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# HETEROFUNCTIONAL FEATURES OF REDUCED AMINATED SUPPORTS MODIFIED WITH GLUTARALDEHYDE MAKES THE ENZYME RELEASE DIFFICULT

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## ABSTRACT

In this work, we try to understand the impact of the different interactions that can be present in the immobilization of enzymes on aminated supports using glutaraldehyde as reactive agent. To achieve this, we have studied the immobilization of the beta-galactosidase from *Aspergillus oryzae*, on aminated supports (MANAE) and aminated supports modified with one (MANAE-GLU) or two molecules of glutaraldehyde (MANAE-GLU-GLU). To eliminate the chemical reactivity of the glutaraldehyde, the supports were reduced using sodium borohydride. After the beta-galactosidase adsorption on the different supports, we studied the release of the enzyme from the supports using different concentrations of NaCl, Triton X100, ionic detergents (SDS and CTAB), and different temperatures (4 °C to 55 °C). When using the MANAE support, at 0.3 M of NaCl almost all the immobilized enzyme was released. Using MANAE-GLU, the incubation in 0.3 M, and 0.6 M NaCl gave similar results, with all enzymes being released from the support. However, the incubation in 1 M or 2 M NaCl, many enzyme molecules were not released from the support. For the MANAE-GLU-GLU support, none of the tested concentrations of NaCl was sufficient to release all enzyme bound to the support at room temperature. Only using high temperatures combined with 0.6 M NaCl, and 1 % CTAB or SDS, could the totality of the proteins be released from the support. The results shown in this paper confirm the heterofunctional character of aminated supports modified with glutaraldehyde and can be used to explain some of the pre-existing results found when immobilizing enzymes on aminated supports activated with glutaraldehyde.

**Keywords:** Enzyme release. Heterofunctional supports. Mixed adsorption.

## 1 INTRODUCTION

Enzyme immobilization has become a powerful tool to improve many enzyme features, such as activity, specificity, selectivity, and stability[1,2]. How good the final biocatalyst is will depend on various aspects of the immobilization, such as the support features, the active groups that are present on the support surface and the immobilization protocols used[3]. However, this is not a simple objective and may be much more complex when using supports bearing several moieties able to interact with the enzyme[4].

Immobilization of enzymes on supports containing primary amino groups via the glutaraldehyde chemistry is one of the most utilized immobilizations strategies[5]. This support is a heterofunctional one and it is highly versatile[4,6]. The researchers can immobilize the enzyme via ion exchange and then treat the enzyme and the support with glutaraldehyde under conditions where just one glutaraldehyde molecule attaches to each external amino group. Another option, the aminated support can be pre-activated using glutaraldehyde. In this case, the cationic interaction of the support is maintained, but the amino group is masked by a larger moiety, more hydrophobic.[4,7]. Therefore, the immobilization of enzymes on aminated supports using the glutaraldehyde chemistry may involve three different interactions, cationic, hydrophobic, and covalent interactions between the support and the enzyme[8]. However, this heterofunctionality is ignored in many works[3].

Given these considerations, this work is focused on understand the impact of the modification of aminated supports with one or two molecules of glutaraldehyde on physical adsorption of the beta-galactosidase from *A. oryzae*, as model enzyme.

## 2 MATERIAL & METHODS

The β-galactosidase activity was determined by measuring the increase in absorbance at 380 nm produced by the release of o-nitrophenol[9]. Protein concentration was calculated using the method reported by Bradford[10], using bovine serum albumin as a standard. MANAE support was prepared as previously described[11]. This aminated support was treated with glutaraldehyde under two different conditions: 1 % (v/v) glutaraldehyde (MANAE-GLU), and 10 % (v/v) glutaraldehyde (MANAE-GLU-GLU)[7]. Reduced supports were obtained by incubating 10 mg.mL$^{-1}$ of solid sodium borohydride on 25 mM sodium bicarbonate at pH 10[12].

β-galactosidase immobilization on both supports was carried using 3 mg of protein per g of support in a 5 mM Tris-HCl solution at pH 7 and enzyme immobilization course was followed by measuring the enzyme activity in the supernatant and in the whole suspension at different time intervals using o-NPG as substrate.

Desorption of β-galactosidase from the supports was carried out by incubating in a solution of 5 mM Tris-HCl containing different concentrations of NaCl, Triton X-100, NaCl and some detergents (Triton X-100, CTAB, SDS). The incubations were performed at pH 7.0 and 25 °C for 1 h and the activities of both supernatant and suspension were followed using o-NPG. The SDS-PAGE experiments were carried out following the Laemmli[11] protocol with some modifications.

## 3 RESULTS & DISCUSSION

Using MANAE support, the increase in NaCl concentration promoted a progressive release of the enzyme, at 0.3 M almost all enzyme activity was released and further increases in the ionic strength almost did not affect the results. Using MANAE-GLU, at 0.3 M NaCl and 0.6M there was a clearly higher release of enzyme than using MANAE. However, when using a concentration of NaCl of 1 M or higher, some enzyme molecules were no released from the support. This could be related to the moderate hydrophobicity of the reduced glutaraldehyde moiety, that at this high ionic strength seemed to be able to fix some enzyme molecules to the support. Using MANAE-GLU-GLU, the use of higher concentration of NaCl did not permit to release more galactosidase from the support, maintaining similar values even at 2 M NaCl (Fig. 1).

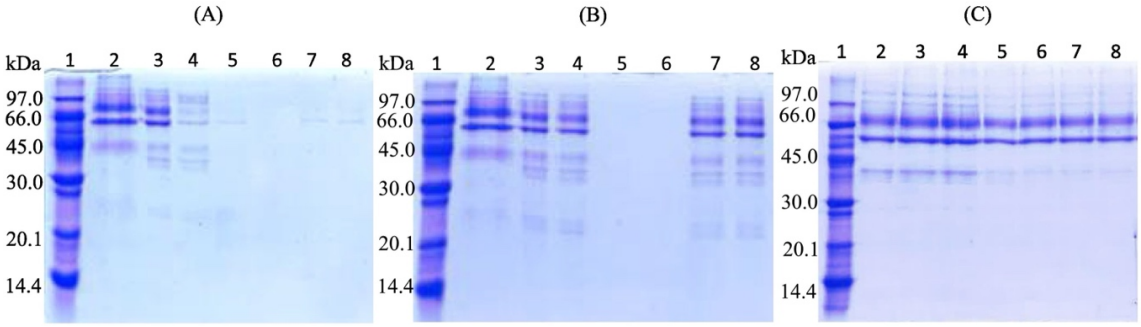


**Figure 1** SDS-PAGE analysis of β-galactosidase preparations submitted to different treatments, immobilized on different supports (A): MANAE, (B): MANAE-GLU support, (C): MANAE-GLU-GLU support. Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: immobilized β-galactosidase (control). Lane 3: enzyme remaining in the support after desorption of β-gal with 0.05 M NaCl. Lane 4: enzyme remaining in the support after desorption of β-gal with 0.15 M NaCl. Lane 5: enzyme remaining in the support after desorption of β-gal with 0.3 M NaCl. Lane 6: enzyme remaining in the support after desorption of β-gal with 0.6 M NaCl. Lane 7: enzyme remaining in the support after desorption of β-gal with 1 M NaCl. Lane 8: enzyme remaining in the support after desorption of β-gal with 2 M NaCl.

Using simultaneously a 300 or 600 mM NaCl and a growing concentration of Triton X100 still a large percentage of enzyme remained on the support (Fig. 2). Next, we tried to release the enzyme using different ionic detergents (SDS and CTAB) and employed different temperatures (from 4 °C to 55 °C) using 0.3 or 0.6 M NaCl (Fig. 3).

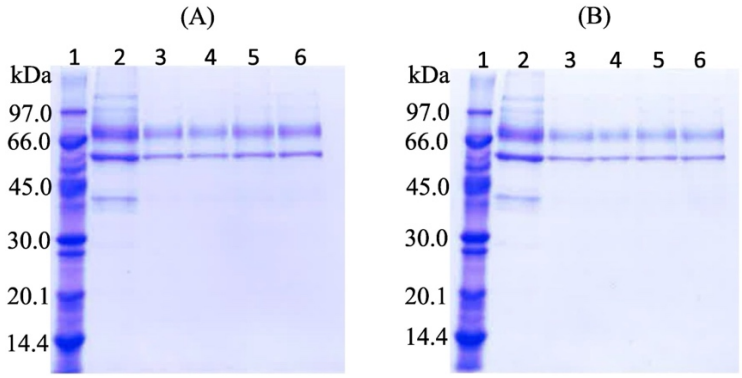


**Figure 2** SDS-PAGE analysis of β-galactosidase immobilized on MANAE-GLU-GLU submitted to different incubations using 0.3 (A) or 0.6 M NaCl (B) and different triton X-100 concentrations at 25 °C. (A): Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: β-galactosidase immobilized (control). Lane 3: enzyme remaining in the support after desorption of β-gal adding 0.1 % triton X-100 and 0.3M NaCl. Lane 4: 0.25 % triton X-100 and 0.3M NaCl. Lane 5: 0.5 % triton X-100 and 0.3M NaCl. Lane 6: 1 % triton X-100 and 0.3M NaCl. (B): Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: β-galactosidase immobilized (control). Lane 3: enzyme remaining in the support after desorption of β-gal adding 0.1 % triton X-100 and 0.6M NaCl. Lane 4: 0.25 % triton X-100 and 0.6M NaCl. Lane 5: 0.5 % triton X-100 and 0.6M NaCl. Lane 6: 1 % triton X-100 and 0.6 M NaCl.

The use of higher temperatures enabled the release of a higher percentage of enzyme, but only using 55 °C, 0.6 M NaCl and 1 % CTAB or SDS all proteins could be released from the support, using Triton X-100 some enzyme molecules still remained in the support (Fig. 3). This shows the very strong adsorption that the MANAE-GLU-GLU was able to promote, a strong mixed ionic-hydrophobic adsorption that permitted establishing many enzyme-support interactions that cannot be easily broken later.

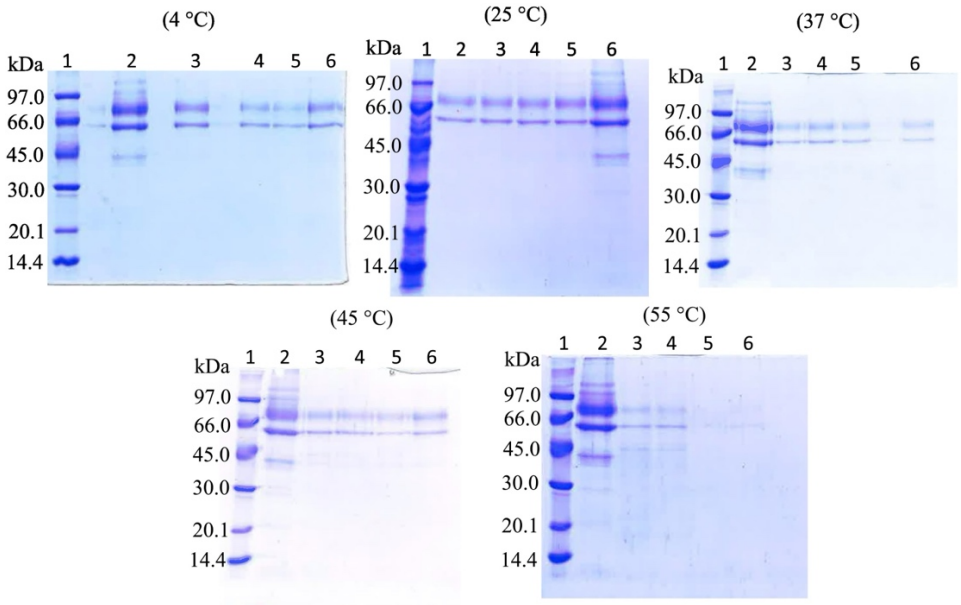


**Figure 3** SDS-PAGE analysis of β-galactosidase desorption with 0.6 M NaCl and different detergents at 4 °C; 37 °C; 45 °C; and 55 °C. Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: MANAE-GLU-GLU-β-gal. Lane 3: enzyme remaining in the support after desorption of β-gal with 0.6 M NaCl. Lane 4: 0.6 M NaCl and 1 % triton. Lane 5: 0.6 M NaCl and 1 % CTAB. Lane 6: 0.6 M NaCl and 1 % SDS.

## 4 CONCLUSION

The results confirm the heterofunctional character of aminated supports modified with glutaraldehyde. Even after eliminating the chemical reactivity of the supports by reduction with borohydride, the glutaraldehyde was able to establish interactions with the immobilized enzyme, making their release complex.

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