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Bovine trypsin immobilization on agarose activated with divinylsulfone: Improved activity and stability via multipoint covalent attachment

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ABSTRACT

Trypsin has been immobilized on divinyl sulfone (DVS) activated agarose at pH 5, 7 and 10. While at pH 5 and 7 immobilization was slow and presented a negative effect on enzyme activity, the immobilization at pH 10 produced a significant increment of activity (by a 24 fold factor). Using this preparation, the effect on enzyme activity/stability of different blocking reagents (used as an enzyme-support reaction end point) were evaluated, selecting ethylenediamine (EDA) because it produced an increase in enzyme activity (a 4 fold factor) and the best results in terms of stability. Next, the effect of alkaline incubation on enzyme activity/stability before the blocking step was analyzed. Activity decreased by 40% after 72 h (but it should be considered that previously it had increased by a 24 fold factor), but the stability significantly improved after this incubation. Thus, after immobilization at different pH values, the immobilized trypsin was submitted to 72 h of alkaline incubation and blocked with EDA. The most active and stable preparation was that immobilized at pH 10. This preparation was less stable than the glyoxyl preparation in thermal inactivations (by less than a twofold factor), but was more stable in organic solvent inactivation (also by less than a twofold factor). The number of groups involved in the enzyme support attachment was 6 Lys using glyoxyl and became a minimum of 13 (including Lys, Tyr and His) using the DVS-activated support (the precision of the method did not permit to analyze the implication of some of the 3 terminal amino groups). Thus, this DVS-agarose support seems to be a very promising support to permit a very intense enzyme-support multipoint covalent attachment.

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1. Introduction

Divinylsulfone (DVS) activated supports have been used to immobilize different enzymes and proteins [1–9]. Recently, DVS activated agarose beads have been proposed as a support suitable to stabilize enzymes via multipoint covalent attachment [10]. Vinylsulfone groups can react with different moieties of aminoacids of proteins without any previous activation of the protein (Lys, Tyr, His, terminal amino group(s)) [10]. The enzyme-support reaction produces stable one point bonds after immobilization, not being necessary to stabilize them to avoid the bond breakage during operation even under the most drastic conditions [10]. Nevertheless, it was also convenient, as reaction end point, to block the remaining vinylsulfone groups by using different nucleophiles (e.g., aminoacids, amino or thiol compounds) to prevent enzyme-support uncontrolled reactions [10]. The reactive groups are also very stable in storage and immobilization conditions, and are able to immobilize proteins in a broad range of pH values (in [10] chymotrypsin was immobilized at pH values from 5 to 10), although at alkaline pH value the immobilization is faster. The further long term incubation of the immobilized chymotrypsin at alkaline pH

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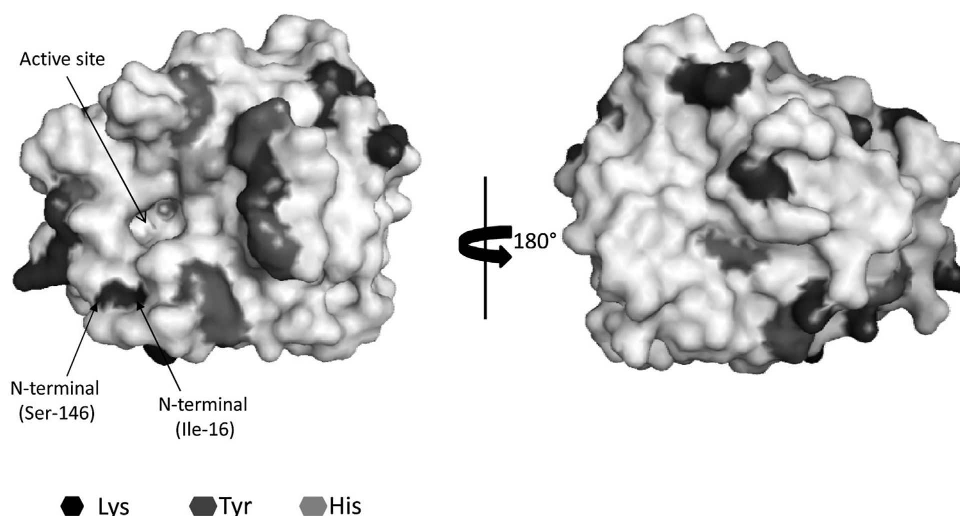

Fig. 1. *3D surface structure model of trypsin.* The trypsin structure with the external DVS-reactive groups marked. (Lys, Tyr, His, terminal amino acids). The 3D surface structure was obtained using PyMol version 0.99. The 3D structure of trypsin was obtained from the Protein Data Bank (PDB). For trypsin pdb code is 2PTN.

values enabled the achievement of higher stabilization factors, even surpassing the results obtained with glyoxyl agarose [11], via multipoint covalent attachment [10].

Compared to other supports that have been successfully employed to stabilize enzymes via multipoint covalent attachment (epoxy [12–14], glutaraldehyde [15] or glyoxyl [16] activated supports), DVS activated supports offer some advantages.

For example, epoxy and DVS activated supports can react with the same range of groups (epoxy groups can also react with carboxylic moieties, but very slowly) [17]. However, DVS-agarose is much more reactive than epoxy, they can directly immobilize enzymes via covalent attachment [10], while the epoxy groups require the previous adsorption of the enzyme on the support surface [18].

Compared to glyoxyl supports, the main advantage of DVS supports to produce an intense multipoint covalent attachment is that the support may react with other groups different to primary amino groups as it occurs using glyoxyl supports [19]. Moreover, just thinking in a support to immobilize biomacromolecules and not to stabilize them, it is possible to use lowly activated supports (the unstable imine bonds of glyoxyl-supports make the use of relative highly activated supports necessary even to get the enzyme immobilization) [19] and furthermore it is not necessary to perform the immobilization at alkaline pH value, nor is it necessary to use a reduction step (e.g., using sodium borohydride) like when using glyoxyl supports [20]. The main drawback is that the spacer arm is longer (to the agarose-CH_2OH groups, 4 C atoms and 1 S must be added (see Scheme 1)). And that may reduce the rigidification achieved by the multipoint covalent attachment [21–24]. However, this longer spacer arm may also permit to involve a larger percentage of the enzyme surface in the enzyme-support reaction [21–24].

The low reactivity of epoxy and glyoxyl groups under certain circumstances has permitted the development of heterofunctional support to immobilize enzymes via different orientations promoted by other groups (immobilized metals, ionic groups) but via the same chemistry and under the same immobilization conditions [25]. DVS-supports may be too reactive with enzymes to use this strategy to direct the enzyme immobilization, as they can covalently immobilize proteins in the range of pH 5–10. However, the reactivity of the different enzyme groups at different pH values is different, and this may permit to alter the enzyme orientation just altering the immobilization pH [10].

In this new paper, DVS-agarose support has been used to immobilize one of the most utilized proteases, trypsin [26]. The properties of this immobilized enzyme will be compared to those obtained via immobilization in glyoxyl support (a very stable preparation) [27,28] that has been utilized in different protein hydrolytic processes [29–32].

2. Materials and methods

2.1. Materials

Divinylsulfone, bovine trypsin (E.C. 3.4.21.4), benzoyl-arginine *p*-nitroanilide (BANA), ethylenediamine (EDA), ethanolamine, glycine (Gly), aspartic acid (Asp), and 2-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Agarose beads 4BCL support was purchased from Agarose Bead Technologies (ABT), Spain. All other reagents were of analytical grade.

All experiments were performed by triplicate and the results are reported as the mean of this value and the standard deviation (usually under 10%).

2.2. Enzymatic assays

The activity of the soluble or suspended enzyme (10 mg/mL) was assayed by determination of the increase in absorbance at 405 nm which accompanies the hydrolysis of the synthetic substrate BANA (100 or 200 μL soluble or suspended enzyme were added to 2.5 mL of 50 mM sodium phosphate containing 30% (v/v) ethanol at pH 7 having 2 mM BANA, at 25 °C [28].

2.3. Preparation of glyoxyl-support

The activation of agarose gels was performed according to the procedure described in [19] the gel was suspended in 1 M NaOH and 0.5 M $NaBH_4$ 2:1 (v/v). These reducing conditions prevent oxidation of the gel. While keeping this mixture in an ice bucket, glycidol was added dropwise in order to reach a 2 M final concentration. The resulting suspension was gently stirred overnight at room temperature. The modified gel was then washed once with abundant distilled water (pH 7), incubated in an aqueous solution (300 mL) containing 60 μmol $NaIO_4$/g gel in order to achieve glyoxyl groups. This oxidative reaction was allowed to proceed for 2–3 h under mild

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