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Effect of the support and experimental conditions in the intensity of the multipoint covalent attachment of proteins on glyoxyl-agarose supports: Correlation between enzyme–support linkages and thermal stability

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Abstract

The immobilization of trypsin, chymotrypsin and carboxypeptidase A using 4 and 10% glyoxyl-agarose beads at different times of incubation was investigated. Enzyme loadings of 30 mg/mL gel for trypsin and chymotrypsin, and 2 mg/mL gel for carboxypetidase A were used. Immobilization rates were very rapid in both supports and reactions were completed after 1 h of reaction. Final residual activities at these concentrations were around 60% for trypsin and chymotrypsin, and 50% for carboxypeptidase A. Comparison of the thermal stability of the soluble and immobilized enzymes revealed that immobilization by binding to 10% glyoxyl-agarose yielded the most stable enzymatic activities. Reaction with this support yielded immobilized trypsin, chymotrypsin, and carboxypeptidase A that were 4700, 10,000, and 1000 times more stable than the soluble enzymes, respectively. It was observed that the number of lysine residues that took part in the immobilization process was a consequence of the type of support and reaction time of the experimental conditions, and that the increasing of the thermal stability of the derivatives was correlated with a increasing number of lysines residues involved in a multipoint covalent attachment.

Keywords: Immobilized enzymes; Glyoxyl-agarose; Thermal stability; Trypsin; Chymotrypsin; Carboxypeptidase A

1. Introduction

Proteases represent a class of enzymes with important roles in physiological process. Besides this, commercially they are extremely important, accounting for about 60% of the total worldwide sale of enzymes. In terms of evolution, these enzymes are responsible of many essential biological processes, but nowadays they are also involved in a wide variety of applications, mainly in detergent and food industries [1–3]. However, most proteolytic enzymes can degrade themselves and therefore, they are difficult to recycle. Any immobilization protocol of proteins inside a porous support can solve these problems because this concerns the fixation of individual molecules of enzyme, which reduces losses of activity by autolysis and increases the half-life of the enzymes [4–7]. Moreover, if the immobilization system is properly designed, can also enhance the stability of enzymes by "rigidification" of their three-dimensional structures, which results in a higher resistance to conformational changes induced by heat, organic solvents or pH [8–11].

Several procedures have been employed to achieve stabilization of enzymes, including genetic and protein engineering techniques [12–17]. However, immobilization of enzymes on porous solid carriers (silica, alumina, glass, agarose and cellulose) [18–20] by different methods (covalent and ionic attachment or physical adsorption) is probably the most used strategy to insolubilize and improve the stability of enzymes [21–23]. In this sense, glyoxyl-agarose beads have been successfully employed for the immobilization–stabilization of many different enzymes, resulting in high stabilization factors and high preservation of enzymatic activities [24].

As commented by Mateo et al. [24] and Grazú et al. [25], the first immobilization in these supports is already a multipoint

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process, implying the area with the highest density of lysine residues more than the area where the more reactive residue (e.g., the terminal amino group) is placed. Following the immobilization reaction by reaction of activated agarose with the enzyme, a reduction with sodium borohydride is usually performed in order to obtain very stable secondary amino bonds and to transform the residual aldehyde groups in the support into very hydrophilic and inert hydroxyl-groups. Thus, molecules having just one amino group do not significantly react with glyoxyl-agarose [24].

It has been proven that the enzymes immobilized on the glyoxyl-supports presented in many instances many enzyme-support linkages. In fact, the increased stability from one preparation to other has been explained by an increase in the number of support-enzyme linkages [26], but without a real evidence of the different multipoint interaction between preparations having different stabilities. Thus, a clear correlation between number of enzyme-support linkages and enzyme and the final protein stabilization have no been established to date. Thus, the effect of time and temperature [27] that increase the final stability of the preparations, have been explained as variables that may increase the intensity of the support-enzyme interaction, but only functional properties of the immobilized enzymes have been used to state this hypothesis [24].

One variable with a great interest is the geometric congruence between enzyme and support. It has been suggested that the greater the enzyme-support congruence, the higher the possibilities to achieve an intense multipoint attachment. In the case of agarose beads, they are formed by a non-covalent association between polymers of agarose forming trunks of a diameter related to the concentration of agarose [28]. Higher the concentration of the agarose during gelification, thicker the agarose trunks formed, and at first glance, higher the possibilities to achieve an intense multipoint covalent attachment.

In this sense, advancing in the effect of the variables in the stabilization and/or multipoint covalent attachment, we have analyzed for first time the effect of the agarose utilized, comparing agarose beads with 4% and 10% of agarose presenting a low or high activation. In supports with a high activation, in both cases the number of glyoxyl groups per 1000 $Å^2$ is around 20 [29,30].

However, agarose forms "trunks" during polymerization, and the thickness of these trunks is larger as the percentage of agarose increases [28]. Thus, 4% agarose has larger pores but thinner "trunks" than 10% agarose beads, implying a lower "geometrical congruence" between the support and the enzymes.

The main goal of this work was the study of the correlation between the thermal stability of the immobilized derivatives and the number of lysine residues involved in the immobilization process. Variables such as temperature, time of incubation or type of activated agarose were modified with the aim of establishing this possible direct correlation between them. This study was performed bearing in mind the high stability of the secondary amine bonds in the enzyme-support linkages against the protein acidic hydrolysis that permitted to the lysine residues remained modified after the hydrolysis of the protein.

2. Materials and methods

2.1. Materials

Bovine trypsin (E.C. 3.4.21.4), α-chymotrypsin (E.C. 3.4.21.1), benzoyl-L-arginine *p*-nitroanilide (BANA), and benzoyl-L-tyrosine *p*-nitroanilide (BTNA) were purchased from Sigma Chemical Co. (St. Louis, MO). Carboxypeptidase A (E.C. 3.4.17.1) was purchased from Serva (Heidelberg, Germany) and hyppuril-L-phenylalanine (HP) was purchased from Fluka (Buchs, Switzerland). Cross-linked agarose beads 4% and 10% (w/v) were donated by Hispanagar S.A. (Madrid). Organic solvents and all other reagents were of analytical grade.

2.2. Methods

All experiments were performed at least three times. Data shown correspond to mean values. Experimental error was never higher than 5%.

2.2.1. Activation of agarose gels

The activation of 4% and 10% agarose gels was done according to the procedure previously described by Guisán [31] with slight modifications. The gels were suspended in 1 M NaOH and 0.5 M $NaBH_4$ 2:1 (v/v) (0.7 g swelling agarose is roughly equivalent to 1 mL). These reducing conditions prevent oxidation of the gel. While keeping this mixture on an ice bucket, glycidol was added drop wise in order to reach a 2 M final concentration. The resulting suspension was gently stirred overnight at room temperature. The activated gel was then washed once with abundant distilled water (pH 7), and then washed again with distilled water (300 mL) containing 5 or 300 μmoles $NaIO_4$/mL gel in order to achieve one-point or multi-point attachment, respectively. This oxidative reaction was allowed to proceed for 2–3 h under stirring at room temperature. Using this procedure the glyceryl groups obtained in the etherification reaction with glycidol are oxidized specifically by periodate mole to mole. This reaction is very important because subsequent measurement of excess $NaIO_4$ by titration with KI is used to determine the number of aldehyde groups that have been produced by the reaction of agarose with glycidol [32].

2.2.2. Immobilization of the enzymes

Immobilization of the enzymes on 4% or 10% activated agarose gels was done according to procedures previously described with slight modifications [33–35]. The gels (30 mg enzyme/mL gel for trypsin and chymotrypsin and 2 mg enzyme/mL gel for carboxypeptidase A) were suspended in 0.2 M sodium bicarbonate, pH 10.0, 1:10 (v/v) and gently stirred at room temperature for 180 min. Derivatives were then reduced by addition of $NaBH_4$ (0.1% (w/v)). After gentle stirring for 30 min at room temperature, the resulting derivatives were washed with abundant distilled water to eliminate residual sodium borohydride.

2.2.3. Enzymatic assays

α-chymotrypsin: The activity of the soluble or suspended enzyme (30 mg/mL) was assayed by determination of the increase in absorbance at 405 nm which accompanies the hydrolysis of the synthetic substrate BTNA (75 μL soluble or suspended enzyme were added to 2.5 mL 50 mM sodium phosphate 40% ethanol, pH 7, containing 25 μL BTNA 40 mM in hexane:dioxane 1:1 (v/v) at room temperature).

Trypsin: The activity of the soluble or suspended enzyme (30 mg/mL) was assayed by determination of the increase of absorbance at 405 nm which accompanies the hydrolysis of the synthetic substrate BANA (75 μL soluble or suspended enzyme were added to 2.5 mL 50 mM sodium phosphate 40% ethanol, pH 7, containing 200 μL BANA 1 mM in sodium phosphate 40% ethanol, pH 7, at room temperature).

Carboxypeptidase A: The activity of the soluble or suspended enzyme (2 mg/mL) was assayed by determination of the increase of absorbance at 254 nm which accompanies the hydrolysis of the synthetic substrate HP (75 μL of sample were mixed with 2.5 mL of 1 mM HP in 50 mM sodium phosphate, pH 7, at room temperature).