAE-EVR-GLU-ß-galactosidase reuse was incubated in 10 mM Na_2CO_3 buffer at 55 °C and pH 8.0. Residual activities were calculated as the percentage of the biocatalyst initial activity.

3 RESULTS & DISCUSSION

Eversa® Transform 2.0 was covalently immobilized on the support (MANAE agarose beads, subsequent treatment with glutaraldehyde), given that not protein band could be observed in the SDS-PAGE analysis (Figure 1A). Then, the ß-galactosidase was immobilized on the MANAE-Eversa-GLU biocatalyst via ion exchange. Analyzing the inactivation course of the combibiocatalyst, Eversa® Transform 2.0 maintained over 90 % of the initial activity after 75 minutes of inactivation, whereas the ß-galactosidase lost almost all its activity (Figure 1B).

Then, we checked the possibility of releasing the ß-galactosidase from the combibiocatalyst. For this we incubated the combibiocatalyst in 300 mM NaCl. Figure 2A showed that all the ß galactosidase enzyme was released from the combibiocatalyst. Furthermore, the activity of Eversa® Transform 2.0 was unchanged after this incubation.

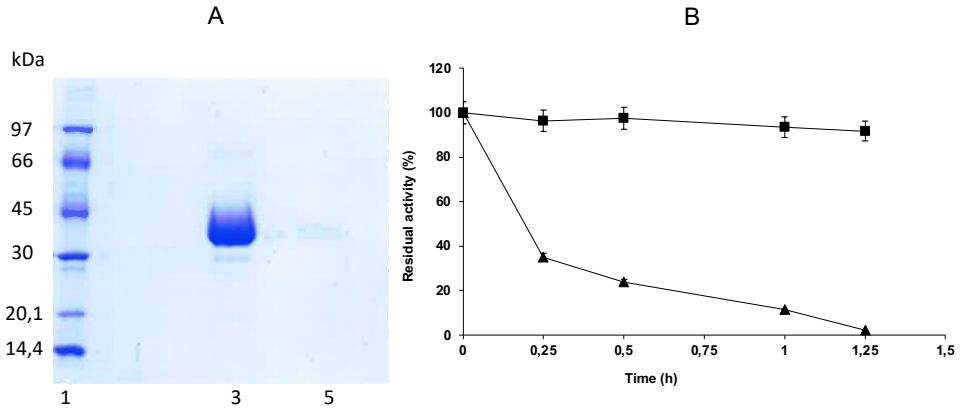

Figure 1 A) SDS-PAGE analysis of different Eversa® Transform 2.0 preparations. Lane 1: Low Molecular Marker, Lane 3: MANAE-EVR, and lane 5: MANAE-EVR-GLU (1%). B) Inactivation course of MANAE-EVR-GLU-ß-Galactosidase. The reaction was performed using 10 mM of carbonate buffer at pH 8.0 and 55 °C. Solid triangles: ß-galactosidase, and solid squares: Eversa® Transform 2.0.

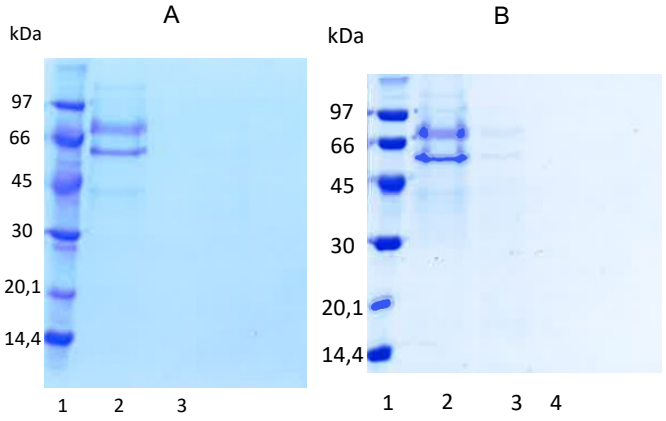

Figure 2 A) SDS-PAGE analysis of MANAE-EVR-GLU-ß-galactosidase after incubation and washing with 0.3 M of NaCl at pH 7.0. Lane 1: Low Molecular Marker; Lane 2: initial combi-biocatalyst; Lane 3: Combi-biocatalyst after incubation in 0.3 M of NaCl. B) SDS-PAGE analysis of MANAE-EVR-GLU-ß-galactosidase after 3 cycles of thermal inactivation at 55 °C and pH 8.0 and incubation and washing with 0.3 M NaCl at pH 7.0. Lane 1: Low Molecular Marker, Lane 2: initial combi-biocatalyst, Lane 3: Combibiocatalyst after incubation in 0.3 M of NaCl. Lane 4: Combibiocatalyst after incubation with 0.6 M of NaCl.

Figure 3 presents several cycles of combibiocatalyst MANAE-EVR-GLU-ß-Galactosidase incubation at 55ºC and pH 8. After the ß-galactosidase inactivation, the combibiocatalyst was washed with 0.3 M NaCl, and next a new load of ß-galactosidase was immobilized on the biocatalyst. After this new load the activity of Eversa® Transform 2.0 recovered the initial values and several cycles could be repeated with similar results (Figure 2A). Figure 2B shows that after the 3 use cycles of the combibiocatalyst, the washing with 0.3 M NaCl was not able to eliminate all the inactivated ß-galactosidase from the biocatalyst. However, when we wash with 0.6 M NaCl achieved an almost fully clean lane after 3 reaction cycles.

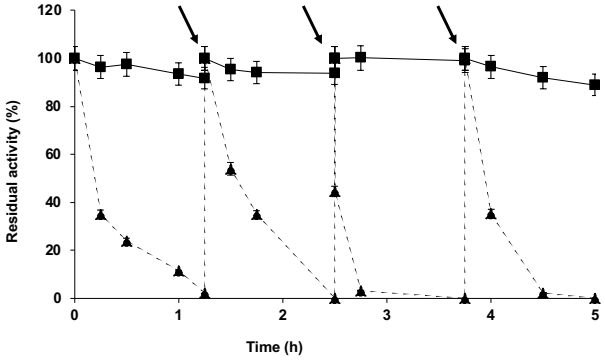

Figure 3 Cycles of thermal inactivation at 55 ºC and pH 8.0 of MANAE-EVR-GLU- ß-galactosidase and ß-galactosidase desorption by incubation and washing with 0.3 M NaCl and immobilization of a new batch of ß-galactosidase (indicated with an arrow). Solid symbols and dashed lines: ß-galactosidase, and solid symbols and solid lines: Eversa® Transform 2.0.

4 CONCLUSION

Therefore, we were able to immobilize the more stable enzyme covalently to the support after the biocatalyst is treated with glutaraldehyde. After borohydride reduction to eliminate chemical reactivity of the support, the low stable enzyme may be coimmobilized in the biocatalyst via ion exchange. In addition, the enzyme more stable could be reused, after the least stable enzyme is fully inactivated and released of the support by incubation in solutions of a high ionic.

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