ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 7 (1999) 181–189

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Stabilization of multimeric enzymes via immobilization and post-immobilization techniques

Roberto Fernández-Lafuente [a], Verónica Rodríguez [a], Cesar Mateo [a], Guadalupe Penzol [a], Odette Hernández-Justiz [b], Gabriela Irazoqui [c], Andrea Villarino [c], Karen Ovsejevi [c], Francisco Batista [c], José M. Guisán [a,*]

[a] *Department of Biocatalysis, Instituto de Catálisis, CSIC, Campus Universidad Autónoma, 28049 Madrid, Spain*
[b] *Department of Biotechnology, Technical University of Havana, Havana, Cuba*
[c] *Cátedra de Bioquímica, Universidad de la República, Montevideo, Uruguay*

Abstract

Controlled and directed immobilization plus post-immobilization techniques are proposed to get full stabilization of the quaternary structure of most multimeric industrial enzymes. The sequential utilization of two stabilization approaches is proposed: (a) Multi-subunit immobilization: a very intense multi-subunit covalent immobilization has been achieved by performing very long immobilization processes between multimeric enzymes and porous supports composed by large internal surfaces and covered by a very dense layer of reactive groups secluded from the support surface through very short spacer arms. (b) Additional cross-linking with poly-functional macromolecules: additional chemical modification of multi-subunit immobilized derivatives with polyfunctional macromolecules promotes an additional cross-linking of all subunits of most of multimeric enzymes. A number of homo and hetero-dimeric enzymes has been stabilized by the simple application of multi-subunit immobilization but more complex multimeric enzymes (e.g., tetrameric ones) were only fully stabilized after the sequential application of both strategies. After such stabilization of the quaternary structure these three features were observed: no subunits were desorbed from derivatives after boiling them in SDS, thermal inactivation becomes independent from enzyme concentration and derivatives became much more stable than soluble enzymes as well as than non-stabilized derivatives. For example, thermal stability of D-amino acid oxidase from *Rhodotorula gracilis* was increased 7.000 fold after stabilization of its quaternary structure.

Keywords: Multimeric enzymes; Protein immobilization; Chemical cross-linking of proteins; Dextrans; Stabilization of enzymes

1. Introduction

Controlled and directed immobilization techniques are not only useful to allow the re-use or continuous use of industrial enzymes. In addition to that, immobilization and subsequent post-immobilization techniques can be very useful to greatly increase activity–stability properties of industrial enzymes [1,2]. In this communication, the application of such techniques for the development of a general strategy to stabilize the quaternary structure of multimeric enzymes is discussed.

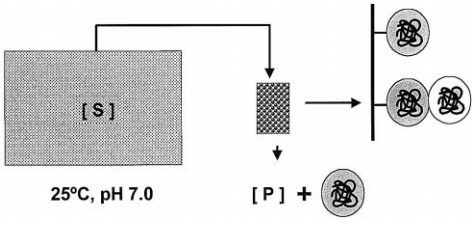
Inactivation of multimeric enzymes may be strongly influenced by the dissociation of subunits. Multipoint non-covalent assemble between monomers could stabilize the structure of

* Corresponding author. Tel.: +34-15-854800; Fax: +34-91-585-47-60; E-mail: jmguisan@icp.csic.es

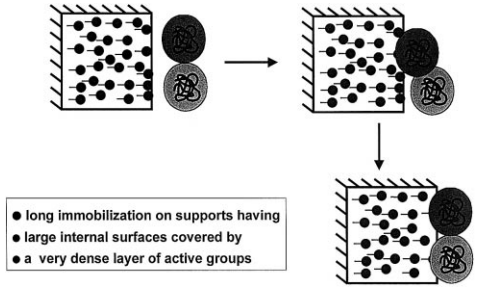
1381-1177/99/$ - see front matter
PII: S1381-1177(99)00028-4

each individual monomer correctly assembled in the multimer. On the contrary conformational changes promoted by any denaturing agent (heat, pH, organic solvents) on the small fraction of dissociated monomers (no stabilized by these interactions) could be much more rapid and intense [3]. Dissociation of subunits may become even more relevant when working at industrial scale under very mild experimental conditions. In general, we will try to use large volume of reagents for many reaction cycles (Scheme 1). The dissociation of a small fraction of subunits per reaction cycle could promote a very rapid deactivation of the enzyme bio-reactor. At first glance, we may assume that stabilization of the quaternary structure of multimeric enzymes may have very profitable effects on their industrial performance.

A very intense chemical cross-linking between subunits should be one of the best approaches to get both the chemical stabilization of the quaternary structure as well as the stabilization of the correct enzyme assembly. However, this is a very difficult practical approach: the reaction of enzymes with cross-linking agents may only promote a slight intense inter-subunits cross-linking plus a high number of undesirable side reactions [4,5]. In this way, we propose the multi-subunit covalent attachment of multimeric enzymes to very rigid supports through very short spacer arms as a way to get the stabilization of assemble and quaternary structure with no side modifications. Of course, the design of such type of multi-subunit immobilization is not a trivial problem and it may require a careful selection of the immobilization



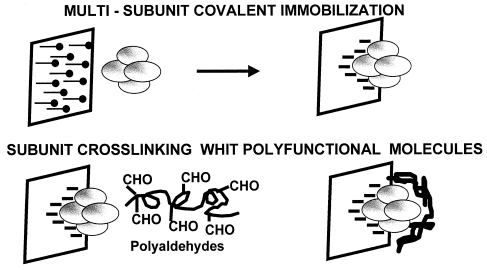
Scheme 1. Multimeric enzymes in industrial reactors.



Scheme 2. Stabilization of multimeric enzymes via multi-subunit immobilization.

systems. At first glance, the performance of long immobilization processes between multimeric enzymes and highly activated supports containing large internal surfaces would seem a likely suitable technique to achieve multi-subunit immobilizations (see Scheme 2) [6].

On the other hand, stabilization of quaternary structure of tetrameric or more complex multimeric enzymes may be much more difficult than the stabilization of dimeric enzymes. For geometrical reasons may be impossible to get all enzyme subunits covalently immobilized on a planar support surface. In this way, we propose a dual strategy to stabilize such complex enzymes. After a very intense multi-subunit covalent immobilization, enzyme derivatives were further modified with polyfunctional macromolecules (see Scheme 3). As compared to bifunctional reagents, polyfunctional macromolecules may exhibit advantages as general reagents for cross-linking of enzyme subunits.



Scheme 3. An integrated approach to the stabilization of multimeric enzymes.

Such cross-linking with macromolecules should be easier to design because now cross-linking may occur between groups placed at very different distances. In addition to that, side-reactions may be very reduced (for example, a minimal chemical modification of immobilized enzymes with poly-aldehydes yields; after mild borohydride reduction; a final hydrophilic and inert poly-alcoholic structure) [7]. Finally, multipoint cross-linking with improvement of the stability of the enzyme assembly could be easily achieved. Useful polyfunctional macromolecules can be obtained from commercial dextran. Dextran can be oxidized by periodate yielding a poly-aldehyde structure suitable to react with amino groups of proteins. The reaction between poly-aldehydes and proteins occurs via the formation of Schiff's bases. The reaction is finalized by borohydride reduction and Schiff's bases are reduced to secondary amino groups and poly-aldehydes are converted in poly-alcohols. On the other hand, poly-aldehydes can be further modified with different reagents [7].

In this paper we discuss the applicability of such sequential combination of multi-subunit immobilization plus cross-linking with polyfunctional macromolecules as a general strategy to stabilize the quaternary structure of most of multimeric enzymes.

2. Materials and methods

2.1. Materials

D-Amino acid oxidases from *Rodotorula gracilis* and from *Trigonopsis variabilis*, glutaryl acylase from *Acinetobacter* sp., alpha-amino acid ester hydrolase from *Acetobacter turbidans*, and crude extracts from *A. turbidans* were kind gifts from Antibioticos. Crude extract from *E. coli* was a gift from Dr. Jose L. Garcia (CIB, CSIC, Spain). Other enzymes, products and substrates were from Sigma.

Toyopearl was from Tosoh (Japan) and Sepabeads was a kind gift from Resindion S.R.L. (Mitsubishi Chemical Corporation) (Milano, Italy).

2.2. Preparation of glyoxyl-Toyopearl

Toyoperal was activated as previously described for agarose [6]. 100 ml of Toyopearl was suspended in distilled water up to a total volume of 120 ml in an ice bath. Then 34 ml of 1.7 N NaOH solution containing 0.95 g of sodium borohydride was added, and this suspension was very gently stirred. After, 6.7 ml of glycidol were added very slowly to prevent an undesired increase of temperature. After 2 h, take the vessel from the ice bath and continue the stirring for 16 h at room temperature. Then, wash the glycidyl-support with an excess of distilled water. Then the glycidyl-support was oxidized at the desired degree with sodium periodate as previously described for agarose [6]. This step is the one that permits the final control of the activation degree.

2.3. Preparation of glyoxyl-Sepabeads

192 g of Sepabeads was suspended in 820 ml of distilled water containing 312 ml of acetone and 370 g of NaOH. The suspension was very gently stirred and 10.8 g of sodium borohydride were added. Then, 132.5 ml of epiclorohydrine was slowly added. After 8 h, 132 ml of acetone and 312.5 ml of epyclorohydrine were added, and again after 24 h. After a total reaction time of 110 h, the support was washed with an excess of distilled water. The glycidyl-Sepabeads support was oxidized with sodium periodate at the desired degree as previously discussed for agarose [6].

2.4. Preparation of amino-supports

The quantitative modification of the aldehyde groups introduced in Toyopearl or Sepabeads by

the previous protocols was performed by using 2 M ethylenediamine pH 10.05 and further reduction with 10 mg/ml of sodium borohydride as previously reported [8].

2.5. Preparation of glutaraldehyde supports

The quantitative modification of the primary amino groups of both supports was performed by adding 10 ml of support to 90 ml of 1 M glutaraldehyde in 0.1 M sodium phosphate pH 7 at room temperature. This suspension was very gently stirred for 12 h, then washed with an excess of distilled water. These supports were used immediately after preparation

2.6. Preparation of aldehyde dextran

A 100 ml solution containing 3.33 g of dextran (MW 6000 Da) in distilled water was prepared. Then, 8 g of solid sodium periodate was added (this permitted the full oxidation of the dextran molecule) and this solution was stirred for 3 h. After, this solution was 4 times dialyzed against 50 volumes of distilled water to eliminate the formaldehyde produced during the oxidation.

2.7. Protein immobilization protocol

The immobilizations were carried out at 25°C, the enzymes or proteins were dissolved in 0.1 M sodium phosphate/250 mM NaCl at a concentration of 2 mg/ml. Immobilizations were performed utilizing 10 ml of the desired glutaraldehyde-support and 90 ml of enzyme or protein solution. Sepabeads or Toyoperal activated with different concentrations of reactive groups (15 or 75 μmol/ml) were used, and the immobilization proceeded for different times (from 2 h to 24 h). As an end point to the immobilization reaction, the enzyme derivatives were reduced by increasing the pH at 8.5 and by adding sodium borohydride (200 mg). In this way, all the enzyme-support bonds were transformed into very stable secondary amine bonds. The immobilized enzyme derivatives were washed with 0.25 M NaCl and distilled water at 4°C.

2.8. Modification of the immobilized enzymes with dextran–aldehyde

10 ml of immobilized protein derivative was suspended in 60 ml of sodium phosphate at pH 7 and 4°C. Then, 30 ml of aldehyde dextran (prepared as previously described) was added to the suspension. This suspension was very gently stirred for 12 h. Then, 900 ml of 0.1 M sodium borate at 4°C and pH 8.5 containing 2 g of sodium borohydride was added to reduce the remaining aldehyde groups as well as the aldehyde–amine bonds. After 1 h, the enzyme derivative was washed 4 times with 3 volumes of 0.1 M phosphate. In this way, all the protein–dextran bonds are transformed in highly stable secondary amine bonds. Similar washes and treatments were performed with immobilized enzyme no modified with aldehyde–dextran to verify that these treatments do not release the subunits of multimeric enzymes.

2.9. Analyses of the stabilization of the quaternary structure of the immobilized proteins by SDS-PAGE

The enzyme derivatives were boiled in the presence of mercaptoethanol and SDS. This treatment releases from the support any protein molecule that was not covalently bond (directly or indirectly) to the support. In this way, by checking the presence or absence of the different bands in the SDS-PAGE, we can verify the stabilization of the quaternary structure of the whole pool of multimeric proteins presented in the different extracts.

3. Results

3.1. Stabilization of the quaternary structure of multimeric proteins via multi-subunit immobilization

3.1.1. Effect of the activation degree

Crude extract from *E. coli* was immobilized on two Sepabeads supports, one with a high degree of activation and the other with a low activation degree. Fig. 1 shows the SDS-PAGE of the supernatants obtained after boiling the immobilized derivatives in the presence of SDS and mercaptoethanol. In these supernatants, all the subunits of the multimeric proteins present in the extract but not covalently immobilized on the support should be presented.

It is remarkable the high number of bands that appeared in the SDS-PAGE of the poorly activated support. Bearing in mind that protein molecules attached to the support even via a single bond can not be desorbed by the treatment, all these bands may be subunits of multimeric proteins that were not covalently attached to the support, although a certain percentage may be proteins just non-covalently associated tightly with other protein or substances able to be covalently immobilized on the support. Thus, a great percentage of proteins in *E. coli* extracts seemed to be multimeric proteins, and many of them were not stabilized by immobilization on poorly activated supports. The high number of desorbed proteins gave an idea of the importance of the problem of the stabilization of multimeric enzymes. Also, this result suggests that poorly activated supports can not be used to stabilize multimeric enzymes via multi-subunit immobilization.

However, when highly activated Sepabeads was used as immobilization support, only a few proteins were released after boiling the support in the presence of SDS. This suggested that the quaternary structure of a great percentage of the pool of multimeric enzymes presented in the

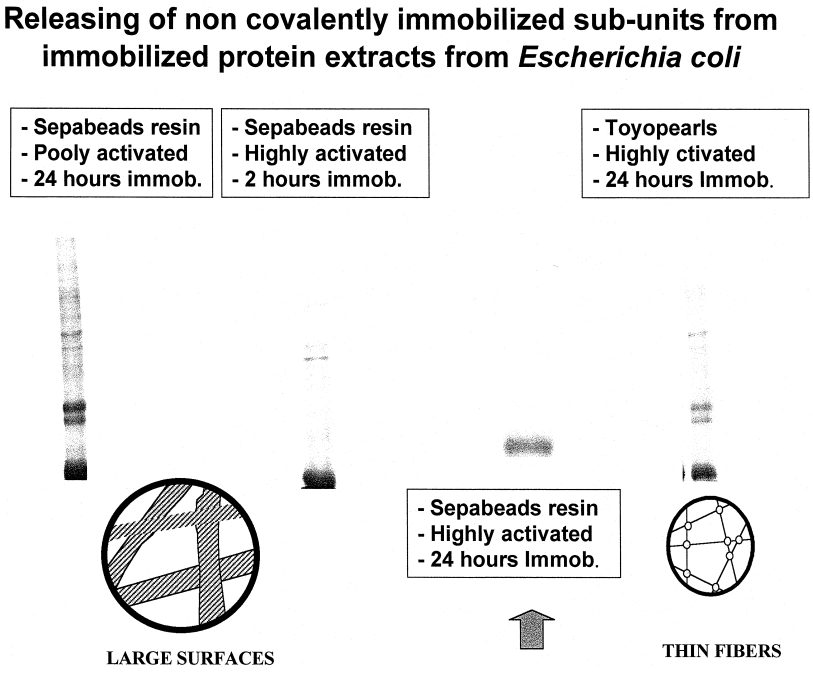


Fig. 1. SDS-PAGE analyses of the proteins released from different derivatives. After immobilization for different times, derivatives were reduced with sodium borohydride. Then, enzyme derivatives were boiled in the presence of SDS as described in Section 2.

extract were stabilized via multi-subunit immobilization when using highly activated supports. This result is more significant if considering that the type of multimeric enzymes may be very wide (from dimeric to much more complex multimeric enzymes) and that using crude preparations it is not possible to discard the presence of other types of non-covalently bind proteins.

3.1.2. Effect of the internal morphology of the support

We have immobilized *E. coli* extracts on Toyopearl (support formed by very thin fibers) and Sepabeads, (support having large internal surfaces) highly activated. Sepabeads derivatives release only some protein subunits after the desorption treatment (Fig. 1), suggesting that supports having large internal surfaces were suitable to stabilize a great number of multimeric enzymes. However, Toyopearl derivatives release many proteins to the supernatant after boiling in the presence of SDS (Fig. 1). This suggested that supports composed by thin fibers (compared to the protein size) may be not suitable for multi-subunit immobilization of multimeric proteins.

3.1.3. Effect of the immobilization time

E. coli extracts were immobilized on highly activated Sepabeads. After 2 h, most proteins had been immobilized on the support. A portion of the enzyme derivative was reduced at that moment. After boiling in the presence of SDS, SDS-PAGE (Fig. 2) shows that many protein subunits were released to the medium. However, if the reaction between the enzyme and the support was let to continue for 24 h, the number of subunits released to the medium after desorption treatment were strongly reduced. This result suggests that, while the immobilization of a monomeric or multimeric protein (via a single point of a single protein subunit) is a very rapid process, the establishment of new bonds be-

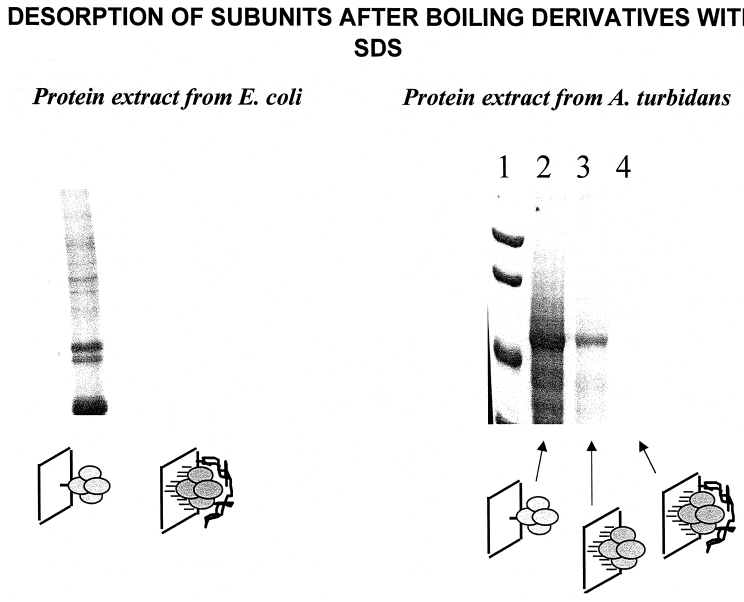


Fig. 2. Stabilization of quaternary structures of multimeric proteins via immobilization and post-immobilization techniques. Derivatives were prepared as described in Section 2. Figure shows the SDS-PAGE analyses of the supernatants obtained after boiling different derivatives in the presence of SDS as described in Section 2. On the left it is shown the protein desorbed after immobilizing an *E. coli* extract under very mild conditions or after multi-subunit immobilization plus chemical cross-linking. On the right, it is shown the results obtained with the extract from *A. turbidans*, lane 1 are the weight markers, lane 2 the limited linked derivative, lane 3 the multi-subunit immobilized derivative and lane 4 the multi-subunit immobilized plus cross-linked derivative.

tween two complex structures such as a protein and a support surface require longer reaction times, very likely because of the difficulties in the correct alignment between the enzyme and the support groups. Similar results were obtained for the stabilization of monomeric proteins via multipoint covalent attachment to activated supports [9].

3.1.4. Discussion

From the results previously shown, it is evident that the stabilization of the quaternary structures of proteins requires long immobilization times on supports having large internal surfaces and a high activation level. On these supports, most multimeric enzymes bond all of the subunits to the support. In a crude extract of *E. coli*, a high percentage of proteins having very complex quaternary structure (tetrahedrical enzymes, hexameric proteins, etc) may be present,. Some of these proteins, by geometrical reasons, can not be attached via all of their subunit on a planar surface. However, (Fig. 1), even thus, most of the quaternary structure of proteins were stabilized by using the optimal immobilization system. Therefore, we can expect that this system may allow the stabilization of most of the most simple multimeric enzymes: the dimeric ones. Table 1 shows that, in fact, many enzymes composed by two subunits may have their quaternary structure directly stabilized via multi-subunit immobilization under suitable supports and conditions. In all cases, immobilization yields were 100%, activities losses during optimized immobilization protocols were under 20%. Optimization of the different systems will be subject of forthcoming papers.

The stabilization of the most complex multimeric enzymes may require the use of post-immobilization techniques, such as the cross-linking with poly functional macromolecules (e.g., aldehyde– dextrans).

3.1.5. Stabilization of the quaternary structure of immobilized multimeric proteins via multi-subunit cross-linking with dextran–aldehyde

Immobilization of crude extracts from *E. coli* and *A. turbidans* under optimal conditions is not enough to fully stabilize the quaternary structure of all multimeric enzymes (or adsorbed proteins to immobilized molecules) that are presented in the extracts, as it is showed by the SDS-PAGE of the supernatants obtained by boiling the derivatives in the presence of SDS and mercaptoethanol. However, after cross-linking of the immobilized proteins with aldehyde–dextran, it is not possible to detect any protein desorbed from the support after boiling in the presence of SDS. A protein derivative that was treated in the same way but where inert dextrans were used instead of aldehyde–dextran kept the same SDS-PAGE pattern as before the treatment. This showed that the subunits were not desorbed during the different steps of the treatment, but the chemical cross-linking had been able to bond the free subunits to some units previously immobilized on the support.

Table 1
Dimeric enzymes stabilized directly via multi-subunit immobilization

Enzyme	Source	Support	Activation
Penicillin G Acylase	*E. coli*, [12]	Agarose, Sepabeads	Glyoxyl, glutaraldehyde
Penicillin G acylase	*K. citrophila*, [13]	Agarose, Sepabeads	Glyoxyl, glutaraldehyde
D-amino acid oxidase	*R. gracilis*, [14]	Agarose, Sepabeads	Glyoxyl glutaraldehyde
D-amino acid oxidase	*T. variabilis*, [14]	Agarose, Sepabeads	Glyoxyl, glutaraldehyde
β-galactosidase	*K. lactis*, [14]	Agarose	Glyoxyl
Glutaryl acylase	*Acinetobacter sp.*, [14]	Agarose	Glutaraldehyde

Stabilization of the quaternary structure was checked by SDS-PAGE.

Table 2
Some tetrameric enzymes stabilized by multisubunit immobilization plus chemical cross-linking with poly-aldehyde dextran

Enzyme	Source	Support	Activation
alpha-amino acid ester hydrolase	*A. turbidans*, [11]	Agarose	Glutaraldehyde
β-galoctosidase	*E. coli*, [14]	Agarose	Glutaraldehyde

This result suggests that the integration of immobilization and post-immobilization strategies has been enough to fully stabilize the quaternary structure of most multimeric enzymes present in the crude extracts of both microorganisms, that is, even the most complex ones. Also, any protein that is indirectly immobilized via any type of non-covalently interaction with other proteins or substances (e.g., carbohydrates) has been finally indirectly or directly attached to the support.

In fact, this strategy has been utilized for stabilizing some tetrameric enzymes (Table 2) whose quaternary structure could not be stabilized by 'mere' multi-subunit immobilization but where the further modification with aldehyde–dextrans yielded fully stabilized multimeric enzymes. In the case of the amino acid ester hydrolase from *A. turbidans*, the immobilization yield was 100% and the whole treatment left more than 85% of the initial activity

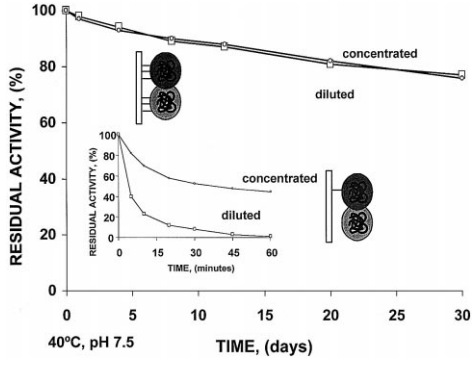


Fig. 3. Time course of thermal inactivation of D-amino acid oxidase from *R. gracilis* derivatives. In the main figure it is shown the multi-subunit immobilized derivative inactivation at 0.05 and 0.005 mg/ml suspension. The inserted figure shows the inactivation of one-subunit immobilized derivative, at the same protein concentrations as indicated above.

of the enzyme. Optimization of the different derivatives will be subject of a forthcoming paper.

3.1.6. Effect of the stabilization of the quaternary structure on enzyme thermal stability

The prevention of enzyme dissociation permitted to greatly improve the enzyme stability when the volume of enzyme derivative is very small related to the volume of the reactor. Fig. 3 shows the dramatic stabilization obtained by stabilizing the quaternary structure of D-amino acid oxidase from *R. gracilis*. Now, the enzyme stability is fully independent from the enzyme concentration. Thus, the stabilization reached a 7000-fold factor (compared to one subunit immobilized enzymes). Thus, this enzyme derivative could be utilized in the production of alpha-keto acids under very diluted conditions with high stability [10].

The stabilization of the quaternary structure of multimeric enzymes allowed the use of these enzymes under conditions where the non-stabilized enzyme is rapidly inactivated by dissociation of subunits. For example, alpha-amino acid ester hydrolase from *A. turbidans* has been described as a very suitable catalyst in the synthesis of ampicillin, but the results were only relevant in absence of phosphate ions [11]. This ion was very related to the maintenance of the quaternary structure, therefore only multi-subunits attached plus cross-linked derivatives could be used [11].

4. Conclusions

The stabilization of multimeric proteins is relatively simple using immobilization and

post-immobilization techniques. Multimeric enzymes with the quaternary structure stabilized under the following conditions.

(1) Do not desorb any protein molecule to the reaction medium, even after conditions so drastic as boiling in the presence of detergents. This prevents the contamination of the products by the proteins and the continuous losses of protein in the product solution.

(2) The enzyme stability does not depend on the dilution of the enzyme (a direct consequence of the no desorption of enzyme molecules), in this way, these enzyme derivatives may be used even in continuous reactors.

(3) Their operational stability is greatly improved, enabling the use of multimeric enzymes in many reaction of high relevance.

Almost fully stabilization of crude extracts shows the power of this technique, because these extracts may contain very complex multimeric enzymes and even some non-covalently associated protein complex present in the extracts have been finally directly or indirectly attached to the support.

Acknowledgements

This work has been partially supported by grants from Resindion S.R.L. (Mitsubishi Chemical Corporation), from Comunidad Autonoma de Madrid and from Agencia Española de Cooperacion Internacional. We are very grateful to V. Cuomo, Miyata and M. Daminati for suggestions and support. We are also very grateful to Antibioticos for supply of enzymes and substrates.

References

[1] G.F. Bickerstaff (Ed.), Immobilization of Enzymes and Cells, Humana Press, Totowa, NJ, 1997.
[2] J.M. Guisan, R.M. Blanco, R. Fernandez-Lafuente, C.M. Rosell, G. Alvaro, A. Bastida, in: V.J.J. van der Tweel, A. Harder, R.M. Buittelar (Eds.), Stability and Stabilization of Enzymes, Elsevier, Amsterdam, 1993, pp. 55–62.
[3] V.P. Torchilin, V.S. Trubetskoy, V.G. Omelýananko, K.J. Martinek, Mol. Catal. 19 (1983) 291–301.
[4] R. Fernnandez-Lafuente, C.M. Rosell, V. Rodriguez, J.M. Guisan, Enzyme Microb. Technol. 17 (1995) 517–523.
[5] R. Fernandez-Lafuente, C.M. Rosell, G. Alvaro, J.M. Guisan, Enzyme Microb. Technol. 14 (1992) 489–495.
[6] J.M. Guisan, Enzyme Microb. Technol. 10 (1988) 375–382.
[7] J.M. Guisan, G. Penzol, P. Armisen, A. Bastida, R. Blanco, R. Fernandez-Lafuente, E. Garcia-Junceda, in: G.F. Bickerstaff (Ed.), Methods in Biotechnology 1, Immobilization of Enzymes and cells, Humana Press, Totowa, NJ, 1997, pp. 261–276.
[8] R. Fernandez-Lafuente, C.M. Rosell, V. Rodriguez, M.C. Santana, G. Soler, A. Bastida, J.M. Guisan, Enzyme Microb. Technol. 15 (1993) 546–550.
[9] J.M. Guisan, A. Bastida, C. Cuesta, R. Fernandez-Lafuente, C.M. Rosell, Biotechnol. Bioeng. 38 (1991) 1144–1152.
[10] R. Fernandez-Lafuente, V. Rodriguez, J.M. Guisan, Enzyme Microb. Technol. 23 (1998) 28–33.
[11] R. Fernandez-Lafuente, O. Hernandez-Justiz, M. Terreni, J.M. Guisan, Biotechnol. Bioeng., submitted.
[12] G. Alvaro, R. Fernandez-Lafuente, R.M. Blanco, J.M. Guisan, Appl. Biochem. Biotechnol. 26 (1990) 181–195.
[13] J.M. Guisan, G. Alvaro, R. Fernandez-Lafuente, C.M. Rosell, J.L. Garcia, A. Tagliani, Biotechnol. Bioeng. (1993) 455–464.
[14] R. Fernandez-Lafuente, V. Rodríguez, C. Mateo, G. Penzol, O. Hernandez-Justiz, G. Irazoqui, A. Villarino, K. Ovsejevi, F. Batista, J.M. Guisán, Manuscript in preparation.