One-Point Covalent Immobilization of Enzymes on Glyoxyl Agarose with Minimal Physico-Chemical Modification: Immobilized "Native Enzymes"

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Abstract

The immobilization of soluble enzymes inside the porous structure of a preexisting support is one of the most interesting techniques to prepare heterogeneous biocatalysts. The main cause of inactivation of these biocatalysts is the distortion of the tridimensional structure of the immobilized enzymes. In some cases, immobilization of enzymes on preexisting supports can be used in order to improve its functional properties: stabilization by multipoint covalent immobilization, hyper-activation, and stabilization of lipases by interfacial adsorption on hydrophobic supports, etc. In other cases, the properties of the enzyme can be modified by additional interactions of the enzyme surface with the support surface: hydrophobic or electrostatic interactions.

In all cases, it would be very interesting to evaluate the intrinsic tridimensional stability of native industrial enzymes. Under drastic experimental conditions, soluble enzymes may undergo undesirable aggregations, and the tridimensional stability of one enzyme is more accurately evaluated by using immobilized native enzymes. That is, immobilized derivatives associated to a minimal chemical modification of the enzyme surface placed in the proximity of a fully hydrophilic and inert support surfaces. In this chapter, the immobilization of enzymes with minimal physicochemical modification on glyoxyl agarose supports is proposed. At pH 8.5, the unique reactive amino group on the enzyme surface is the N-terminus. At the end of the immobilization, mild borohydride reduction, the primary amino terminus is simply converted into a secondary amino group, with similar physical properties, and aldehyde groups on the supports are converted into fully inert hydroxyl groups. The preparation of immobilized derivatives of penicillin G acylase (PGA) with identical properties (activity and stability) that one of the soluble enzyme is reported: preparation of immobilized native PGA.

Key words Covalent immobilization, Glyoxyl agarose, Native enzymes, N-terminus, Thiolated compounds, 2-picoline borane

1 Introduction

1.1 Immobilization of Enzymes inside Porous Supports: Stabilizing Effects

Immobilization of enzymes via a firm attachment to the internal surface of porous supports may exert interesting stabilizing effects regarding the corresponding soluble enzymes [1]. The immobilized enzyme molecules cannot undergo aggregations (at high temperatures, in the presence of organic cosolvents, etc.), and they cannot interact with interfaces in stirred reactors (oxygen bubbles, immiscible solvents in biphasic reactors, etc.) (Fig. 1) [2]. Immobilized enzyme molecules can be only inactivated by distortions of their tridimensional structure. Under rare very drastic conditions (e.g., very high temperatures), they could also be inactivated by chemical modification of key residues.

On the contrary, soluble enzymes can be inactivated by aggregation and by interaction with interfaces. Very mild enzyme aggregations may even be stabilizing ones but drastic aggregations (heat, solvents) are highly destabilizing ones [3]. In this way, enzyme immobilization inside porous supports can be associated with interesting stabilizations. This stabilization is common to any immobilization protocol of a given enzyme inside a porous structure, and it does not reflect the accuracy of a given immobilization protocol. Different immobilization protocols should be compared between themselves and, if possible, an immobilized derivative of the "native enzyme" should also be compared. In this way, the structural stabilization/destabilization of different derivatives of a given

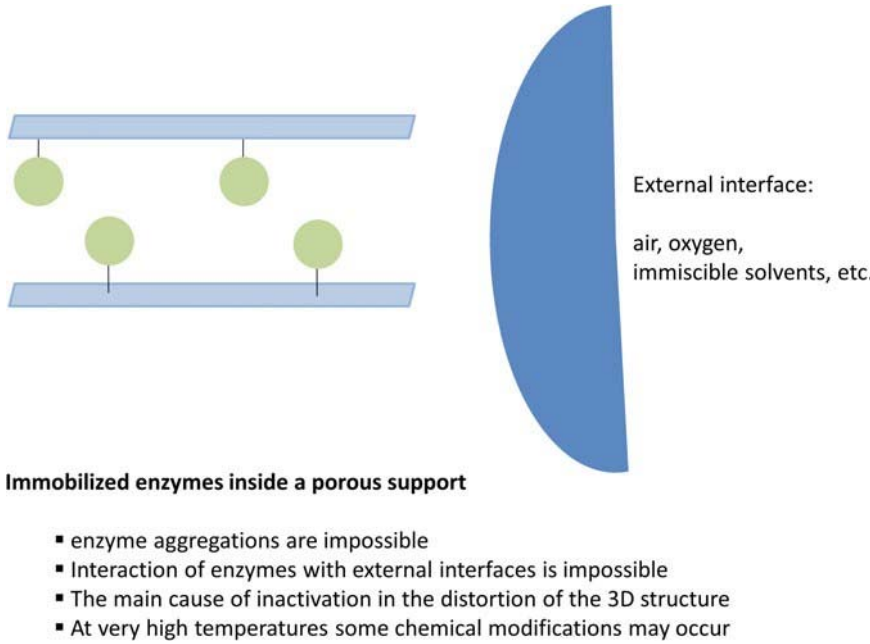

Fig. 1 Immobilized enzyme molecules inside the porous structure of a support

enzyme could be correctly evaluated. The best immobilization protocol would be the one promoting the most intense structural stabilization of a given enzyme [2].

1.2 The Use of Soluble Enzymes as "Blanks" to Test the Functional Properties of Enzymes Immobilized Inside Porous Supports

In many cases, immobilized enzyme derivatives are compared to soluble enzymes in order to test the stabilizing effect of a given immobilization protocol [4]. This could be fairly correct when the blank is a very diluted solution of a pure enzyme incubated at moderate temperatures in the absence of stirring (aggregations are minimized). However, when the blank is a concentrated crude extract incubated at high temperatures and/or in the presence of organic cosolvents, the comparison soluble vs. immobilized enzyme may be clearly incorrect as commented above. A suitable blank of an immobilized "native enzyme" would be very convenient. Obviously, immobilization of an enzyme inside a porous supports implies a certain modification of the native enzyme [5]. However, it is possible to immobilize the enzyme with minimal chemical and physical modification.

1.3 Immobilized "Native Enzymes"

One-point covalent immobilization of enzymes on aldehyde-agarose supports (at slightly alkaline pH) is here proposed as a suitable protocol to prepare immobilized "native enzymes" (Fig. 2). After immobilization, derivatives are reduced with sodium borohydride. In this way, the primary amino terminus of the native enzyme is simply converted into a secondary amino group (with very similar physical properties), and the enzyme surface remains unaltered in the proximity of a fully inert and highly hydrophilic internal surface of agarose gels.

1.4 Immobilization of Enzymes on Glyoxyl Supports

Highly activated glyoxyl supports rapidly immobilize enzymes at pH 10 (where the ε-amino groups of the Lys residues placed on the enzyme surface are very reactive), and stabilize them by multipoint covalent attachment (*see* Chapter 5) [6]. However, glyoxyl supports do not immobilize enzymes at pH 8.5 where the N-terminus is the unique reactive amino group on the enzyme surface (non-protonated form) [7]. One-point attachment between one amino group and one linear aldehyde group yields a very unstable Schiff's base and the enzymes do not become immobilized on the support (Fig. 3).

However, these single Schiff's bases can be stabilized in order to promote stable one-point enzyme-agarose immobilizations (Fig. 3):

(a) By selective reduction of Schiff's bases to very stable secondary amino bonds in the presence of very mild reducing agents such as cyanoborohydride or 2-picoline borane (*see* Note 1) [8].

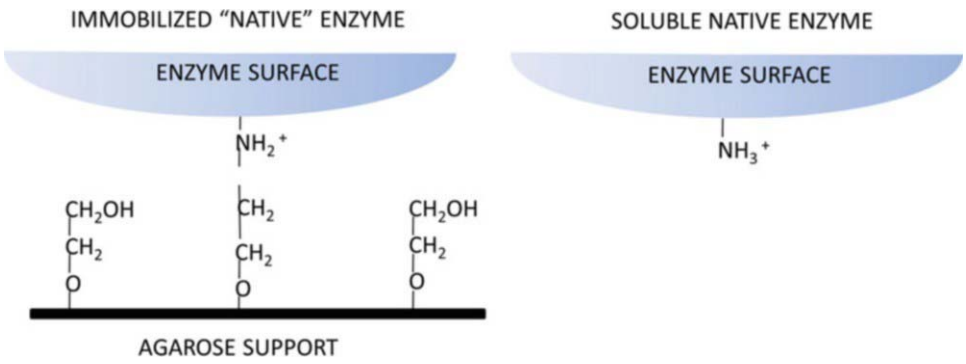

Fig. 2 Immobilized native enzymes may be almost identical to soluble native enzymes

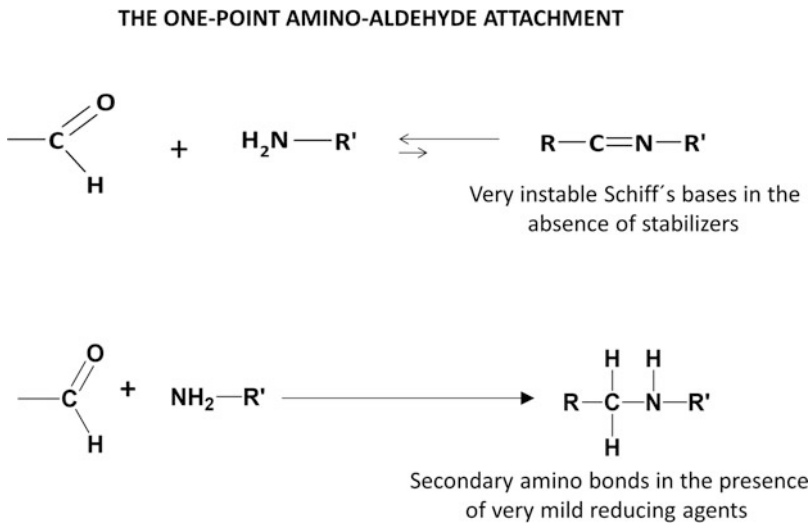

Fig. 3 One-point amino-aldehyde attachment in the absence or in the presence of stabilizers

(b) By the formation of stable complexes between Schiff's bases and thiolated compounds such as acetyl-cysteine, mercapto-ethanol, or dithiothreitol (DTT) [7].

After the one-point covalent immobilization, the immobilized derivatives have to be reduced with sodium borohydride in order to reduce Schiff's bases to secondary amino groups and to reduce remaining aldehyde groups into hydroxyl ones [7].

1.5 Structural Studies of Native Enzymes Under Nonconventional Conditions

Nowadays, in addition to their key physiological role, enzymes have also become relevant industrial catalysts. In this way, structure–function studies of enzymes under a great variety of experimental conditions, not only physiological ones, have become very interesting: high temperatures, presence of cosolvents, anhydrous solvents, solvent-free reaction systems (e.g., ethanolysis of oils), ionic liquids, etc. Some experimental techniques, solid-state nuclear magnetic

resonance (NMR), circular dichroism, fluorescence, etc., could be very useful to study conformational studies of immobilized enzymes. For example, agarose gels are transparent supports (they only promote slight "light scattering" but they do not adsorb radiations, except IR radiation) [9, 10]. Agarose gels seem very suitable for structural studies of immobilized enzymes. Most of these techniques require the use of high enzyme concentrations (e.g., NMR) and, in some case, soluble enzymes could not be used because of aggregation problems. A suitable derivative of a highly concentrated immobilized "native enzyme" would be necessary to perform solid-state NMR [11].

2 Materials

1. Commercial highly activated glyoxyl-6% agarose gels (High Density Glyoxal 6BCL, Agarose Bead Technologies, Madrid, Spain).
2. Highly activated glyoxyl-6% agarose gels can be also prepared as described in [12] (or *see* Chapter 5).
3. Commercial penicillin G acylase (penicillin amidase), PGA, from *Escherichia coli*.
4. Reducing agents: 2-picoline borane (2-methylpyridine borane complex) and sodium borohydride.
5. DL-Dithiothreitol (DTT, Cleland's reagent).
6. 6-nitro-3-phenylacetamidobenzoic acid (NIPAB).

3 Methods

3.1 PGA Activity Assay

1. Principle. Activity was determined by measuring the increase in absorbance in the reaction mixture promoted by the release of 3-amino-6-nitrobenzoic acid (molar extinction coefficient 8980 M^{-1} cm^{-1} at 405 nm, pH 7.5, and 25 °C).
2. Add a suitable amount of enzyme to a spectrophotometric cell containing 2 mL of 0.2 mM NIPAB dissolved in sodium phosphate buffer at pH 7.5.
3. Monitor absorbance increase and calculate activity using the initial linear reaction rate. One NIPAB unit was defined as the amount of enzyme required to hydrolyze 1 μmol of NIPAB per minute at pH 7.5 and 25 °C. This unit corresponds to 2.4 IU of PGA: this amount of enzyme is able to liberate 2.4 μmols of phenyl acetic acid by hydrolysis of NIPAB by penicillin G at pH 8.0 and 37 °C. 1 mg of commercial PGA preparation has 4 NIPAB units per mg of protein (25% purity).

3.2 One-Point Covalent Immobilization of Penicillin G Acylase (PGA) on Glyoxyl Agarose

PGA is a heterodimeric enzyme containing two N-terminus. However, one of them is buried inside the active center and it is not exposed to the medium. Hence, PGA behaves as a monomeric enzyme with only one exposed amino terminus [13].

3.2.1 Standard Immobilization in the Absence of Stabilizers

1. 3 g of wet glyoxyl-6% agarose gel was added to 30 mL of PGA solution (0.2 mg of commercial enzyme per mL of 0.1 M bicarbonate buffer pH 8.5). This is eight NIPAB units were offered per wet gram of support (in order to prevent diffusional limitations).
2. Leave the suspension under very gentle stirring (*see* Note 2) (e.g., in a rotating shaker) for 10 h at 4 °C. Take samples of supernatant and suspension for follow immobilization (Subheading 3.3).
3. After 10 h, the suspension was reduced by adding 11 mg of sodium borohydride, and the reaction was performed under very gentle stirring for 30 min.
4. Then derivatives were filtered and washed with distilled water and finally with 50 mM phosphate at pH 8.0. The wet immobilized derivatives were stored at 4 °C.

3.2.2 Standard Immobilization in the Presence of 2-Picoline Borane

1. 3 g of wet glyoxyl-6% agarose gel was added to 30 mL of PGA solution (0.2 mg of commercial enzyme per mL of 0.1 M bicarbonate buffer pH 8.5 containing 20 mM of 2-picoline borane).
2. Leave the suspension under very gentle stirring for 10 h at 4 °C. Take samples of supernatant and suspension for follow immobilization (Subheading 3.3).
3. After 10 h, the suspension was reduced by adding 30 mg of sodium borohydride, and the reaction was performed under very gentle stirring for 30 min.
4. Then, derivatives were filtered and washed with distilled water and finally with 50 mM phosphate pH 8.0. The wet immobilized derivatives were stored at 4 °C.

3.2.3 Standard Immobilization in the Presence of DTT

1. 3 g of wet glyoxyl-6% agarose gel was added to 30 mL of PGA solution (0.2 mg of commercial enzyme per mL of 0.1 M bicarbonate buffer pH 8.5 containing 50 mM of DTT).
2. Leave the suspension under very gentle stirring for 10 h at 4 °C. Take samples of supernatant and suspension for follow immobilization (Subheading 3.3).

3. After 10 h, the suspension was reduced by adding 30 mg of sodium borohydride, and the reaction was performed under very gentle stirring for 30 min.
4. Then, derivatives were filtered and washed with distilled water and finally with 50 mM phosphate pH 8.0. The wet immobilized derivatives were stored at 4 ºC.

3.3 Time-Course of One-Point Covalent Immobilizations

3.3.1 Principle

Time-courses of immobilization were followed by measuring the enzymatic activity of the supernatants of the immobilization suspensions (*see* Fig. 4).

3.3.2 Steps

1. Incubate blanks of enzyme solutions without activated supports under the same conditions of immobilization (Subheading 3.2).
2. Measure enzymatic activity from blanks and samples from immobilization (Subheading 3.2).

3.3.3 Results Analysis

Figure 4 shows the output of the analysis. These blanks remain fully active during the whole immobilization and hence the decrease in the activity of the supernatants of the immobilization suspensions exactly represents the percentage of enzyme that has been incorporated into the solid support at different times.

The activity of the supernatant of immobilization suspension in the absence of stabilizers remains fully active during 4 h. This means that immobilization is not possible because of the instability of one-point Schiff's base.

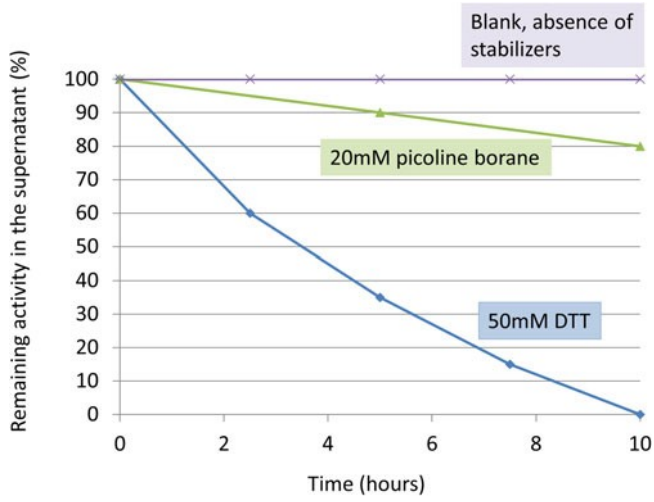

Fig. 4 Time-course of one-point covalent immobilizations of PGA on glyoxyl agarose at pH 8.5 and 4 ºC

The activity of the supernatant of immobilization suspension in the presence of 2-picoline borane slightly decreases down to 80% after 10 h. It seems that reduction of Schiff's bases by 2-picoline borane is possible, but it is very slow. A low loaded one-point covalent immobilized derivative can be obtained.

The activity of the supernatant of the immobilization suspension in the presence of DTT decreases down to less than 5% after 10 h. It seems that the stabilization of Schiff's bases by complexation with DTT is possible, and it is fast enough (*see* Note 3). This is the optimal protocol to get one-point covalent immobilization of monomeric enzymes.

3.4 Thermal Inactivation

3.4.1 Steps

1. One wet gram of each immobilized derivative was suspended in 10 mL of phosphate 50 mM at pH 8.0, and 1 mg of a commercial sample of the soluble enzyme was diluted in 10 mL of phosphate 50 mM at pH 8.0.
2. Measure the initial activity of all preparations.
3. Incubate the immobilized derivatives and the soluble enzyme at 50 °C. At different times, aliquots (100 μL) of the suspensions and of the solution were withdrawn and assayed.

3.4.2 Analysis

The time-courses of thermal inactivation of both derivatives and of soluble enzyme were identical (Fig. 5). The one-point covalently attached derivatives exhibit the same activity and stability of soluble enzyme under moderate experimental conditions. They seem to be immobilized "native enzymes," and they are very useful to test the intrinsic 3D stability of native enzyme against any type of denaturing agent under more drastic experimental conditions (temperature, pH, cosolvents, ionic liquids, organic solvents, etc.). By using highly loaded immobilized derivatives of "native enzymes," a number of interesting structural studies could also be performed.

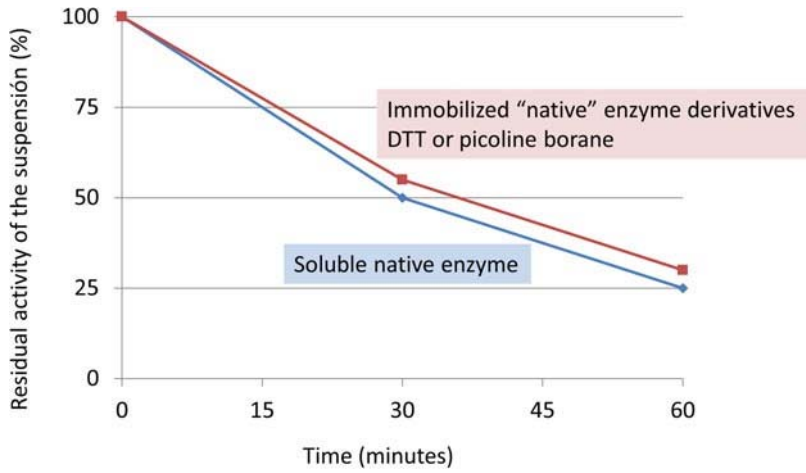

Fig. 5 Time-course of thermal inactivation at pH 8.0 and 50 °C

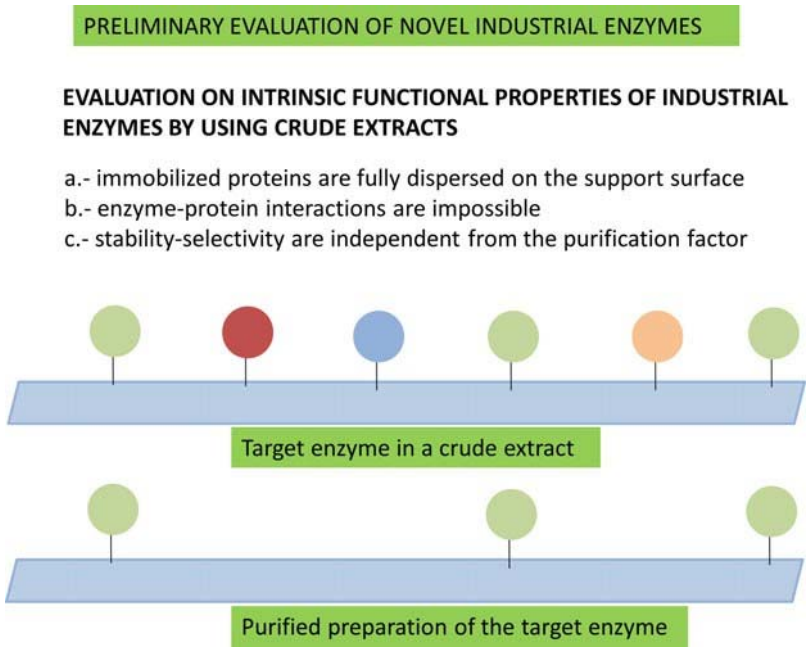

Fig. 6 Immobilization of crude extracts prevents any aggregation process

3.5 Evaluation of Novel Potential Industrial Enzymes by Using Crude Protein Extracts

The one-point covalent immobilization proposed here can also be used to immobilize protein crude extracts (Fig. 6). After this immobilization, all proteins (including the target enzyme) are fully dispersed on the support surface, and they cannot interact ones with others. In addition, they undergo minimal physicochem- ical modifications. In this way, the observed stability and selectivity of the target enzyme exactly represent the intrinsic properties of the native enzyme without the need of a preliminary complex purification.

4 Notes

1. In addition to 2-picoline borane, other mild reducing agents could be used (e.g., cyanoborohydride and triacetoxyborohydride). However, most of them are highly toxic and poorly soluble in water [14]. On the contrary, 2-picoline borane is not toxic, and it can be dissolved up to 20 mM in water.
2. Agarose gels are very resistant to mechanical stirring, but they are easily broken under magnetic stirring. Very gentle sitting of agarose suspensions should be done. Rotating shakers can be very useful.
3. Multimeric enzymes may have two or more amino terminus within of one plane and hence they could be attached to the support, in the absence of stabilizers, via a simultaneous

two-point covalent attachment associated to the stabilization of the quaternary structure [15]. One-point covalent immobilization can be achieved by using DTT and an excess of the soluble enzyme with very short immobilization times (30 min–1 h) at 4 °C in order to prevent the two-point immobilization. Another alternative may be the use of lowly activated supports (e.g., 20 μEqs of aldehyde groups per gram of support).

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