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Multifunctional Epoxy Supports: A New Tool To Improve the Covalent Immobilization of Proteins. The Promotion of Physical Adsorptions of Proteins on the Supports before Their Covalent Linkage

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Multifunctional supports containing epoxy groups are here proposed as a second generation of activated supports for covalent immobilization of enzymes following the epoxy chemistry on any type of support (hydrophobic or hydrophilic ones) under very mild experimental conditions (e.g., low ionic strength, neutral pH values, and low temperatures). These multifunctional supports have been easily prepared by modifying a small fraction (10−20%) of the epoxy groups contained in commercial epoxy supports. In this way, additional groups that were able to physically adsorb proteins (e.g., cationic or anionic groups, metal chelate, phenyl boronate) are generated on the support surface. The covalent immobilization of proteins on these supports proceeds via their initial physical adsorption on the supports (via different structural features). Then, "intramolecular" covalent linkages between some nucleophilic groups of the adsorbed enzyme (e.g., amino, thiol, or hydroxy groups) and the dense layer of nearby epoxy groups on the support are established. This two-step covalent immobilization dramatically improves the very low reactivity of epoxy groups toward nonadsorbed proteins. In this way, all other relevant practical advantages of epoxy groups for protein immobilization (their high stability and their ability to form very strong linkages with several nucleophilic enzyme residues with minimal chemical modification) can be an object of universal exploitation. The use of these new multifunctional supports exhibits important advantages regarding immobilization of enzymes previously adsorbed on hydrophobic homofunctional epoxy supports: (i) hydrophilic supports can also be used for immobilization of industrial enzymes; (ii) immobilization can also be carried out at low ionic strength; (iii) every protein contained in crude extracts from *Escherichia coli* and *Acetobacter turbidans* can be immobilized by sequentially using a set of different supports; (iv) in most cases, each enzyme has been immobilized on different supports, orientated through different structural features and very likely involving different areas of its surface. For example, three industrial enzymes (penicillin G acylase, lipase, and β-galactosidase) could be immobilized through different strategies yielding immobilized derivatives with very different activities. The best derivatives preserved 75−100% of activity corresponding to the soluble enzymes used for immobilization, while in some cases a particular immobilization protocol promoted the full inactivation of the enzyme.

Introduction

Epoxy Supports for Covalent Immobilization of Proteins. Many protocols for covalent immobilization of enzymes have been already reported. Many of them are very useful, at least to immobilize small amounts of proteins per milliliter of support, when performed at the laboratory by researchers with some expertise in both support activation and protein immobilization methods.[1−9] In fact, enzyme immobilization is considered as a very well developed technique. However, most of these protocols for covalent protein immobilization may have some drawbacks when trying to quantitatively immobilize, under mild experimental conditions, large amounts of enzyme per milliliter of support with a long-term handling of the activated supports, e.g., when immobilization is carried out at an industrial level. Thus, there are no methodologies that may be applied to immobilize large amounts of any enzyme in any solid under mild experimental conditions.[1−9] Having in mind these considerations, epoxy-activated supports seem to be almost ideal systems to develop very easy protocols for enzyme immobilization. Epoxy groups are very stable at neutral pH values even in wet conditions, and hence commercial supports can be stored for long periods of time and they can be prepared quite far from the place where the enzyme has to be immobilized. Moreover, these epoxy supports may be utilized to stabilize enzyme through multipoint covalent attachment by controlling the enzyme−support interactions.[10]

Moreover, while other popular immobilization protocols may promote great alterations in the protein surface, yielding

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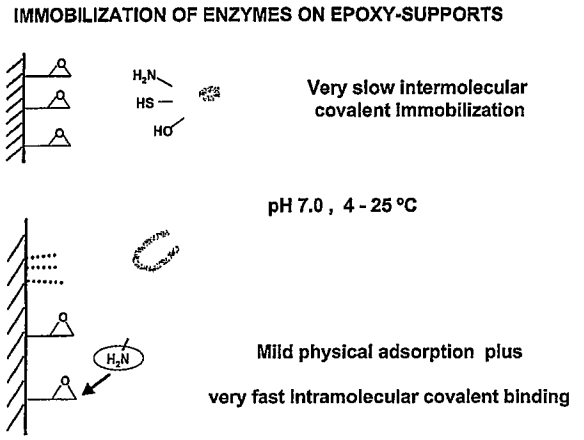

Figure 1. Mechanism of immobilization of proteins on epoxy supports. The covalent reaction between soluble enzyme and epoxy support is extremely slow. However, the covalent reaction between previously adsorbed proteins and the support proceeds at very high apparent concentrations of reactive groups on the support and on the protein surface (an "intramolecular" reaction) giving a high covalent immobilization rate.

labile enzyme–support attachments (e.g., when using BrCN-activated supports),[11–12] epoxy supports are able to react with different nucleophilic groups on the protein surface (e.g., amino, hydroxy, or thiol moieties) to form extremely strong linkages (secondary amino bonds, ether bonds, thioether bonds) with minimal chemical modification of the protein (e.g., p*K* values of the new secondary amino groups are very similar to those of the pre-existing primary amino ones).

At the end of the immobilization process, epoxy groups can be easily blocked by reaction with very different thiol or amine compounds under mild conditions,[13] preventing further uncontrolled reaction between the support and the enzyme that could decrease its stability.

Enzyme Immobilization on Hydrophobic Homofunctional Epoxy Supports. Successful immobilizations of enzymes at neutral pH values have been achieved by using fairly hydrophobic epoxy supports (e.g., epoxy–acrylic resins) in the presence of very high concentrations of salt. Such immobilizations have been recently reviewed, and a very interesting mechanism for such covalent immobilizations has been proposed.[14–17] Enzymes are first hydrophobically adsorbed on the epoxy supports, and then a covalent attachment between nucleophile groups of the enzyme (amino, thiol, or hydroxy groups) and a high density of nearby epoxy groups in the same support is strongly favored (Figure 1). In fact, epoxy groups are hardly reactive for enzyme immobilization under mild experimental conditions (neutral pH, low ionic strength).[18–20] There is a clear relationship between reactivity of activated support toward nucleophilic compounds and the stability of these supports in water (water is also able to act as a weak nucleophile). From, this point of view, the high stability of epoxy groups can be logically related to their low reactivity with proteins.

Multifunctional Epoxy Supports for Protein Immobilization. Having in mind this two-step mechanism for covalent immobilization of proteins on epoxy supports, multifunctional supports are here proposed as a second generation of activated supports that are able to covalently immobilize enzymes, antibodies, and other proteins under very mild experimental conditions. In general, these multifunctional supports should contain two types of functional groups: (i) groups that were able to promote the physical adsorption of proteins (e.g., by ionic exchange, by metal–chelate adsorption); (ii) groups that were able to covalently immobilize the enzyme (e.g., epoxy groups).

In this way, enzymes could be first adsorbed on the support and then some covalent linkages between nucleophilic groups of the protein (amino, thio, hydroxyl ones) and a dense layer of epoxy groups on the support could take place. Obviously this "intramolecular" covalent immobilization could be much more rapid than the corresponding "intermolecular" covalent immobilization between nonadsorbed proteins and monofunctional epoxy-activated supports (Figure 1). This two-step mechanism of immobilization allows the use of epoxy groups (or similar ones) having a very high stability and the formation of very stable covalent linkages with the enzyme but also having a very low reactivity toward soluble nucleophiles. The use of these multifunctional supports and these immobilization protocols may have very important advantages for the preparation of biocatalysts (e.g., enzymes) and bioadsorbents (antibodies, protein A, etc.). For example, because of the high stability of the groups involved in the physical adsorption and the high stability of the groups involved in the covalent immobilization, these very stable multifunctional supports could become commercially available and they would be directly used for long immobilization of large amounts of enzymes and proteins on large amounts of activated supports, even at an industrial scale, without any kind of additional activation procedure.

In this paper, some limitations of covalent immobilization of proteins on different commercial epoxy supports under different experimental conditions were first evaluated. Then, the preparation of different multifunctional epoxy supports was also evaluated: amino–epoxy supports, carboxyl–epoxy supports, copper chelate–epoxy supports, and boronate–epoxy supports. In each case the optimal combination of the concentration of both functional groups was previously studied. To evaluate the overall limitations and possibilities of the different supports, a crude extract of proteins from *E.coli* and *A. turbindans* was used as "substrate" for covalent immobilization. Finally, some industrial enzymes were also immobilized on different multifunctional supports.

Materials and Methods

Materials. Epoxy-Sepabeads (EP-HG-15) supports were kindly donated by Resindion S.R.L. (Mitsubishi Chemical Corp.). Penicillin G acylase from *Escherichia coli*, crude protein extracts from *Escherichia coli* and *Acetobacter turbidans,* and penicillin G were kind gifts from Antibioticos S.A. Iminodiacetic acid, ethylenediamine, *m*-aminophenylboronic acid, *p*-nitro-phenylpropionate, *o*-nitro-phenyl-β-D-galactopyranoside (oNPG), β-galactosidase from *Aspergilus oryzae* (Grade XI) and lipase from *Candida rugosa* (type VII) were purposed from Sigma Chemical Co. Eupergit C was obtained from Rohm Pharma. Epoxy-agarose gels were a generous gift from Hispanagar S.A. (Burgos, Spain) and

they were prepared as previously described.[21] Other reagents were of analytical degree.

Methods. (a) Partial Modification of Eupergit C Supports. The epoxy groups of Eupergit C were partially modified with different moieties. Optimization of the supports was carried out by studying the immobilization rate of some proteins (that is, the rate of adsorption of the proteins) and the covalent immobilization rate of those enzymes (that is, the fraction of enzymes that remained immobilized in the support when incubated under desorption conditions (see below)).

(1) Aminated Support. A 10-g portion of Eupergit C was incubated in 60 mL of 2% v/v ethylenediamine at pH 8.5 for different times (from 15 min to 24 h) under very gently stirring. Then, the support was washed with an excess of distilled water and stored at 4 °C. Modification degree was quantified by titration of the amino groups introduced in the support.[22]

(2) Iminodiacetic Acid (IDA) Supports. A 10-g portion of Eupergit C was incubated in 18 mL of 0.1 M sodium borate/2 M iminodiacetic acid pH 9 at 25 °C under very gently stirring.[21] At different times (from 15 min to 24 h), the support was washed with an excess of distilled water and stored at 4 °C.

(3) Copper−IDA Supports (Cu Supports). A 10-g portion of IDA-Eupergit C was incubated in 60 mL of distilled water containing 2 g of $CuSO_4$ under very gently stirring. After 2 h, the support was washed with an excess of distilled water. This treatment should modify 100% of the IDA groups in the support. After the Cu was released from the support by treatment with EDTA, the quantification of the copper atoms by atomic absorption spectroscopy was utilized to quantify the degree of modification of the epoxy groups with the IDA groups.

(4) Boronate Supports. A 10-g portion of Eupergit C was incubated in 33 mL of 5% w/v *m*-aminophenylboronic acid in 20% dioxane at pH 8 and 25 °C.[23] At different times (from 30 min to 24 h) samples were washed with 0.1 M borate pH 6 and water and stored at 4 °C. Quantification of the modification degree was performed by elemental analysis detecting the atoms of boron.

(b) Immobilization of Proteins and Enzymes on Epoxy Supports. A 5-g portion of support was suspended in 45 mL of solutions of proteins or enzymes (maximum protein concentration was 1 mg/mL) in sodium phosphate pH 7, using different concentrations of buffer (from 5 mM to 1 M) at 20 °C. Periodically, samples of the supernatants were withdrawn and analyzed for protein concentration (Bradford's method[24] and densidometry analyses of SDS-PAGE) or/and enzyme activity. In some cases the immobilized enzyme was incubated under conditions where the physically adsorbed protein molecules were released, to check that the immobilization was actually covalent. After immobilization, derivatives were washed under those conditions to eliminate any protein molecule noncovalently attached to the support.

(c) End Point to the Support−Enzyme Reaction. To completely block the epoxy groups, 5 g of the support or enzyme−support derivative was incubated in 25 mL of 50 mM phosphate pH 7.75 containing 5% mercaptoethanol for 16 h at 20 °C.[13] Then, the derivatives were washed with an excess of distilled water. Mercaptoethanol was found to be able to release all Cu^{2+} from the Cu supports.

(d) Desorption of the Proteins Adsorbed but Not Covalent Attached on the Support. To test the covalent attachment of the proteins, the conditions of desorption of the proteins adsorbed on the different supports with the epoxy groups completely modified with the different reagents that promoted the physical adsorption were studied. Under these conditions the different supports were incubated to ensure that the protein molecules that remained bound to the support were really covalently attached. These conditions were as followed:

Eupergit C (blocked with mercaptoethanol). Most of 95% of the proteins adsorbed in this support resulted released to the medium by incubation in 25 mM phosphate, pH 7.

Fully modified aminated-Eupergit C released more than 95% of the adsorbed proteins by incubation in 500 mM sodium chloride, pH 7.[25]

Fully modified IDA-Eupergit C released more than 95% of the adsorbed protein by incubation in sodium chloride, 500 mM, pH 7.

Fully modified Cu−Eupergit C released more than 95% of the adsorbed protein by incubation in 100 mM imidazole.[21]

Fully modified phenylboronate supports released more than 95% of the adsorbed protein by incubation in 500 mM manitol.[23]

(e) Determination of the activity of the enzymes. (1) Penicillin G acylase from *E. coli*. Activity was measured by titration of the phenyl acetic acid released during the hydrolysis of 30 mM penicillin G at pH 8 and 25 °C using a pHstat.

(2) Lipase from *C. rugosa*. Activity was determined by the increase in the absorbance at 348 nm promoted by the hydrolysis of 0.4 mM *p*-nitrophenyl propionate in 25 mM sodium phosphate pH 7 and 25 °C.[26]

(3) *β*-Galactosidase from *A. oryzae*: Activity was assayed by controlling the increase in the absorbance at 405 nm caused by the hydrolysis of 10 mM *o*NPG in 0.1 M sodium acetate pH 4.5 at 25 °C.

Results

Immobilization of Proteins on Commercial Epoxy Supports. A crude protein preparation was incubated in the presence of epoxy−agarose at high or low ionic strength. Negligible immobilization of the protein was found in both conditions (Figure 2A). This result points out that the previous hydrophobic adsorption of proteins on the supports at high ionic strength (described as necessary for the protein immobilization on these supports[16,17]) is a consequence of the hydrophobicity of the core of the supports and not of the presence of the epoxy groups covering the support surface. This result also suggests some limitations to the currently employed methodology for immobilization on epoxy supports: only fairly hydrophobic supports may be utilized to immobilize proteins via the classic epoxy chemistry.

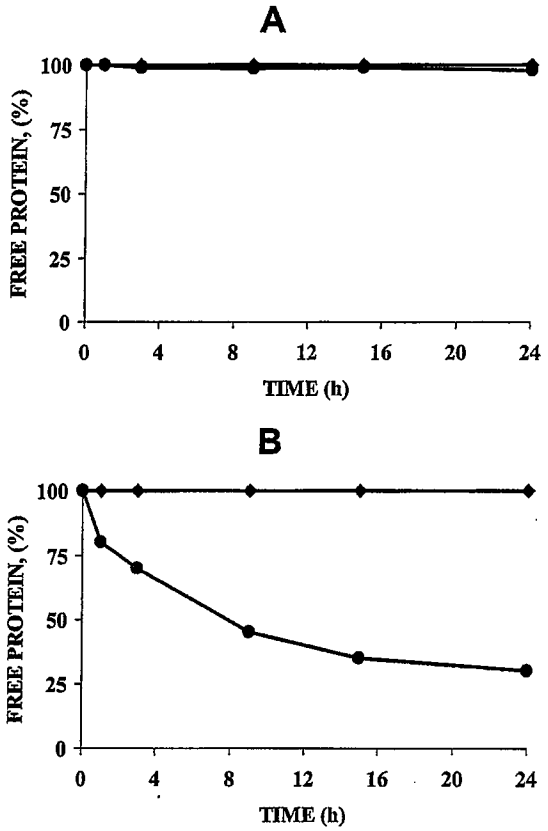

Figure 2. Immobilization of crude protein extracts from *E. coli* on epoxy–agarose (A) and Eupergit C (B) at different ionic strengths: rhombus, supernatant of the immobilization suspension in 5 mM sodium phosphate; circles, supernatant of the immobilization suspension in 1 M sodium phosphate. Immobilizations were carried out at pH 7 and 20 °C as described in Methods. Determination of the protein in the supernatant was performed by Bradford's method.

On the other hand, the incubation of crude protein extracts from different microorganisms in the presence of epoxy-SEPABEADS or Eupergit C at high ionic strength promoted the covalent immobilization of around 70% of the proteins contained in different crude extracts on these supports after only 15–20 h (Figure 2B). Moreover, immobilization of the enzymes at low ionic strength was negligible even using these supports (Figure 2B). This result suggests a second limitation of the current methods of immobilization on epoxy supports: it is necessary to use a high ionic strength (and some proteins cannot be exposed to these conditions), and not all proteins seem to be immobilized even under those conditions.

Therefore, the preparation of new multifunctional supports was intended in order to promote other types of physical adsorption of the proteins on the support prior to the covalent immobilization (e.g., ionic). At first glance, it seems that this modification must keep a compromise solution, on one hand it must be high enough to promote the physical adsorption of the proteins, while on the other hand it must leave a high enough amount of reactive epoxy groups in order to enable the covalent immobilization.

Optimization of the Partial Chemical Modification of the Epoxy Groups of the Supports. The modifications of the epoxy supports with ethylenediamine, iminodiacetic acid, or *m*-aminophenylboronic acid (Figure 3) were used to give

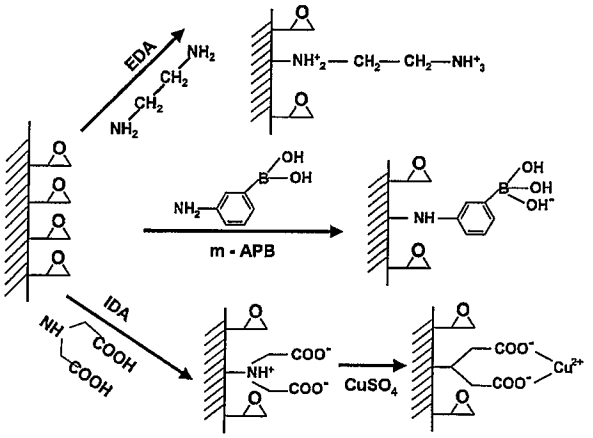

Figure 3. Different multifunctional epoxy supports utilized in this work.

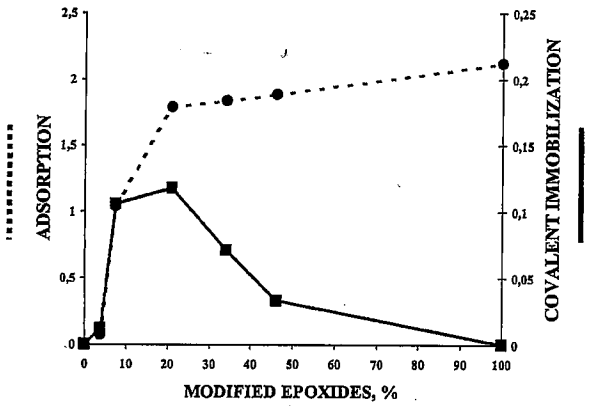

Figure 4. Optimization of the modification with ethylenediamine of the epoxy groups of Eupergit C for the covalent immobilization of proteins. The figure represents the immobilization of β-galactosidase from *Aspergillus oryzae* at pH 7 and 5 mM sodium phosphate. Experiments were carried out as described in Methods. The dotted line curve represents the rate of adsorption of the enzyme to the support. The solid line curve shows the rate of covalent immobilization of the support. That was determined by adding 0.5 M of NaCl to a sample of the immobilization suspension. This treatment releases no covalently bound enzyme molecule from the support. Later, the activity of the supernatant was assayed.

bifunctional supports, with moieties that were able to physically adsorb the proteins and groups that enable covalent immobilization of the protein (the remaining epoxy groups). Figure 4 shows the effect of the modification degree of the epoxy groups of Eupergit C on the rate of protein adsorption and covalent immobilization of a model enzyme, β-galactosidase from *A. oryzae*, on amino-epoxy-Eupergit. The initial rate of adsorption of the protein on the support continuously increased when increasing the amount of amino groups in the support. However, the initial rate of covalent immobilization only increased until 10–20% modification of the epoxy groups (where the rate of physical adsorption is already quite high) and then started to decrease. This shows that the covalent immobilization of the proteins on epoxy supports needs a minimal modification of the epoxy support with groups that were able to adsorb the proteins, and then the rate of covalent immobilization decreases because of the decrement of the reactive epoxy groups in the support. In these first stages of the protein immobilization, the supports incubated in 500 mM sodium chloride released a significant

Table 1. Immobilization of a Crude Protein Preparation from *E. coli* on Differently Modified Eupergit C[a]

	support				
	Eupergit C	Cu–Eupergit C	boronate-Eupergit C	IDA-Eupergit C	amine-Eupergit C
immobilized protein	70%	>85%	75%	<10%	65%

[a] Crude protein extract from *E. coli* was incubated in the presence of the different supports at pH 7 for 24 h. When using nonmodified Eupergit C, 1 M sodium phosphate was used. In the other cases, 5 mM of sodium phosphate was utilized as buffer. Percentage of immobilized protein was quantified by Bradford's method.

percentage of the immobilized protein, showing that a certain percentage of the protein was just physically adsorbed on the support. After 24 h, most proteins remained immobilized in the optimal support even after incubation at high ionic strength. These results showed that the covalent immobilization was significantly slower than the adsorption rate.

Similar results were obtained when using the other supports described in this paper (Figure 3), obtaining in all cases the maximum rate of protein covalent immobilization at a degree of substitution of 10–20% of the epoxy groups.

Immobilization of Proteins on Multifunctional Epoxy Supports. Table 1 shows the percentage of proteins (from a crude protein extract from *E. coli*) immobilized on the different supports. The commercial support and those having amines or phenyl boronic acid are able to immobilize around 70% of the proteins, while the Cu-IDA-Eupergit C was able to immobilize more than 85% of the proteins. Less than 10% of the proteins could be immobilized on IDA supports under the immobilization conditions (around 80–85% of the proteins have negative charge at pH 7[25]). Of course, immobilization at lower pH values enables the immobilization of more proteins on this support (results not shown). Similar results were obtained when using crude protein extracts from *A. turbidans*.

In all cases, after 24 h of enzyme-support interaction, most proteins remained bound to the support even when incubated under conditions where all the proteins physically adsorbed were released to the supernatant. This means that the proteins were covalently attached to the support.

These results implied that most proteins could be immobilized on several of the epoxy-modified supports; therefore the support could be chosen bearing in mind the stability of the target enzyme and the activity of the final derivative.

When these new multifunctional supports were used, the physical adsorption of the protein was also the first step of the immobilization. For example, IDA-Eupergit (without Cu adsorbed on the support) was not able to immobilize penicillin G acylase at pH 7 (Figure 5A). The same support, but with copper adsorbed on the IDA groups, was also unable to immobilize the enzyme in the presence of imidazole. However, the immobilization of the enzyme was quantitative when the enzyme was offered to the Cu support at pH 7 and in the absence of imidazole (Figure 5A).

Similarly, when the other supports were used under conditions where the proteins were not adsorbed on the supports (see methods), protein immobilization on the epoxy supports was always found to be negligible.

Immobilization of Enzymes on Multifunctional Hydrophilic Epoxy Supports. Bearing in mind that now the first adsorption of the protein on the support may be not

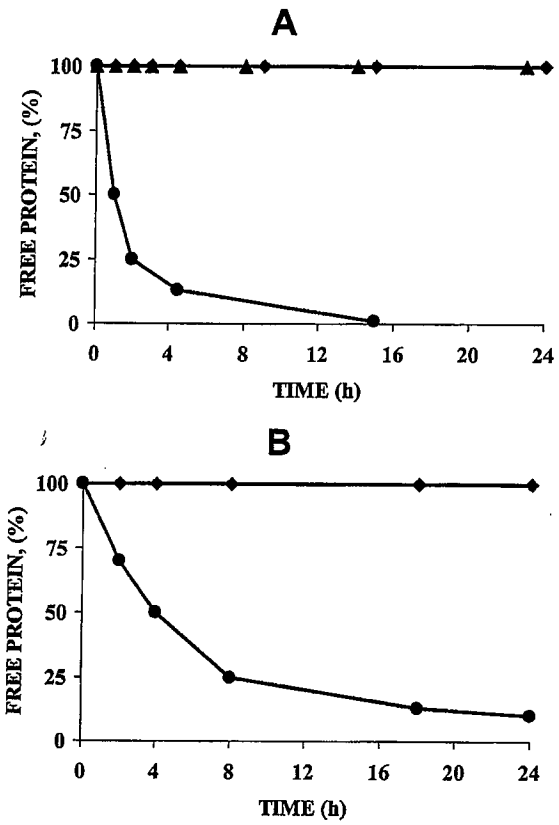

Figure 5. Immobilization of penicillin G acylase from *E. coli* on Cu-IDA-Eupergit C (A) and Cu-IDA-epoxy-agarose (B) under different conditions: triangles, IDA support (without Cu) was incubated with the enzyme; rhombus, IDA–Cu support was incubated with the enzyme (Figure 3) and incubated with the enzyme in the presence of 50 mM imidazole; circles, the enzyme was incubated with the previous support in the absence of imidazole. Immobilizations were carried out at pH 7 and 20 °C as described in Methods.

hydrophobic, it may be expected that some hydrophilic supports may be used to immobilize proteins.

By modification of 20% of the epoxy groups with the different compounds proposed in this paper, proteins have been immobilized on substituted epoxy-agarose. For example, Figure 5B shows that Cu-epoxy-agarose is able to immobilize penicillin G acylase. The slightly slower immobilization rate when using agarose compared to that observed when using Eupergit C is caused by the lower concentration of epoxy groups of agarose (Eupergit C has 200–220 μmol of epoxy groups/g of wet support, while agarose presents 55–60 μmol of epoxy groups/g of wet support). This possibility enlarges the range of supports to be utilized for enzyme and proteins immobilization, in function of other requirements of the application of the immobilized preparation (such as mechanical properties,

Table 2. Immobilization of Different Proteins on Differently Modified Eupergit C

enzyme	immobilization yield[a] (%) (intrinsic activity[b] (%))		
	EC[c]	EDA-EC[d]	Cu-EC[e]
lipase from *C. rugosa*	100 (100)	95 (95)	70 (100)
PGA from *E. coii*	100 (75)	nd	100 (75)
β-galactosidase from *A. oryzae*	100 (0)	100 (95)	100 (0)

[a] Immobilization yield: percentage of offered enzyme that is immobilized on the support. [b] Intrinsic activity: percentage of activity exhibited by the immobilized enzyme when compared to the soluble form. [c] EC: commercial-Eupergit C. Immobilizations were performed in 1 M sodium phosphate pH 7 at 20 °C for 20 h. [d] EDA-EC: Ethylenediamine-Eupergit C. Immobilizations were performed in 5 mM sodium phosphate pH 7 at 20 °C for 20 h. [e] Cu-IDA-EC: Copper chelate-Eupergit C. Immobilizations were performed in 5 mM sodium phosphate pH 7 at 20 °C for 20 h.

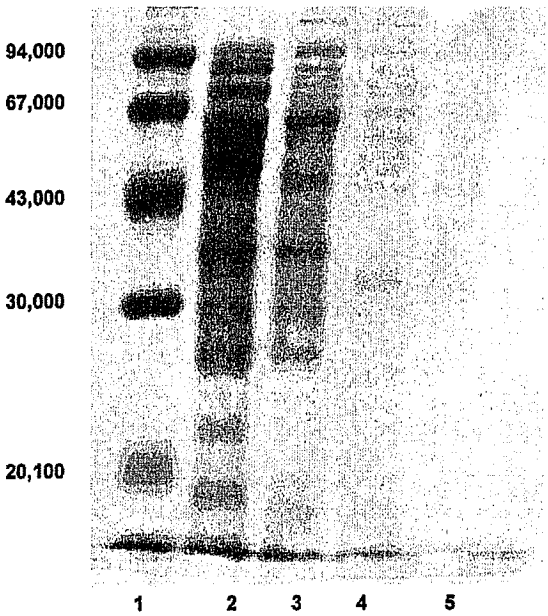

Figure 6. SDS–PAGE analyses of the immobilization of a crude extract of *E. coli* on different epoxy supports: lane 1, molecular weight markers; lane 2, crude protein extract from *E. coli*; lane 3, supernatant (I) after immobilization on aminated-Eupergit C of crude protein extract from *E. coli*; lane 4, supernatant (II) after immobilization on Cu-IDA-Eupergit C of the supernatant (I); lane 5, supernatant after immobilization on boronate-Eupergit C of the supernatant (II). Immobilizations were performed in 5 mM sodium phosphate at pH 7. Other conditions were as described in Methods. Sequential immobilization on different supports.

compatibility with the enzyme, physical properties of the support surfaces, etc.).

General Applicability of the Immobilization of Proteins on Epoxy Supports. The sequential use of some of the different epoxy supports presented in this paper enables the quantitative immobilization of all the proteins contained in crude preparations from different microorganisms. For example, the sequential immobilization on aminated-Eupergit C, Cu-chelate-Eupergit C, and boronate supports (at 5 mM sodium phosphate pH 7 and 20 °C) permitted the complete covalent immobilization of *ALL PROTEINS* contained in different crude protein preparations (Figure 6 shows the results obtained for crude preparations from *E. coli*).

In this way, the use of the battery of epoxy supports presented in this paper enables the immobilization of any protein and in many cases using different protocols that permit different immobilization conditions.

Immobilization of Industrial Enzymes on Different Epoxy Supports. β-Galactosidase from *A. oryzae*, lipase from *C. rugosa*, and penicillin G acylase from *E. coli* were immobilized in some of these epoxy supports. Table 2 summarizes the results. The three enzymes could be immobilized in several of the supports presented in this paper. However, some significant differences in the percentage of activity recovery of the derivatives were found. For example, β-galactosidase from *A. oryzae* was fully inactivated as a consequence of the immobilization by using the commercial support, while keeping almost unaltered the activity when the first adsorption was via a ionic interaction on aminated support.

Discussion

The immobilization of proteins in supports activated with epoxy groups proceeds via a previous "rapid" physical adsorption of the proteins on the support surface. Then, the high "apparent" concentration of reactive groups in the protein and of epoxy groups in the support makes possible a rapid "intramolecular" covalent reaction among the reactive groups of the enzyme and the epoxy groups of the support (Figure 1).[14–17]

The use of conventional homofunctional epoxy supports makes necessary the use of a hydrophobic support and high ionic strength to immobilize proteins, and that may not be convenient in many cases. Also, this method did not enable the immobilization of all proteins contained in a crude protein preparation and can yield inactive enzyme preparations in some cases (e.g., β-galactosidase from *A. oryzae*).

However, the new generation of epoxy supports presented in this paper is able to adsorb the proteins via different structural features. This enables the immobilization of all the proteins contained in crude preparations (in some instances using differently modified supports) and opens the opportunity to use any epoxy support (e.g., a hydrophilic one with the most suitable mechanical properties, loading capacity, and price) and reaction conditions (e.g., low ionic strength) to immobilize the enzymes. This may be achieved by introducing an amount of new groups that were able to promote the adsorption of the protein, leaving most of the epoxy groups available to react with the adsorbed protein.

This battery of epoxy supports has permitted to immobilize several enzymes, in all cases giving at least one derivative with high activity recovery (over 70%).

Some examples of the new opportunities opened by this second generation of epoxy supports will be subject of forthcoming papers (Guisan et al., in preparation).

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