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A new heterofunctional amino-vinyl sulfone support to immobilize enzymes: Application to the stabilization of β-galactosidase from A*spergillus oryzae*

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ABSTRACT

The paper shows the preparation of a new heterofunctional agarose support: amino-vinylsulfone. This has been employed to immobilize the interesting enzyme β-galactosidase from *Aspergillus oryzae*. The enzyme cannot be immobilized on just vinylsulfone activated support a pH values ranging from 5.0 to 9.0. Neither the enzyme was immobilized using 200 mM of NaCl on amino-vinylsulfone support. However, the enzyme was readily immobilized at moderate ion strength at pH values from 5.0 to 9.0 via ion exchange on amino-vinylsulfone support, and later some covalent enzyme-support bonds could be formed, more rapidly at alkaline pH value. After optimization of immobilization pH, incubation pH and time, and blocking reagent, several immobilized biocatalysts on amino-vinylsulfone support having 50–80% of the initial activity and a stabilization factor of around 8–15 were prepared, depending on the exact immobilization conditions.

1. Introduction

Immobilization of enzymes is a powerful tool to improve enzyme properties [1–4]; a properly designed enzyme immobilization may greatly improve enzyme stability [1–5], but also activity, selectivity [6,7], and resistance to inhibitors or even protein purity [8]. To optimize the stabilization results, a maximum number of bonds between the enzyme and the support must be established [9,10]. This way, all enzyme groups linked to the support reduce their mobility (to the length of the support spacer arm), maintaining their relative positions under any distorting condition.

However, to achieve a stabilizing multipoint covalent attachment is precise to select a good support (having large surfaces and a high superficial density of active groups), a good protocol that maximizes the prospects of enzyme-support reaction (that is, favoring enzyme conformation mobility and support/enzyme reactivity) and a proper group in the support [9,10]. An ideal group to get an intense multipoint covalent attachment should be stable under conditions where the enzyme/support reaction is favored (usually alkaline pH value), and also should present low steric hindrances for the reaction with the enzyme groups and react with the maximum variety of nucleophiles of the enzyme surface [11]. The ε-amino of Lys uses to be one of the target groups to this goal, because it is a cationic group frequently placed in the enzyme surface, and it is reactive with many other groups when it is in not ionized form [12].

Agarose is an adequate support to evaluate any immobilization protocol, as the support is fully inert if it is not modified, and that is an important feature of a support to immobilize proteins [12]. Glutaraldehyde [13], epoxy [14–17] and glyoxyl [18] supports have been utilized to immobilize-stabilize enzymes via multipoint covalent attachment. The activation of supports with divinylsulfone (DVS) to immobilize proteins and enzymes have been described for a long time [19–27]. However, it has been only recently when DVS activated supports have been described as very suitable supports to produce an extremely intense multipoint covalent attachment using proper immobilization protocols (incubation at alkaline pH value, blocking with suitable nucleophiles), improving the results achieved using glyoxyl supports because vinylsulfone can react with primary amino groups and also with imidazole, thiol or phenol groups. Vinylsulfone groups are also very stable under a wide range of pH values, have not steric problems for reaction with the protein groups, etc. [28]. The only problem is that the spacer arm is longer than that of the glyoxyl group, and the rigidification effect of each additional bond is therefore lower, and in some instances even having more enzyme-support bonds, the stabilization obtained is lower than using glyoxyl supports [29,30]. Moreover, in some instances some proteins that become immobilized on glyoxyl supports are not immobilized on supports activated with DVS, or they are rapidly inactivated, without any clear reason [31].

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The concept of heterofunctional supports has opened new possibilities to the enzyme immobilization [11]. The idea is to develop supports having two different kinds of groups, one whose function is to produce the first enzyme immobilization (usually a physical adsorption), and other groups that are the responsible ones for an intense enzyme-support multipoint covalent attachment. In some cases, this is inherent to the support activation protocol (e.g., glutaraldehyde that is prepared using an anion exchanger support) [32]. In other cases, they are prepared with this objective, like epoxy-amino [33], glyoxyl-amino supports [34], or in the case of lipases, octyl-glyoxyl, octyl-epoxide or octyl-glutaraldehyde supports [35–42]; even a mix of octyl moieties with cationic or anionic groups has been proposed to get mixed lipases adsorptions [43,44]. Octyl-DVS has been also presented, with very good results, enhancing the stabilization results obtained using octyl-glyoxyl [45]. Interestingly, some lipases that cannot be immobilized on supports activated with DVS were successfully covalently immobilized on these heterofunctional DVS activated supports, after the lipase adsorption via interfacial activation [45].

In this new paper, the preparation of amino-DVS activated agarose beads and its use in the immobilization of the enzyme β-galactosidase from *Aspergillus oryzae* will be studied. This glycosylated enzyme [46] is difficult to stabilize via multipoint immobilization; the best results have been reported using amino-epoxy supports and the stabilization was a moderate factor of 12 after optimization [47]. The enzyme has interest in hydrolysis of acid milk whey, and also in the production of galacto-oligosaccharides or modification of galactose with other alcohols [48–53].

It has been shown that this enzyme may be immobilized via ion exchange at different pH values. This alters the enzyme orientation on the support and therefore their final features, e.g. stability [54]. This immobilization pH has been shown to have a great impact in the enzyme stability when immobilized using just aminated supports via ion exchange, now that covalent immobilization is intended, this immobilization pH affect may be quite different as the number of enzyme-support covalent linkages or the enzyme area involved in the covalent immobilization may play a significant role on the final enzyme stability.

Thus, the protocol of covalent immobilization of the enzyme will be optimized on supports activated with divinylsulfone (monofunctional and heterofunctional amino-DVS): immobilization pH value, incubation pH value and time and, finally, blocking agent nature will be the studied variables. The results obtained using agarose activated with DVS and agarose-amino activated with DVS will be compared, using the enzyme immobilized on aminated supports as a reference.

2. Materials and methods

2.1. Materials

β-Galactosidase from *A. oryzae* (20 Units oNPG/mg of protein), o-nitrophenyl-β-galactopyranoside (oNPG) and o-nitrophenol (oNP) were purchased from Sigma–Aldrich (St. Louis, USA). Divinylsulfone, ethylenediamine, glycine, aspartic acid, histidine, L-cysteine and 2-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, MO). Agarose beads 4% (w/v) matrix was from Agarose Bead Technologies (ABT, Spain) and MANAE-agarose [55] support was prepared as previously described with some modifications (reaction time was prolonged to 24 h), as this permitted increasing the amount of amino groups by a 30%. All other reagents were of analytical grade.

2.2. Standard determination of enzyme activity

This assay was performed by measuring the increase in absorbance at 380 nm produced by the release of oNP in the hydrolysis of 10 mM oNPG in 100 mM sodium acetate at pH 4.5 and 25 °C (the calculated extinction coefficient was 10,493 $M^{-1}cm^{-1}$ under these conditions [54]). To start the reaction, 50–100 μL of the enzyme solution or suspension were added to 2.5 mL of substrate solution. One unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1 μmoL of oNPG per minute under the conditions described previously.

2.3. Preparation of divinylsulfone activated agarose beads

A volume of 7.5 mL of divinylsulfone was added to 200 mL of 333 mM sodium carbonate buffer at pH 11.0 and vigorously stirred until the solution became homogeneous, then 10 g of agarose or MANAE-agarose beads was added and let under gentle stirring for 60 min. Then, the support was washed with an excess of distilled water and stored at 4 °C.

2.4. Immobilization of the enzyme

The immobilization was carried out employing 20 oNPG units of free beta-galactosidase activity per g of wet support (1 mg of enzyme per gram of support). This low loading was used to prevent diffusion limitations that could distort the results. The commercial samples of the enzyme were dissolved in the corresponding volume of 50 mM sodium acetate at pH 5.0, sodium phosphate at pH 7.0 or sodium carbonate at pH 9.0. Using DVS activated supports, after the immobilization, the insolubilized enzymes were incubated at different pH values and finally the remaining vinyl sulfone groups were blocked by incubation for 24 h at room temperature in 2 M of different nucleophiles (ethylenediamine, glycine, aspartic acid, histidine, cysteine or mercaptoethanol) at pH 8.0. Finally, the immobilized enzyme preparations were washed with an excess of distilled water and stored at 4 °C.

2.5. Thermal inactivations of the different enzyme preparations

To check the stability of the different enzyme immobilized derivatives, 1 g of each immobilized enzyme preparation was suspended in 10 mL of 25 mM sodium acetate at pH 5.0, sodium phosphate at pH 7.0 or sodium carbonate at pH 9.0 at different temperatures. Periodically, samples were withdrawn and the activity was measured using oNPG. Half-lives were calculated from the observed inactivation courses.

3. Results

3.1. Immobilization of the β-galactosidase from Aspergillus oryzae on DVS activated supports

Fig. S1 shows that the β-galactosidase from *Aspergillus oryzae* was not immobilized on agarose activated with DVS at all the studied pH values (5.0, 7.0 and 9.0). Considering the high reactivity of the support with different lateral chains of aminoacids [28] this was an unexpected result, but the failure of immobilization on this support has been previously reported with other enzymes [31]. However, the enzyme was readily immobilized on MANAE-agarose at the 3 studied pH values (Fig. S2) [54]. The enzyme may be released from MANAE-agarose using 250 mM NaCl, and the enzyme was not immobilized on the support under these ion strength.

Therefore, we decided to try the heterofunctional DVS-MANAE-agarose to immobilize the β-galactosidase from *Aspergillus oryzae*. This support efficiently immobilizes the β-galactosidase (Fig. 1) at the 3 pH values. If the immobilized enzyme was incubated in 250 mM NaCl immediately after immobilization, more than 90% of the enzyme was desorbed at pH 5.0, 30–40% of the enzyme was desorbed if the immobilization was performed at pH 7.0, while less than 5% of the enzyme was released from the support if the immobilization was performed at pH 9.0. These results suggested that there were a percentage of just ionically adsorbed enzyme molecules, and that after the ionic adsorption some enzyme molecules established a (or several) covalent bond(s), more efficiently at alkaline pH value, less efficiently at acid pH value, in accordance with the reactivity of vinylsulfone groups with the