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Affinity chromatography of polyhistidine tagged enzymes New dextran-coated immobilized metal ion affinity chromatography matrices for prevention of undesired multipoint adsorptions

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Abstract

New immobilized metal ion affinity chromatography (IMAC) matrices containing a high concentration of metal–chelate moieties and completely coated with inert flexible and hydrophilic dextrans are here proposed to improve the purification of polyhistidine (poly-His) tagged proteins. The purification of an interesting recombinant multimeric enzyme (a thermo-resistant β-galactosidase from *Thermus* sp. strain T2) has been used to check the performance of these new chromatographic media. IMAC supports with a high concentration (and surface density) of metal chelate groups promote a rapid adsorption of poly-His tagged proteins during IMAC. However, these supports also favor the promotion of undesirable multi-punctual adsorptions and problems may arise for the simple and effective purification of poly-His tagged proteins: (a) more than 30% of the natural proteins contained in crude extracts from *E. coli* become adsorbed, in addition to our target recombinant protein, on these IMAC supports via multipoint weak adsorptions; (b) the multimeric poly-His tagged enzyme may become adsorbed via several poly-His tags belonging to different subunits. In this way, desorption of the pure enzyme from the support may become quite difficult (e.g., it is not fully desorbed from the support even using 200 m*M* of imidazole). The coating of these IMAC supports with dextrans greatly reduces these undesired multi-point adsorptions: (i) less than 2% of natural proteins contained in crude extracts are now adsorbed on these novel supports; and (ii) the target multimeric enzyme may be fully desorbed from the support using 60 m*M* imidazole. In spite of this dramatic reduction of multi-point interactions, this dextran coating hardly affects the rate of the one-point adsorption of poly-His tagged proteins (80% of the rate of adsorption compared to uncoated supports). Therefore, this dextran coating of chromatographic matrices seems to allow the formation of strong one-point adsorptions that involve small areas of the protein and support surface. However, the dextran coating seems to have dramatic effects for the prevention of weak or strong multipoint interactions that should involve a high geometrical congruence between the enzyme and the support surface.

Keywords: Immobilized metal ion affinity chromatography; Dextran-coated supports; Enzymes; Galactosidases

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1. Introduction

The use of immobilized metal ion affinity chromatography (IMAC) to purify proteins fused with polyhistidine (poly-His) tags is a popular technology for the simple and cheap production of large amounts of pure industrial enzymes [1–12]. In most cases, it is possible to insert at the terminal amino or carboxyl positions a small tag composed of six His molecules with a negligible effect on the enzyme stability–activity features. Poly-His tagged proteins may be easily purified by IMAC. They are quite strongly adsorbed on these columns via interaction of the poly-His tag with just one chelate [13–18]. They may then be easily desorbed using imidazole or by lowering the pH.

At first glance, the use of highly activated IMAC matrices strongly improves the rate of adsorption of poly-His tagged proteins. However, by using such highly activated matrices, many of the natural proteins present in crude protein extracts may also be adsorbed on the matrices via multi-point weak adsorptions (Fig. 1) [13–17]. This undesired adsorption is a drawback for a good IMAC performance (e.g., the necessity of increasing the size of the affinity column, the possibility of co-desorption of contaminant proteins with the poly-His tagged protein). The design of tailor-made IMAC matrices with less ability to promote these multi-point interactions would solve this problem.

On the other hand, poly-His tagged homo-multimeric proteins have one poly-His tag per enzyme subunit. Hence, there is the possibility that these proteins will become very strongly adsorbed to the column by multi-tag adsorption. In these cases, the desorption of multi-point adsorbed tagged proteins may become too difficult. Again, the design of tailor-made supports with less ability to form multi-point adsorption would be very valuable.

The simplest way to reduce the ability of IMAC matrices to promote multi-point adsorption is to reduce the concentration (and therefore the surface density) of metal chelate moieties (Fig. 1) [18]. However, the use of these lowly derivatized matrices also promotes a significant decrease in the one-point adsorption rate of poly-His tagged proteins [18].

In this paper, the utilization of a new class of tailor-made matrices to improve the purification of poly-His tagged proteins is proposed. These novel matrices are prepared with a high concentration of metal chelate moieties, but they are further covered by one or two dense layers of dextrans. In this way a

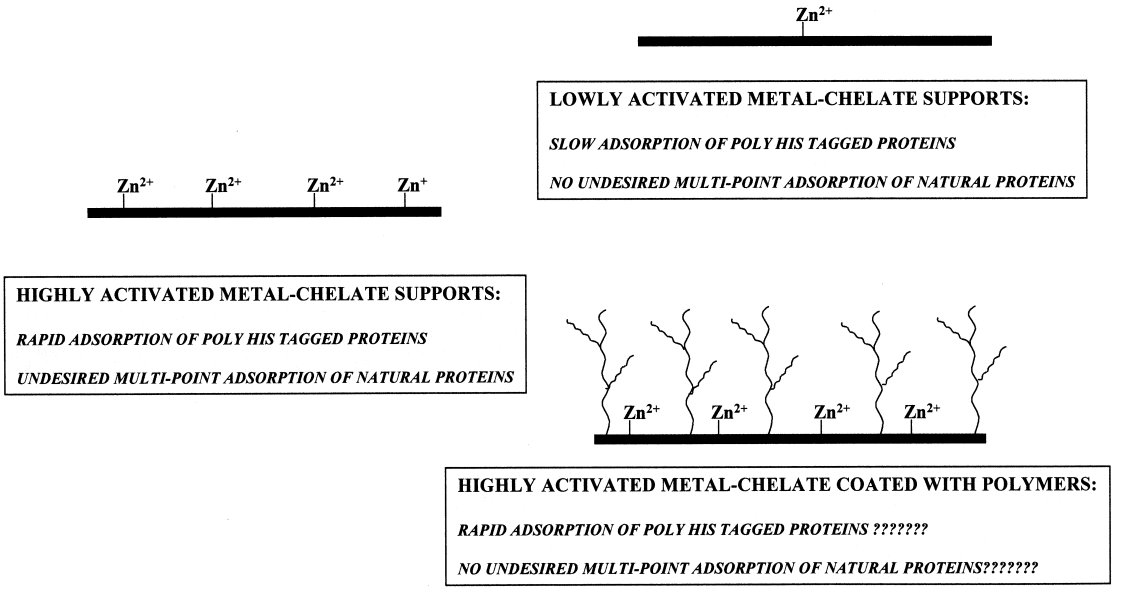

Fig. 1. Different options for the preparation of IMAC matrices compared in this paper.

significant decrease in the possibility of forming an intense multi-point adsorption between the matrix and the adsorbed proteins (Fig. 1) can be expected. Because of the very open structure of the dextran shell, a slight decrease in the one-point adsorption rates may also be possible.

Dextrans were chosen for the coating because they are random coil, flexible, hydrophilic and inert polymers [19–27], therefore ensuring that they cannot promote undesired interactions with the proteins.

A recombinant poly-His tagged multimeric β-galactosidase from *Thermus* sp. strain T2 [28–30] was cloned in *E. coli* [31] and used to evaluate the prospects of these novel dextran-coated IMAC supports. This enzyme is very stable at high temperatures and is an excellent industrial enzyme for the hydrolysis of lactose in milk and cheese whey.

2. Materials and methods

2.1. Materials

Epoxy-Sepabead (EP-HG-15) supports were kindly donated by Resindion (Mitsubishi). Crosslinked agarose 6% was donated by Hispanagar. Agarose supports with different activation degree with epoxy groups were produced using epichlorohydrin as previously described [18]. Poly-His tagged β-galactosidase from *Thermus* sp. strain T2, cloned and over-expressed in *E. coli*, was produced as published elsewhere [31]. *o*-Nitrophenyl-β-D-galactopyranoside (*o*-NPG) was from Sigma (St. Louis, MO, USA). Iminodiacetic acid disodium salt monohydrate (IDA) was from Fluka (Buchs, Switzerland). Epichlorohydrin and imidazole were purchased from Merck (Darmstadt, Germany). Dextrans (33 mg/ml) were fully oxidized with periodate as previously described [32]. All other reagents were analytical grade.

2.1.1. Preparation of standard IDA (iminodiacetic acid) matrices

The preparation of IDA from the different epoxy supports (agarose or Sepabeads) was carried out according to the method described previously [18]. Activated matrices were prepared as follows: a 10 ml volume of epoxy supports was suspended in 14 ml of 0.1 *M* Na_2CO_3 buffer, containing 0.9 g of IDA and adjusted to pH 11.0 with NaOH. The flask was gently stirred at 25°C for 12 h. The IDA supports were then washed with distilled water.

2.1.2. Coating of IDA matrices with dextrans

2.1.2.1. Amination of IDA supports

The glyceryl groups of IDA supports (agarose or Sepabeads) were oxidized with sodium periodate to obtain an aldehyde support. Using agarose, 45 μmol of periodate per milliliter of support were utilized to completely oxidize the glyceryl groups on the support [33]. When using Sepabeads, 60 μmol of periodate per milliliter of support was used (we use only a small fraction of the available glyceryl groups).

These aldehyde supports were then aminated with ethylenediamine as described elsewhere [34].

2.1.2.2. Chemical modification of the IDA amino supports with oxidized dextrans

A volume of 330 ml of aldehyde dextrans in 100 m*M* sodium phosphate at pH 8.0 was added to 10 g of the different aminated supports and very gently stirred for 16 h. Then, 330 ml of 200 m*M* sodium hydrogencarbonate pH 10 containing 6.6 g of solid sodium borohydride was added. After 2 h, the supports were washed with an excess of distilled water. Two different supports were prepared:

(i) Coated with dextran of M_r 282 000.

(ii) Coated first with dextran of M_r 282 000 and further with dextran of M_r 72 000.

The double coating of the support blocked most of the primary amino groups in the support [35].

2.1.3. Preparation of Zn^{2+}-chelate supports

The IDA supports (standard or coated) were incubated in distilled water containing 5 mg/ml of $ZnCl_2$ for 2 h under very gently stirring [18]. Finally, the supports were washed thoroughly with distilled water.

2.1.4. Adsorption of proteins on IMAC matrices

One milliliter of Zn^{2+}-agarose or Sepabeads support was added to 20 ml of protein (maximum concentration used was 2.5 mg/ml). Three different protein extracts were used:

(a) crude extract of natural proteins from *Escherichia coli*;

(b) pure poly-His tagged β-galactosidase;

(c) crude protein extract from *E. coli* containing poly-His tagged β-galactosidase (degree of purity around 10%) [31].

The proteins were dissolved in 50 m*M* sodium phosphate buffer pH 7.0 containing 0.15 *M* NaCl, at 25°C. In all cases, the enzyme was incubated with the support for 24 h to study the possible effects of the enzyme–support multi-interaction. The amount of adsorbed natural proteins was analyzed by Bradford's method [36]. The amount of poly-His tagged β-galactosidase adsorbed on the supports was measured by evaluating the remaining catalytic activity in the supernatant. This catalytic activity was followed as described below. Experiments were carried out in triplicate and the experimental error was less than 7%.

2.1.5. Analysis of adsorbed proteins

A 100 μl sample of metal chelate support with adsorbed proteins was suspended in 100 μl of 0.125 m*M* Tris–HCl, pH 6.8, containing 10% bromophenol, 10% mercaptoethanol, 5% glycerol and 4% sodium dodecyl sulfate (SDS) and the mixture was boiled for 5 min. This treatment released all adsorbed proteins from the support to the supernatant [37–39]. The soluble samples were treated following the same procedure. Then, electrophoretic analyses were performed using a modification of Laemmli's method [40] and gels were stained with Coomassie blue.

2.1.6. Desorption of the poly-His tagged β-galactosidase from IMAC supports

Adsorbed protein was resuspended in 50 m*M* sodium phosphate buffer pH 7.0 containing 0.15 *M* NaCl at 25°C. Then, the derivatives were incubated in increasing concentrations of imidazole. At each concentration, the activity was determined in the supernatant and the suspension after 30 min of gently stirring.

2.1.7. Assay of enzyme activity

The β-galactosidase activity was evaluated by recording the increment in absorbance at 405 nm produced by the hydrolysis of *o*-NPG. The reaction mixture consisted of 10 m*M* *o*-NPG in 50 m*M* sodium phosphate at pH 7 and 25°C.

3. Results

3.1. Adsorption of natural proteins and poly-His tagged β-galactosidase on highly concentrated IMAC matrices

Recombinant poly-His tagged β-galactosidase is fully adsorbed on the highly activated uncoated support under the experimental conditions. In fact, the adsorption of the enzyme occurs at high speed. Thus, 50% of the target protein becomes adsorbed on the support after only 15 min using 1 ml of support and 20 ml of enzyme suspension, and more than 95% of the enzyme was adsorbed after 1 h. However, a great percentage (more than 30%) of natural proteins become adsorbed on this uncoated highly activated Zn^{2+}-IDA-agarose support (30 μmol/ml) (Table 1).

When this support is coated with a layer of aldehyde dextran (M_r 282 000), the adsorption of native proteins is strongly reduced to 10% of the total natural proteins from a crude extract from *E. coli*. This adsorption of natural proteins becomes almost negligible (less than 2%) when the support is further coated with a second layer of a smaller dextran (M_r 72 000) (Table 1). In spite of this dramatic reduction of the adsorption of natural proteins, the double coating of IMAC supports hardly affects the immobilization rate of the poly-His protein (reduced only to 80% of the rate corresponding to the uncoated support) (Table 1).

3.2. SDS–polyacrylamide gel electrophoresis (PAGE) of adsorbed enzymes and proteins

The results reported above were further confirmed by performing the adsorption (on the uncoated and both coated IMAC supports) of a crude extract of proteins of *Escherichia coli* containing both natural proteins and recombinant poly-His tagged β-galactosidase. The matrices with the adsorbed proteins were washed with buffer and then completely desorbed from the support and analyzed by SDS–PAGE (see Materials and methods).

Table 1
Adsorption of natural proteins and poly-His tagged β-galactosidase on coated and non-coated Zn^{2+}-chelate matrices

IMAC matrix	Adsorption of natural proteins[a] (%)	Adsorption of pure poly-His tagged enzyme[b] (%)	Relative adsorption rate: poly-His tagged enzyme[c] (%)
Standard	32	>95	100
Dextran coated (one layer)	10	>95	90
Dextran coated (two layers)	<2	>95	80

Supports activated with 30 μmol of Zn^{2+}-chelate groups per milliliter were utilized following the protocols described in Materials and methods.

[a] Crude protein extract from *E. coli* was incubated for 24 h with the different IMAC matrices, and the amount of adsorbed protein was determined by Bradford's method.

[b] Pure poly-His tagged enzyme was incubated for 24 h with the different IMAC matrices. The percentage of adsorbed enzyme was determined by measuring the activity of the supernatant.

[c] Relative adsorption rate refers to the rate of adsorption. The value was calculated when 20% of the enzyme was immobilized, and the unit was the rate of immobilization on the standard support.

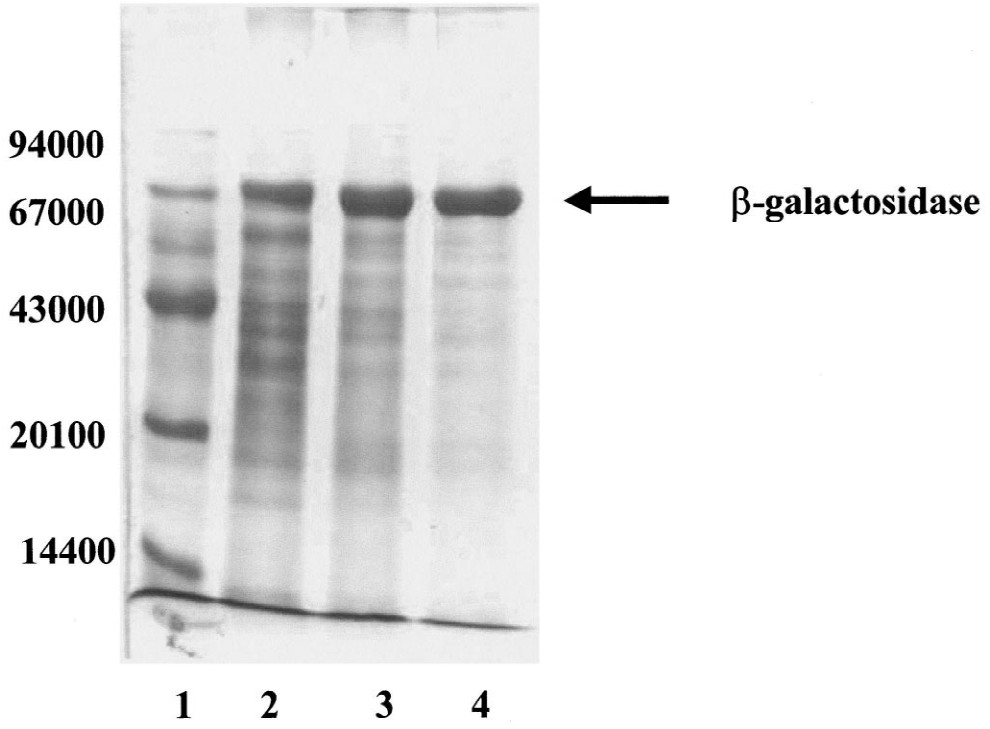

Fig. 2. Analysis by SDS–PAGE of proteins adsorbed on different Zn^{2+}-agarose (30 μmol/ml). Adsorption experiments of the crude extract containing poly-His tagged β-galactosidase and preparation of the samples were carried out as described in Materials and methods. Lanes: 1=molecular mass markers, 2=proteins desorbed from standard supports, 3=proteins desorbed from supports coated with one layer of dextrans, 4=proteins desorbed from supports coated with two layers of dextrans.

Uncoated IMAC agarose gels were able to adsorb a number of native proteins in addition to the poly-His tagged target enzyme (see Fig. 2). Coating of the supports with a layer of dextran strongly reduces the adsorption of natural proteins. Finally, the second coating with dextrans almost completely eliminates the adsorption of natural proteins yielding an almost pure poly-His β-galactosidase adsorbed on the support.

Very similar results were obtained with IMAC-Sepabeads supports. Sepabeads is a trade mark of very robust epoxy-acrylic resins fabricated by Mitsubishi and they are quite different from agarose gels as chromatographic supports (e.g., morphology, structure). However, the effect of dextran coating on Sepabeads is identical to that observed with agarose gels. For example, the double-coated support hardly adsorbs natural proteins from the crude extract of proteins of recombinant *Escherichia coli*. A very pure single band corresponding to the monomer of poly-His β-galactosidase appears to corresponds to the main protein adsorbed on these interesting chromatographic matrices (Fig. 3).

3.3. Desorption of poly-His tagged β-galactosidases from IMAC matrices

A significant percentage of the target enzyme still remained adsorbed on the support even after incubation in 200 m*M* of imidazole when using an uncoated highly activated standard support (see Fig. 4). Similar problems for the desorption of multimeric poly-His tagged proteins were previously observed in our laboratory for other multimeric enzymes (e.g., D-amino acid oxidase from *Rhodotorula gracilis* [41]). On the other hand, the optimal double-coated dextran-IMAC matrix permits the full desorption of the poly-His tagged enzyme by only using 60 m*M* imidazole. This concentration of imidazole is very similar to that necessary to desorb enzymes having

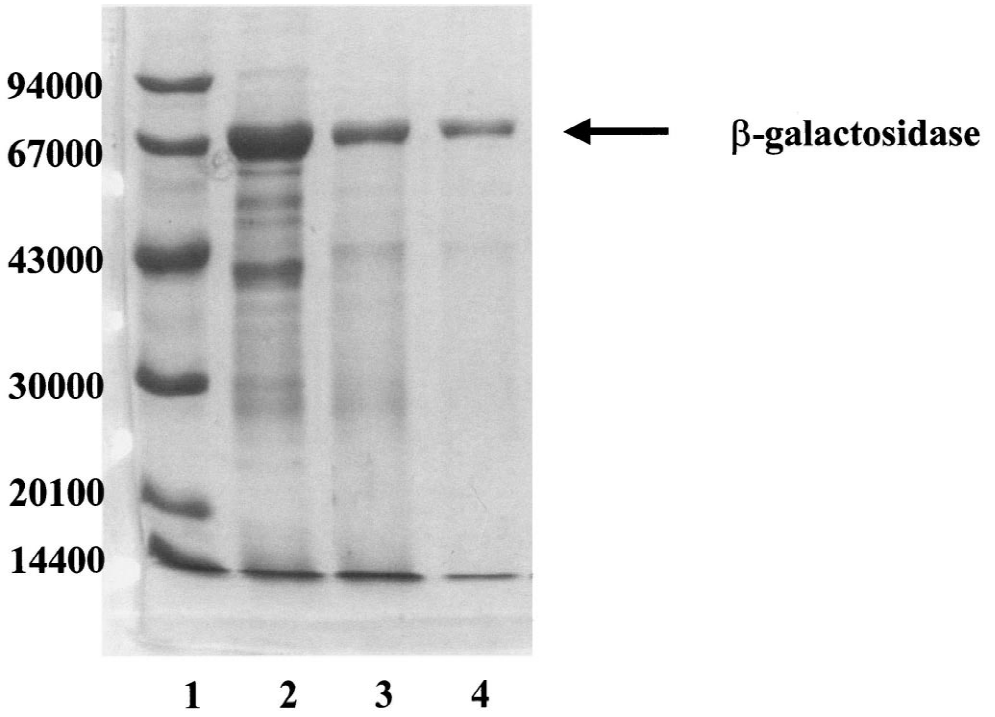

Fig. 3. Analysis by SDS–PAGE of proteins adsorbed on different Zn^{2+}-Sepabeads (30 μmol/ml). Adsorption experiments of a crude extract containing poly-His tagged β-galactosidase and preparation of the samples were carried out as described in Materials and methods. Lanes: 1=molecular mass markers, 2=proteins desorbed from standard supports, 3=proteins desorbed from supports coated with one layer of dextrans, 4=proteins desorbed from supports coated with two layers of dextrans.

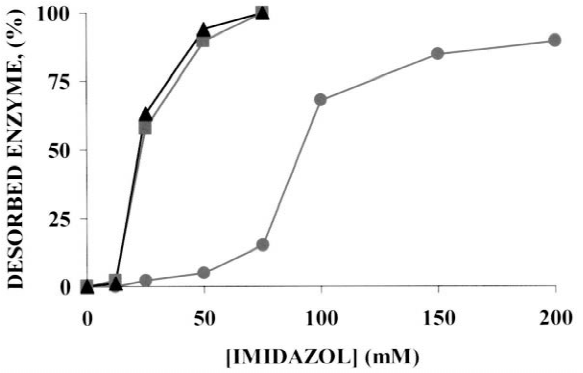

Fig. 4. Desorption of poly-His tagged β-galactosidase and glutaryl acylase adsorbed on different Zn^{2+}-agarose at increasing concentrations of imidazole. Experiments were performed as described in Materials and methods. Triangles: desorption of poly-His tagged glutaryl acylase (protein with just one poly-His tag) adsorbed on a standard agarose activated with 30 μmol/ml; circles: desorption of poly-His tagged β-galactosidase adsorbed on a standard agarose activated with 30 μmol/ml; squares: desorption of poly-His tagged β-galactosidase adsorbed on the double coated support activated with 30 μmol/ml.

only one poly-His tail per enzyme molecule from similar supports (e.g., glutaryl acylase from *Actrobacter* sp. [18]). On the other hand, this concentration of imidazole (60 m*M*) is much higher than the very small concentration required to completely remove the small percentage of natural proteins (less than 2% of the total natural protein content) adsorbed on the support (10 m*M* imidazole).

Thus, after washing the matrices with the adsorbed proteins with 10 m*M* imidazole, it was possible to obtain an enzyme preparation with a degree of purity higher than 98% (specific activity 24 U/mg) from an extract having a specific activity of 2.3 U/mg (degree of purity around 10%) and a recovery activity yield higher than 95%.

The coated matrices could be reused 10 cycles without any change in the performance of the purification. Also, by incubation with EDTA the metal could be eliminated, and simple incubation under adequate conditions enables the preparation of a new IMAC support.

3.4. Evaluation of other alternatives: the use of very low concentrated IMAC matrices

For comparative purposes, we studied the adsorption of the same natural enzymes and the same target protein on different uncoated supports with different concentrations of metal chelates. In this case, we observed an interesting decrease in the adsorption of natural proteins when lowering the chelate concentration and a parallel preservation of the capacity to adsorb our target poly-His tagged protein. In fact, negligible adsorption of natural proteins was observed when using supports having only 3 μmol/ml of chelate groups. However, employing these very slightly activated matrices, the immobilization rate of the poly-His target proteins was 10-fold lower than when using the standard uncoated IMAC supports and, consequently, seven- to eight-fold lower than the adsorption rate obtained using the novel double-coated IMAC support (Table 2).

Table 2
Adsorption of natural proteins and poly-His tagged β-galactosidase on Zn^{2+} matrices with different degrees of activation

$[Zn^{2+}]$ (μmol/ml)	Adsorption of natural proteins[a] (%)	Adsorption of pure poly-His tagged enzyme[b] (%)	Relative adsorption rate: poly-His tagged enzyme[c] (%)
30	32	>95	100
10	9	>95	30
3	<2	>95	10

Experiments were carried out as described in Materials and methods.

[a] Crude protein extract from *E. coli* was incubated for 24 h with the different IMAC matrices, and the amount of adsorbed protein was determined by Bradford's method.

[b] Pure poly-His tagged enzyme was incubated for 24 h with the different IMAC matrices. The percentage of adsorbed enzyme was determined by measuring the activity in the supernatant.

[c] Relative adsorption rate was calculated when 20% of the enzyme was adsorbed on the highly activated matrix, and we considered as the unit the rate of immobilization on the standard support.

4. Discussion and conclusions

The coating of supports with dextrans permits a rapid adsorption of poly-His tagged proteins while drastically reducing the adsorption of natural proteins. Moreover, these new supports are also able to prevent poly-His tagged multimeric proteins adsorbing too strongly.

The adsorption of natural proteins on metal chelate supports occurs via weak multi-point interactions. This phenomenon has been described by Arnold and co-workers [13,14], and may also be deduced from the dramatic influence of the chelate concentration on the rate of adsorption of natural proteins. Thus, supports where multi-interactions are favored (e.g., having a high density of metal–chelate moieties) may not be very suitable for achieving a selective adsorption of poly-His tagged proteins on IMAC matrices (Fig. 5).

On the other hand, the too strong adsorption of poly-His tagged multimeric proteins on IMAC supports also seems to be related to support–enzyme multi-interactions (Fig. 6). Thus, using highly activated standard supports and enzymes with a single poly-His tag (e.g., glutaryl acylase), the enzyme may be desorbed from the support by incubation in 50 m*M* imidazole [18], while in this case it was necessary to use more than 200 m*M* imidazole. Using double-coated supports, the multimeric enzyme used in this study can be desorbed just by incubating the enzyme in 60 m*M* imidazole.

Thus, it seems that the coating of the support

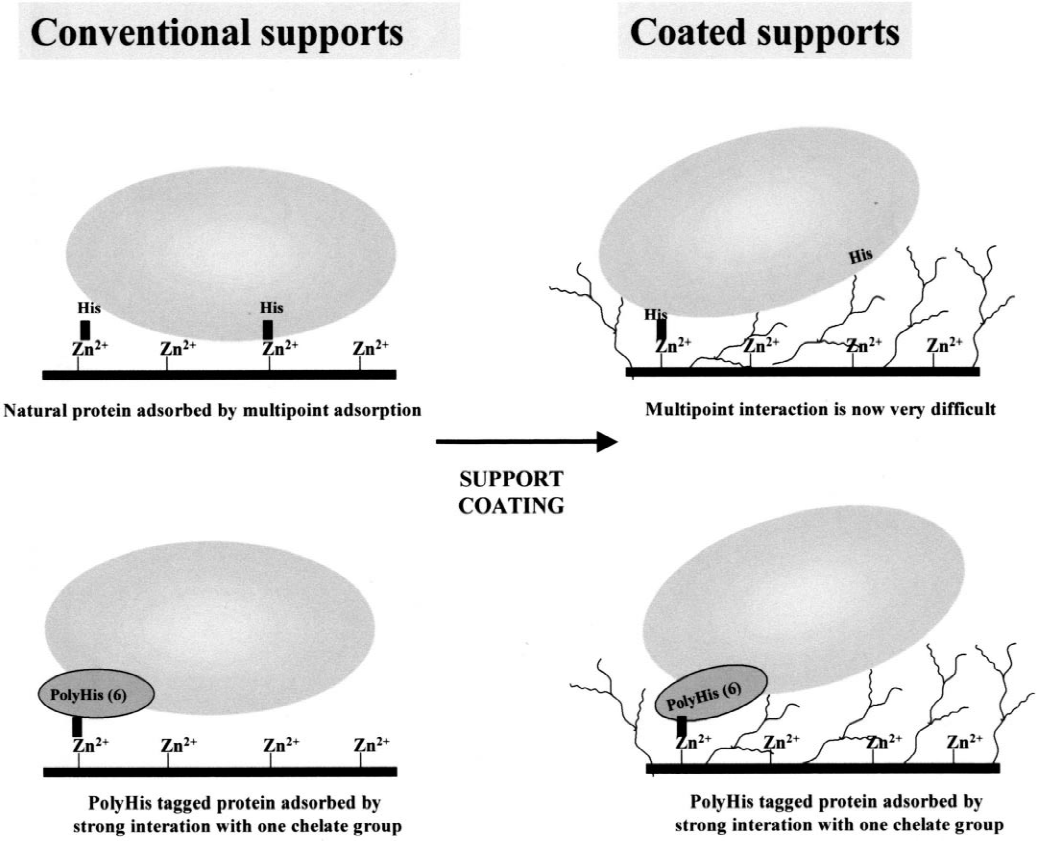

Fig. 5. Possible mechanism of adsorption of natural and poly-His tagged proteins on coated and standard IMAC matrices.

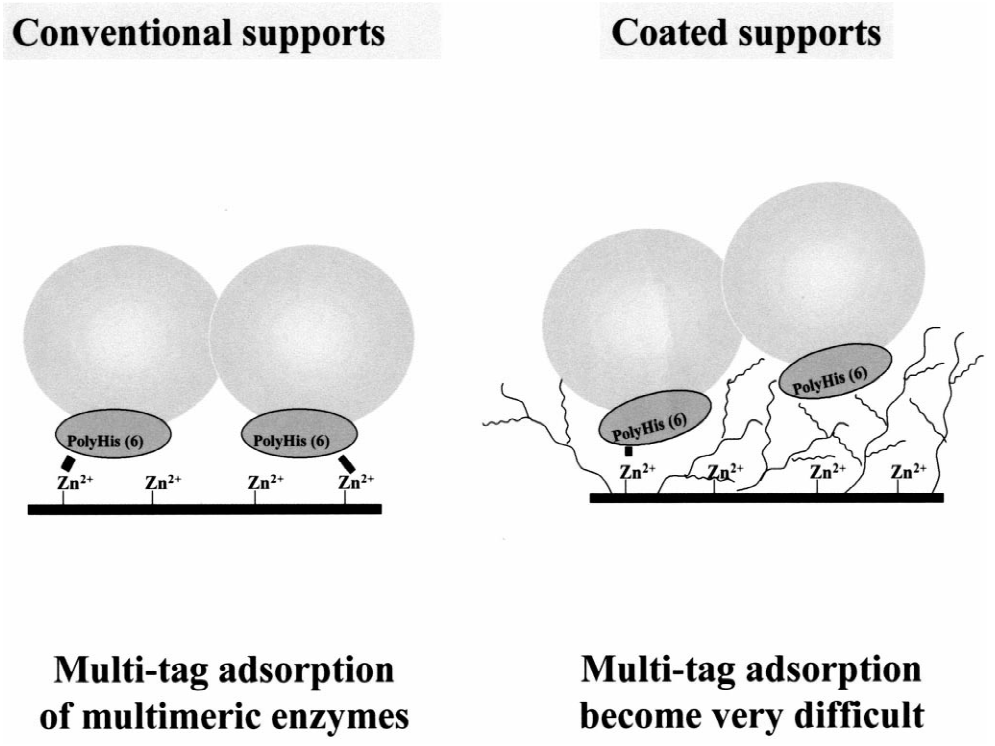

Fig. 6. Possible effect of the dextran coating on the multi-subunit adsorption of multimeric proteins with poly-His tags.

surface with dextrans produces negligible effects on uni-punctual interactions while promoting dramatic reductions in multi-interactions, which implies large areas of the support and protein surfaces.

Dextrans are very flexible structures that do not impede the entry of proteins to the nearby support surface and the promotion of interactions, which implies small regions of the protein surface and just one moiety on the support surface. In this case, the interaction occurs between one poly-His tag that would generally be exposed to the medium and one metal–chelate group on the support surface [13,14,18]. However, the promotion of support–enzyme multipoint interactions implies an intense contact between large areas of the protein and the support, and a support with many groups modified with dextrans surrounding the chelate groups may induce high steric hindrance which will prevent these contacts taking place.

Bearing in mind that most chromatographic techniques are based on the adsorption of proteins via multiple weak interactions (e.g., ionic, hydrophobic adsorptions), this coating strategy, performed in a controlled way, may be useful for modulating the performance of chromatographic matrices, to favor strong one-point interactions over weak multi-point interactions, and even to make the surfaces of different supports inert [35].

In this paper, we have described the purification, in a simple way, of a poly-His tagged multimeric protein (β-galactosidase from *Thermus* sp. strain T2 cloned and over-expressed in *E. coli*) using a new IMAC dextran-coated matrix. The simultaneous adsorption of natural proteins from *E. coli* was greatly reduced and only the target enzyme was adsorbed on the support. Moreover, this enzyme containing multiple poly-His tags could be desorbed from the support using moderate concentrations of imidazole, con-

centrations similar to those necessary to desorb proteins with just one poly-His tag [18].

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