One-Step Purification, Covalent Immobilization, and Additional Stabilization of Poly-His-Tagged Proteins Using Novel Heterofunctional Chelate-Epoxy Supports

Cesar Mateo,1 Gloria Fernández-Lorente,1 Estrella Cortés,2 José L. García,2 Roberto Fernández-Lafuente,1 José M. Guisan1

1Departamento de Biocatálisis, Instituto de Catálisis, CSIC, Campus Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain; telephone: +34-915854809; fax: +34-915854760; e-mail: jmguisan@icp.csic.es
2Centro de Investigaciones Biológicas, CSIC, Madrid, Spain

Received 5 November 2000; accepted 11 May 2001

Abstract: Epoxy supports covalently immobilize proteins following a two-step mechanism; that is, the protein is physically adsorbed and then the covalent reaction takes place. This mechanism has been exploited to combine the selectivity of metal chelate affinity chromatography with the covalent immobilization capacity of epoxy supports. In this way, it has been possible to accomplish, in a simple manner, the purification, immobilization, and stabilization of a poly-His-tagged protein. To fulfill this objective we developed a new kind of multifunctional epoxy support (chelate epoxy support [CES]), which was tested using a poly-His-tagged glutaryl acylase as a model protein (an αβ-heterodimeric enzyme of significant industrial interest). The selectivity of the immobilization in CES toward poly-His-tagged proteins was dependent to a large extent on the density and nature of the chelated metal. The highest selectivity was achieved by using low-density chelate groups (e.g., 5 µmol/g) and metals with a low affinity (e.g., Co). However, the rate of covalent immobilization of the protein by its reaction with the epoxy groups on the support significantly increased at alkaline pH values. The multipoint attachment to the CES also depended on the reaction time. The immobilization of both glutaryl acylase subunits was achieved by incubation of the enzyme derivative at pH 10 for 24 h, with the best enzyme derivative 100-fold more stable than the soluble enzyme. By taking advantage of the selectivity properties of the novel support, we were able to immobilize up to 30 mg of protein per gram of modified Eupergit 250 using either pure enzyme or a very crude enzyme extract. © 2001 John Wiley & Sons, Inc. Biootechnol Bioeng 76: 269–276, 2001.

Keywords: immobilized metal chelate affinity chromatography: epoxy supports; selective immobilization of enzymes; multipoint covalent attachment; glutaryl acylase

INTRODUCTION

The availability of pure enzymes is a key factor in the preparation of very active immobilized biocatalysts, because it allows the entire surface of the support to be occupied by the enzyme. Moreover, the use of pure enzymes avoids undesired side-reactions catalyzed by contaminant enzymes of crude preparations. To overcome the problems derived from enzyme purification (time consuming, high cost, presence of enzyme inactivation, etc.) one suitable solution would be to couple the purification and immobilization steps by designing a highly selective immobilization process.

Immobilized metal-chelate affinity chromatography (IMAC) is a well-developed tool for industrial-scale purification of proteins fused to poly-His-tags (Anspach, 1994; Hemdan et al., 1989; Hubert and Porath, 1980; Porath et al., 1975, 1992; Wang et al., 1994; Woker et al., 1992). In some cases, the adsorption of a poly-His-tagged protein on the chelate support is quite strong and could be used for enzyme immobilization (Beitle and Ataai, 1997; Brena et al., 1994). However, the reversibility of the binding process may be a drawback when used to develop an industrial-scale immobilization procedure. Undesired release of the metals to the reaction media may also become a problem in many cases. For these reasons, IMAC is used mainly for enzyme purification and not for protein immobilization.

On the other hand, one of the most suitable methods for industrial-scale immobilization of proteins is based on epoxy supports (Hartmeier, 1985; Hernaiz and Crout, 2000; Katchalski-Katzir, 2000; Klibanov, 1983; Rosevear, 1984; Royer, 1980). Epoxy supports present many advantages; for example, they are very stable, allowing for long-term storage, prolonged transport from manufacturer to consumer, and extended enzyme-support reaction periods. In addition, they are reactive with different moieties of proteins (amine, thiol, hydroxyl groups), yield-
ing very stable protein-support bonds (secondary amine, thioether, ether). Moreover, the remaining epoxy groups may be easily blocked after enzyme immobilization with different compounds, yielding an inert surface. More recently, we have shown that epoxy supports may be used for enzyme stabilization via an intense enzyme-support multipoint covalent attachment by controlling incubation conditions (Mateo et al., 2000).

The mechanism of enzyme immobilization in epoxy supports (Melander et al., 1984; Smalla et al., 1988; Wheatley and Schmidt, 1993, 1999) provides new opportunities for coupling immobilization to purification. In fact, it has been described that adsorption of the protein on the epoxy resin is necessary to obtain a significant covalent immobilization because of the extremely low reactivity of the epoxy supports with soluble proteins (Melander et al., 1984; Smalla et al., 1988; Wheatley and Schmidt, 1993, 1999). Therefore, due the lower reactivity of the epoxy groups, immobilization of proteins on commercial epoxy supports follows a two-step mechanism: (1) the enzyme is hydrophobically adsorbed on a fairly hydrophobic support (e.g., Eupergit or Sepabeads) at very high ionic strength; and (2) the covalent reaction between the enzyme and the support proceeds. Using this premise, the use of multifunctional epoxy supports to immobilize proteins has been recently reported (Mateo et al., 2000). This second generation of epoxy supports has different moieties that are able to physically adsorb proteins via different structural features, plus a dense layer of epoxy groups able to covalently react with the enzyme. One of the multifunctional supports that may be easily produced are the metal chelate epoxy supports (CES, Mateo et al., 2000). These supports combine the good properties of epoxy supports for enzyme immobilization and stabilization with an increased possibility for IMAC chromatography for purification poly-His-tagged proteins (Scheme 1).

In this study, epoxy chelate supports are optimized to achieve the one step purification, covalent immobilization, and stabilization via multipoint covalent attachment of a model poly-His-tagged protein. The model protein used was poly-His-tagged glutaryl acylase from Acinetobacter sp. (ATCC 53891), overexpressed in Escherichia coli (Armisén et al., 1999). This αβ-heterodimeric enzyme is very important in the pharmaceutical industry as a biocatalyst for the production of 7-aminocephalosporanic acid (7-ACA), which is a key intermediate in the production of many semisynthetic cephalosporins (Croux et al., 1991; Hernández-Justiz et al., 1997).

Although in a conventional IMAC purification process the pure enzyme may be obtained after a controlled desorption of the contaminant proteins, which are more weakly bound to the support, we have shown that an alternative purification method, based on selective adsorption of poly-His-tagged protein on the matrix, may provide some clear advantages (Armisén et al., 1999). Poly-His-tagged proteins can become strongly adsorbed on the support by interaction with just one metal chelate group while natural proteins might require simultaneous interaction with several chelate groups (Johnson and Arnold, 1995). Therefore, it is possible to obtain significant selective adsorption of the poly-His-tagged proteins by using custom-made chelate supports where a multipoint interaction may be difficult (Armisén et al., 1999).

By using epoxy chelate supports for protein purification and immobilization, the adsorption selectivity becomes critical for purification of the protein, because all adsorbed protein molecules may be covalently and irreversibly immobilized on the support. To avoid this problem, we evaluated different CES supports and adsorption conditions in which the binding of contaminant proteins was negligible. Moreover, we studied the incubation conditions that facili-

![Scheme 1](image-url)
tate the stabilization of the enzyme via multipoint covalent attachment. Finally, in a single-step procedure, we prepared an industrial derivative in which the support surface was almost fully loaded with poly-His-tagged glutaryl acylase, using a very crude extract of the enzyme as raw material.

**MATERIALS AND METHODS**

Pure and crude poly-His-tagged glutaryl acylase (prepared at a final concentration of 1 mg/mL) was produced in *E. coli* as described previously (Armisén et al., 1999). Eupergit 250 was from Rohm Pharma (with around 40 μmol of epoxy groups per wet gram of support). Glutaryl 7-ACA was a kind gift from Antibioticos SA. Imidazole were purchased from Merck (Darmstadt, Germany). Iminodiacetic acid di-sodium salt monohydrate (IDA) was from Fluka (Buchs, Switzerland). All other reagents were of analytical grade.

**Preparation of IDA Epoxy Eupergit 250**

Eupergit 250 (10 g) was incubated in 18 mL of 0.1 M sodium borate/2 M iminodiacetic acid (pH 8.5) at 25°C under very gentle stirring. At different timepoints (from 15 min to 24 h), the support was washed with an excess of distilled water and stored at 4°C. The different IDA epoxy supports were then incubated as previously described with different metal salts (Armisen et al., 1999) to obtain the chelate epoxy supports. For example, Cu²⁺ epoxy Eupergit 250 was prepared with the following protocol: Briefly, IDA Eupergit 250 (10 g) was incubated in 60 mL of distilled water containing 2 g of CuSO₄ under very gentle 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 (Scheme 2).

Atomic absorption spectroscopy was used to quantify the degree of modification of the epoxy groups with IDA, after releasing the Cu²⁺ from a sample of the support by treatment with 100 mM ethylene-diamine tetraacetic acid (EDTA) at pH 7.

In some cases, the epoxy groups were blocked with mercaptoethanol (Kramer et al., 1979) or destroyed by acid hydrolysis in 0.5 M sulfuric acid before performing the chelating reaction. These supports were utilized to determine the selectivity of the adsorption of the poly-His-tagged protein on the chelate supports.

**Immobilization of Glutaryl Acylase**

Eupergit 250 (5 g) was suspended in 15 mL of (50 mM or 1 M) sodium phosphate buffer (pH 7) at 25°C. Then, 5 mL of protein extract (1 mg/mL) was added. Periodically, samples were withdrawn and the protein content of the supernatant and glutaryl acylase activity of supernatant and/or suspension were analyzed as described in what follows (Bradford, 1976).

Other samples of the support were incubated in the pres-
ence of 100 mM imidazol and the activities of the suspension and supernatant were analyzed to study the covalent immobilization (this treatment was enough to desorb all of the glutaryl acylase from the fully modified chelate supports, without any remaining reactive epoxy group).

In some cases, the immobilized derivative was incubated at different pH values and, at different timepoints, the epoxy groups were blocked by reaction with 5% mercaptoethanol (pH 7.5) for 16 h (Scheme 2), washed with an excess of distilled water, and stored at 4°C. This treatment also reduces all of the metal in the support, giving free IDA groups.

**Assay of Glutaryl Acylase Activity**

The activity of the glutaryl acylase was evaluated in the hydrolysis of glutaryl 7-ACA using a pH stat. The reaction mixture was 10 mM glutaryl 7-ACA in 50 mM sodium phosphate (pH 7.5), with titration of the glutaric acid released during the reaction by using 25 mM NaOH.

**Study of the Stabilization of Quaternary Structure of Different Derivatives**

To determine the covalent attachment of the α and β subunits of glutaryl acylase, different immobilized derivatives were boiled in 1 vol of 2% sodium dodecylsulfate (SDS) (Bastida et al., 1998; Fernández-Lafuente et al., 1999). In this way, any molecule not covalently attached to the support was released into the medium. Then, SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) analysis of the supernatant was performed and the gel was stained with Coomassie blue and analyzed by densitometry.

**RESULTS**

**Optimization of Adsorption Selectivity**

Studies were performed using chelate supports with the epoxy groups destroyed or blocked (see “Materials and Methods”). The effect of the concentration of chelate groups on adsorption of natural proteins from *E. coli* protein extract and poly-His-tagged glutaryl acylase.

<table>
<thead>
<tr>
<th>Support</th>
<th>Adsorbed natural protein (%)</th>
<th>Adsorbed poly-His-tagged protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu²⁺ (μmol/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>95</td>
<td>&gt;95</td>
</tr>
<tr>
<td>30</td>
<td>89</td>
<td>&gt;95</td>
</tr>
<tr>
<td>10</td>
<td>65</td>
<td>&gt;95</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>&gt;95</td>
</tr>
<tr>
<td>Co²⁺ (μmol/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>24</td>
<td>&gt;95</td>
</tr>
<tr>
<td>30</td>
<td>18</td>
<td>&gt;95</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>&gt;95</td>
</tr>
<tr>
<td>5</td>
<td>&lt;1</td>
<td>&gt;95</td>
</tr>
</tbody>
</table>

Experiments carried out as described in “Materials and Methods” using support without active epoxy groups.

groups (to covalently immobilize the protein), was used to achieve selective immobilization of the enzyme.

**Selective Covalent Immobilization of Poly-His-Tagged Glutaryl Acylase on Epoxy Chelate Supports**

Poly-His-tagged glutaryl acylase was immobilized quantitatively on the optimal Co²⁺ epoxy support after 1 h of incubation at pH 7 and low ionic strength (Fig. 1). Under the same conditions, we did not observe significant immobilization of the enzyme on an IDA epoxy support without Co²⁺ (Fig. 1). Similarly, in the presence of 100 mM imidazol, the poly-His-tagged protein remained in the supernatant even when using the Co²⁺ epoxy support (Fig. 1). These results suggest that previous physical adsorption of the enzyme is necessary to obtain covalent immobilization of proteins in epoxy supports (Scheme 1) (Katchalski-Katzir and Krawietz, 2000; Mateo et al., in press; Melander et al., 1984; Smalla et al., 1988; Wheatley and Schmidt, 1993). In fact, the enzyme derivative in the presence of imidazol.

This demonstrates that, in the first stages of the immobilization, a fraction of the enzyme was physically adsorbed on the support, but not covalently immobilized. This fraction was progressively smaller when increasing the incubation time and when using more alkaline pH values (Fig. 2). However, at pH 7 the complete covalent immobilization of the poly-His-tagged glutaryl acylase required an extremely long incubation period. For this reason, higher pH values were assayed for this immobilization. Thus, after 24 h at pH 8.5, most of the enzyme was covalently attached to the support. Interestingly, the immobilization protocol promoted only a slight decrease in enzyme activity (around 25%).

By boiling the enzyme derivative in the presence of SDS
we were able to release enzyme subunits into the medium that were not covalently attached to the support. This analysis provides some clues about the degree of purity of the immobilized enzyme (Bastida et al., 1998, Fernández-Lafuente et al., 1999). Figure 3 shows that most of the desorbed enzyme subunits belonged to glutaryl acylase, showing the high selectivity of the immobilization protocol. Using other less specific adsorption protocols (e.g., using conventional hydrophobic Eupergit 250 and an immobilization at high ionic strength), many different proteins can be detected in the supernatant after desorption treatment.

Stabilization of Glutaryl Acylase on Epoxy Supports via Multipoint Covalent Attachment

Structural Stabilization

Immobilized enzyme derivatives were submitted to different incubation protocols after the covalent immobilization. They were then boiled in the presence of SDS and the supernatants analyzed by SDS-PAGE. After enzyme immobilization at pH 8.5, and further incubation of the immobilized enzyme for 10 h, it was possible to release most of the large β subunit from the support (Fig. 4), and hence there was a lower proportion of small α subunit (containing the poly-His-tag) accounting for the percentage of enzyme physically adsorbed on the support at that time.

The incubation of the enzyme at pH 10 produced a rapid, progressive decrease in the amount of desorbed enzyme subunits. Thus, after 24 h of incubation, there was no detectable release of the two enzyme subunits after desorption treatment. This result suggests that a multipoint covalent attachment between the enzyme and support was produced; that is, each enzyme molecule was attached by at least one bond per protein subunit to the support (Scheme 3).

Functional Effects of Incubation of Immobilized Glutaryl Acylase

The incubation of the immobilized enzyme at different pH values did not cause significant detrimental effects on the final activity of the derivative. In this sense, the final activity of all derivatives was around 70% to 75% of the activity of the originally immobilized enzyme.
In addition, we also investigated if the structural stabilization of the glutaryl acylase was associated with an increase in enzyme stability. Figure 5 shows that the immobilized derivative further incubated at pH 10 for 24 h (i.e., containing both subunits covalently attached to the support) was not only much more stable than the soluble enzyme but also presented multiphasic inactivation, suggesting some heterogeneity in the degree of enzyme-support attachment. The average increase in the enzyme’s half-life was around 100-fold when compared with the soluble enzyme, although a high percentage of immobilized enzyme presented greatly increased stabilization (>1000-fold more stable than soluble enzyme).

Preparation of an Industrial Derivative of Glutaryl Acylase

We investigated the possibility of preparing an enzyme derivative with the maximum amount of glutaryl acylase per milliliter of support—but by using a crude enzyme extract instead of a pure protein fraction. Figure 6 shows the results obtained when we immobilized increasing concentrations of glutaryl acylase using both the commercial Eupergit 250 at high ionic strength and the Co²⁺ chelate Eupergit 250 described earlier. Using the commercial support we were able to immobilize 100% of the poly-His-tagged glutaryl acylase only when the amount of protein was <30 mg of total crude protein per gram of support (i.e., the maximum loading capacity of the support), because at higher amounts of protein the immobilization yield started to decrease (contaminant and target proteins immobilized). Using our second-generation support, however, it was possible to immobilize 100% of the tagged enzyme contained in 2.5 g of total crude protein per gram of support. In this way, this new support can immobilize up to tenfold more poly-His-tagged glutaryl acylase than the conventional one (results not shown). In fact, the amount of glutaryl acylase immobilized using this new support corresponds almost exactly with the maximum loading capacity of the support, calculated by immobilization of the pure poly-His-tagged glutaryl acylase (i.e., 30 mg of protein per gram of support) (Table II).

DISCUSSION

In this study we have taken advantage of the two-step mechanism of immobilization of proteins in epoxy supports to develop a tool that allows the use of IMAC chromatog-
raphy for protein purification and epoxy supports for enzyme immobilization (Scheme 1). Thus, control of both steps (adsorption and immobilization) permits purification, immobilization, and stabilization (structural and functional) of poly-His-tagged glutaryl acylase to take place in one step (Scheme 3).

By controlling the density of chelate groups and the nature of the metal, selective adsorption of the poly-His-tagged protein has been accomplished. By controlling the immobilization conditions, the stabilization of the enzyme via multipoint covalent attachment has been performed without significant detrimental effects due to enzyme activity. This methodology allows for preparation of an immobilized, stabilized enzyme derivative almost fully loaded with poly-His-tagged protein (>90% of the protein was the tagged protein) using a crude protein extract with <1% enzyme content.

Bearing in mind that these present results are based on the general properties of a protein surface, these methodologies can be utilized easily with any other poly-His-tagged protein to achieve, in a very simple way, the purification, immobilization, and stabilization of recombinant proteins.

The authors thank Mr. Coumo (Resindion), Mr. Daminati (Resindion), Mr. Miyata (Resindion), and Dr. Moreno (Antibiotics SA) for support and interesting suggestions. We also thank Antibióticos SA for donation of substrates.

Table II. Activity of fully loaded derivatives of glutaryl acylase using epoxy chelate supports.

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Activity (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>120</td>
</tr>
<tr>
<td>Pure</td>
<td>130</td>
</tr>
</tbody>
</table>

Experiments performed at pH 8.5 using pure and crude preparations. Other specifications as described in “Materials and Methods.”

References


