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The co-operative effect of physical and covalent protein adsorption on heterofunctional supports

Juan M. Bolivar [a], Cesar Mateo [a], Cesar Godoy [a], Benevides C.C. Pessela [b], Dasciana S. Rodrigues [c], Raquel L.C. Giordano [c], Roberto Fernandez-Lafuente [a,*], Jose M. Guisan [a,*]

[a] Departamento de Biocatálisis, Instituto de Catálisis-CSIC, Campus UAM, Cantoblanco, 28049 Madrid, Spain
[b] Departamento de Microbiología, Instituto de Fermentaciones Industriales-CSIC, C/Juan de la Cierva 3, 2006 CSIC, Madrid, Spain
[c] Federal University of São Carlos, Chemical Engineering Department, Via Washington Luiz, Km 235 - São Carlos-SP - 13 565-905, Brazil

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ABSTRACT

It has been found that the enzymes penicillin G acylase from *Escherichia coli* (PGA) and lipase from *Bacillus thermocatenulatus* (BTL) did not significantly adsorb on highly activated amino-agarose beads at pH 7 (a support where 85–90% of a crude extract of proteins become adsorbed). Moreover, it has been found that these enzymes do not covalently immobilize on highly activated epoxy-agarose beads at pH 7. However, both enzymes slowly immobilize on heterofunctional supports having a high density of amino–epoxy groups. The immobilized enzymes retain a high percentage of activity (more than 90% for PGA and 60% for BTL). On the other hand, the immobilization of a crude extract of proteins on amino–epoxy supports under conditions where only a limited protein ionic exchange was permitted (by using high ionic strength or lowly activated supports), also permitted a similar high immobilization yield of the proteins. Similarly, glutamate dehydrogenase (GDH) and β-galactosidase from *Thermus thermophilus* can be fully immobilized under conditions where less than 20% of these enzymes can be ionically exchanged in the aminated support. The results suggested that the percentage of proteins that may be physically adsorbed on the support becomes irreversibly immobilized by the covalent reaction between the nucleophilic groups in the protein surface and the very near epoxy groups of the support (in an almost intramolecular reaction). Thus, using these supports, it is possible to immobilize almost all the proteins by anionic exchange, that is, the area with the highest density in anionic groups. In many cases, this region could not correspond to the protein regions usually utilized to immobilize proteins. This way, it is possible to achieve, in a very simple fashion and without modifying the protein, new orientations of some immobilized enzymes and proteins.

1. Introduction

The development of strategies to immobilize proteins via different orientations has proven to be very interesting for multiple applications (biocatalysis, biosensors, etc.) [1–5]. The control of the area of the protein in contact with the support may be a key point, for example to decrease steric hindrances when using macromolecular substrates [6], to reduce enzyme inhibition [7,8], to alter the catalytic properties of the enzymes (specificity, regioselectivity, etc. [7,9]), or to immobilize the protein by its surface area/s which is/are the most relevant for enzyme stabilization [10,11]. This may be even more convenient if the support is able to yield an intense multipoint covalent attachment [7,12–14] with the enzyme region involved in the immobilization, therefore rigidifying it, e.g., highly activated epoxy supports [15].

For this reason, the development of new strategies to immobilize proteins may be relevant in enzyme technology, very especially by their surface areas where the immobilization cannot occur when using conventional methodologies.

Epoxy activated supports are quite popular in the immobilization of enzymes [15–17]. The mechanism of immobilization on epoxy supports is via a first incorporation of the enzyme to the support surface (in the case of conventional epoxy supports, that are fairly hydrophobic, it is via a hydrophobic adsorption at high ionic strength [18–21]) followed by the intramolecular reaction between the epoxy groups in the support and many different reactive groups of the enzyme [22]. Using this immobilization mechanism, heterofunctional epoxy supports have been designed to immobilize proteins via different orientations. These supports are based on the co-activation of the support with groups that are able to adsorb the proteins and with a dense layer of epoxy groups [23].

* Corresponding authors. Tel.: +34 91 585 4809; fax: +34 91 585 4760.
E-mail addresses: rfl@icp.csic.es (R. Fernandez-Lafuente), jmguisan@icp.csic.es (J.M. Guisan).

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In the first report using these heterofunctional supports, it was proposed that the proteins must be fully physically adsorbed on the support to be efficiently immobilized in these new supports [23].

Now, we propose that a full physical adsorption of the protein on the support is not ultimately necessary to have a complete protein immobilization. If the first adsorption of the protein on the support surface is very weak, it is possible that only a small, even marginal, percentage of the protein adsorbs on the support surface. However, if a fraction of these adsorbed protein molecules becomes covalently immobilized (because they are near to a dense layer of epoxy groups), they will be eliminated from the protein-support adsorption/desorption equilibrium. This way, a new adsorption equilibrium will be established and more protein molecules can covalently react with the support. Finally, most of the protein molecules will be covalently immobilized on hetero-functional supports, even when they are activated with two kinds of groups that were unable to significantly immobilize the target proteins if used in an independent way. The immobilization will be slower than if the first adsorption was rapid, but the final immobilization yield could be very high.

This hypothesis has been analyzed using amino, amino–epoxy or amino and epoxy activated agarose beads as model supports (Fig. 1) [23]. We have chosen proteins that did not significantly adsorb by ionic exchange on the employed supports (e.g., penicillin G acylase from *Escherichia coli* [24] or lipase from *Bacillus thermocatenulatus*). In other cases, we have selected immobilization conditions where the ionic exchange is difficult (e.g., low activation degree in the support [25], high ionic strength [26]).

Penicillin G acylase (PGA) is very interesting because it is currently used in the production of semisynthetic antibiotics [27,28]. The lipase from *B. thermocatenulatus* (BTL2) presents a high interest due to its thermostability (even in presence of organic cosolvents) and its usefulness as enantioselective biocatalyst [29,30].

Moreover, it has been recently reported that the multimeric structure of glutamate dehydrogenase may be stabilized using amino–epoxy activated support (with a very low amino content) while a highly amino–epoxy activated support did not stabilize it, suggesting that the adsorption on both supports could be different [31]). This enzyme is quite interesting because it may be used as a biocatalyst to regenerate cofactors and as biosensor [32,33].

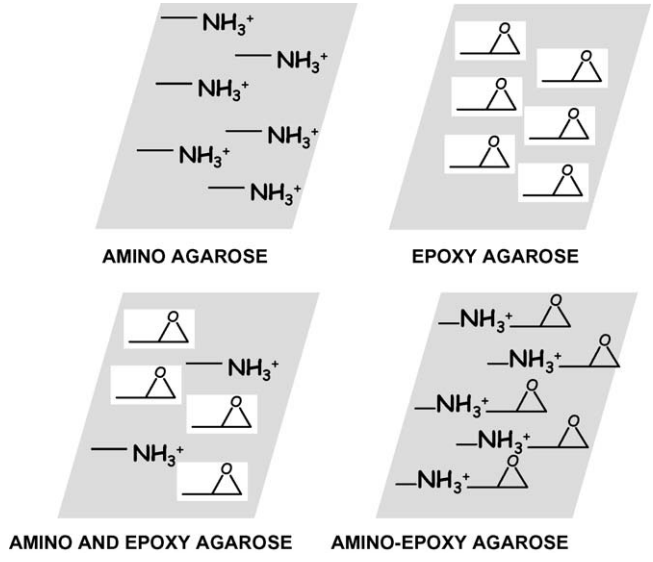

Fig. 1. Scheme of the different supports used in the paper.

2. Materials and methods

2.1. Materials

p-Nitrophenyl butyrate (*p*-NPB), o-nitro-phenylgalactopyranoside (o-NPG), ethylendiamine (EDA), sodium borohydride, 1,4-butanediol diglycidyl ether were from Sigma (St. Louis, MO, USA). Coomassie (Bradford) protein assay kit was purchased from Pierce (USA). Nicotinamide adenine dinucleotide, NAD^+, was purchased from Jülich Fine Chemicals. Crosslinked 4BCL sepharose beads were from GE Healthcare (Uppsala, Sweden). All other used reagents were of analytical grade. Penicillin G acylase from *E. coli* (PGA) and penicillin G were kindly donated by Antibioticos SA. Lipase from *B. thermocatenulatus* (BTL) [34], β-galactosidase from *Thermus* sp. strain T2 [35] and glutamate dehydrogenase from *Thermus thermophilus H8* (GDH) overexpressed in *E. coli* were produced as published elsewhere [36]. Amino supports (monoaminoethyl-N-ethyl (MANAE)-agarose) with different degrees of activation were prepared as previously described [25,37]. Crude protein extract DNA free were kindly donated by Prof. Berenguer (CBM). Protein concentration was determined using Bradford's method [38].

2.2. Determination of enzyme activities

One International Unit (IU) of enzymatic activity was defined as the amount of enzyme that produces 1 μmol of product/min under the specified conditions.

2.2.1. Determination of BTL activity

This assay was performed by measuring the increase in absorbance at 348 nm produced by the release of *p*-nitrophenol in the hydrolysis of 0.4 mM pNPB in 25 mM sodium phosphate at pH 7 and 25 °C. To initialize the reaction, 0.05 mL of lipase solutions or suspensions were added to 2.5 mL of substrate solution.

2.2.2. Determination of PGA activity

Enzyme amidase activity was determined using penicillin G. The hydrolysis of penicillin G was monitored using an automatic titrator (DL50 Mettler Toledo). This way, the release of phenylacetic acid produced by the hydrolysis of 10 mM penicillin G in 0.1 M sodium phosphate/0.5 M NaCl at pH 8.0 and 25 °C was titrated. A 100 mM NaOH solution was used as titrating reagent.

2.2.3. Determination of GDH activity

The activities of the different GDH preparations were analyzed by the increase in absorbance at 340 nm corresponding to the formation of NADH concomitant to glutamic acid oxidation. A sample of enzymatic preparation (25–400 μL) was added to a cell with 2 mL of 250 mM glutamic acid and 100 μL of 100 mM NAD^+ in 100 mM sodium phosphate at pH 8.0 and 66 °C.

2.2.4. Determination of β-galactosidase activity

Activity was followed spectrophotometrically by the increase in absorbance at 405 nm caused by the hydrolysis of o-NPG. The reaction medium was 13.3 mM o-NPG, dissolved in Novo buffer pH 6.5 (2.7 mM sodium citrate; 7.91 mM citric acid; 2.99 mM potassium bi-phosphate; 10.84 mM potassium phosphate; 19.43 mM potassium hydroxide; 4.08 mM magnesium chloride; 5.1 mM calcium chloride; and 3.33 mM sodium carbonate) at 25 °C. The β-galactosidase activity is given in μmol of substrate hydrolyzed per min and per mg of protein under the described conditions.

2.3. Preparation of the supports

The different supports utilized in this paper are shown in Fig. 1.

2.3.1. Preparation of epoxy-agarose

Activation was performed with epichlorohydrin as previously described with some modifications [39]. 10 mL of 4 BCL agarose were washed thoroughly with distilled water and the moist gel was suspended in 30 mL of 0.8 M NaOH containing 340 mg of $NaBH_4$, 11.4 mL of acetone and 5.7 mL of epichlorohydrin. The suspension was stirred for 8 h at 25 °C and finally washed thoroughly with distilled water. Two additions of 5.7 mL of epichlorohydrin were performed after 2 and 4 h of reaction. Determination of epoxy content was determined as previously described [39,40]. This support presented 20 μmol of epoxy groups per gram of support.

2.3.2. Preparation of heterofunctional amino and epoxy-agarose

Controlled amination of the support was performed as previously described [23]. 10 g of wet epoxy-agarose were incubated in 60 mL of 2% (v/v) ethylenediamine at pH 8.5 for different times (from 15 min to 24 h) under very gentle stirring. Then, the supports were washed with distilled water, 1 M NaCl and finally with distilled water. The degree of modification was quantified by titration of the amino groups introduced in the support [41,42].

2.3.3. Preparation of amino–epoxy supports

The support was prepared as previously described with minor modifications [43]. 15 mL of MANAE support activated with 40 μmol/g of support [25,37] was suspended