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Selective adsorption of poly-His tagged glutaryl acylase on tailor-made metal chelate supports

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Abstract

A poly-His tag was fused in the glutaryl acylase (GA) from *Acinetobacter* sp. strain YS114 cloned in *E. coli* yielding a fully active enzyme. Biochemical analyses showed that the tag did not alter the maturation of the chimeric GA (poly-His GA) that undergoes a complex post-translational processing from an inactive monomeric precursor to the active heterodimeric enzyme. This enzyme has been used as a model to develop a novel and very simple procedure for one-step purification of poly-His proteins via immobilized metal-ion affinity chromatography on tailor-made supports. It was intended to improve the selectivity of adsorption of the target protein on tailor-made chelate supports instead of performing a selective desorption. The rate and extent of the adsorption of proteins from a crude extract from *E. coli* and of pure poly-His tagged GA on different metal chelate supports was studied. Up to 90% of proteins from *E. coli* were adsorbed on commercial chelate supports having a high density of ligands attached to the support through long spacer arms, while this adsorption becomes almost negligible when using low ligand densities, short spacer arms and Zn^{2+} or Co^{2+} as cations. On the contrary, poly-His GA adsorbs strongly enough on all supports. A strong affinity interaction between the poly-His tail and a single chelate moiety seems to be the responsible for the adsorption of poly-His GA. By contrast, multipoint weak interactions involving a number of chelate moieties seem to be mainly responsible for adsorption of natural proteins. By using tailor-made affinity supports, a very simple procedure for one-step purification of GA with minimal adsorption of host proteins could be performed. Up to 20 mg of GA were adsorbed on each ml of chelate support while most of accompanying proteins were hardly adsorbed on such supports. Following few washing steps, the target enzyme was finally recovered (80% yield) by elution with 50 m*M* imidazole with a very high increment of specific activity (up to a 120 purification factor).

Keywords: Immobilized metal-ion affinity chromatography; Affinity supports; Glutaryl acylase; Enzymes

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1. Introduction

The use of immobilized metal-ion affinity chromatography (IMAC) to purify proteins fused with poly-His tags is becoming increasingly popular as a tool for the simple and inexpensive production of large quantities of pure industrial enzymes [1,2] and pure enzymes for molecular biology studies. In most cases, the insertion at the terminal amino or carboxy positions of an small tag composed of six histidine residues hardly modifies the activity–stability properties of an enzyme.

The usual protocol of purification for so tagged enzymes is based on their adsorption, along with many other host proteins, on commercial chelating supports. Then, natural proteins (that usually adsorb less intensely than fused proteins) are desorbed by using a gradient or step-wise elution system. Finally, poly-His tagged enzymes are eluted with high purification yields by using more drastic conditions, although in some cases there are some traces of contaminants [2].

This stronger adsorption of the tagged proteins compared to that of natural ones might be a consequence of probable differences in the exact mechanism of adsorption. This may permit the development of new purification protocols including a highly selective adsorption of the target protein on tailor-made chelating supports.

Commercial chelating supports usually contain a high density of iminodiacetic groups (20–40 μmol of chelating groups per ml of support) attached to the support through medium or long spacer arms (7 or 12 carbon atoms) (Sigma and Pharmacia technical information). In this way, several weak specific (chelate) and nonspecific (ionic, hydrophobic) interactions between natural proteins and the support may occur. These supports were previously developed for IMAC of natural proteins [3]. In that case, the requirements were different from the objective of a selective adsorption: this 'first version' of commercial chelating supports was designed to provide supports able to adsorb most proteins. Now, when trying to perform selective adsorptions of poly-His tagged proteins, such versions of commercial supports may be not the best choice. It may be expected that a poly-His tag could be able to promote a strong adsorption of tagged proteins by interacting with only one chelate moiety. Therefore, it may be assumed that chelating supports that were unable to promote multipoint interactions with proteins could be much more suitable for selectively adsorb the tagged proteins. It has been shown that similar adsorptions between isolated histidine residues in natural proteins and single chelate moieties on the supports are very weak. In fact, they are 2 or 3 orders of magnitude less intense than multipoint adsorptions [4].

Glutaryl acylase (GA) from *Acinetobacter* sp. strain YS114 (ATCC 53891) [5,6] is very important in the pharmaceutical industry as a catalyst of the production of 7-aminocephalosporanic acid (7ACA), a key intermediate in the production of semisynthetic cephalosporins, via mild and selective hydrolysis of glutaryl-7ACA [7]. Thus, the large-scale production of inexpensive and pure GA becomes a relevant goal in microbial biotechnology. To construct an active poly-His GA it is necessary to take in account that GA is a heterodimeric enzyme (α and β subunits). The precursor polypeptide has a signal peptide to allow its translocation to the periplasmic space. Here, the single precursor is proteolytically processed to its active form [8]. Therefore, the position of the poly-His tag has to be carefully selected, taking into account both a correct maturation plus a fair exposure to the medium in order to facilitate the subsequent purification of the enzyme.

Having in mind the above factors, the preparation of different chelating supports is now described. A number of variables have been studied: the concentration of ligand moieties, the length of spacer arms, the metal cation forming the chelate. Then, adsorptive properties of such tailor-made supports were further compared to those of commercial ones. Parallel experiments following the adsorption of proteins from crude extracts from *E. coli* as well as the adsorption of a poly-His tagged glutaryl acylase are described. Selectivity of different chelating supports was evaluated in terms of preservation of a high binding capacity for poly-His GA associated with a minimal adsorption of natural proteins. Finally, by using the most selective chelating supports, an improved protocol for one-step purification of such an industrial enzyme was developed. The design of such selective adsorption depends on the understanding of the differences between the mechanisms

involved in the adsorption of host and poly-His fused proteins on metal-ion chelate supports.

2. Experimental

2.1. Materials

Crosslinked 6% agarose gels were generously donated by Hispanagar (Burgos, Spain). Chelating Sepharose Fast Flow (Sepharose 6%) was obtained from Pharmacia Biotech (Stockholm, Sweden) (23–30 μmol/ml of support, spacer arm 7 atoms) and iminodiacetic acid immobilized on crosslinked 4% beaded agarose was from Sigma (St. Louis, MO, USA) (30–40 μmol/ml of support, spacer arm 12 atoms). Epichlorohydrin and imidazole were purchased from Merck (Darmstadt, Germany). Iminodiacetic acid disodium salt monohydrate (IDA) and $CuSO_4$ were from Fluka (Buchs, Switzerland). 1,4-Butanediol diglycidyl ether (95%), $NiCl_2$, $ZnCl_2$, $CoCl_2$ and reagents for electrophoresis were obtained from Sigma All other reagents were analytical grade. Pure poly-His-tagged glutaryl acylase and crude protein extract from *E. coli* TG1 were kindly supplied from Antibióticos (Leon, Spain).

2.2. Strains, plasmids and DNA manipulations

The *E. coli* strain used was TG1 (Amersham, Rainhan, UK). The plasmids utilized were: pBCKS+ (Stratagene, La Jolla, CA, USA) and pJC200 [5] that contains the gene *gla* from *Acinetobacter* sp. strain YS114 (ATCC 53891) encoding the GA [6]. Isolation of plasmid DNA was carried out by the alkaline procedure [9]. Site-directed mutagenesis was performed using the mutagenesis system from Amersham following the procedures recommended by the supplier. Bacteriophage M13tg131 (Amersham) was employed as a cloning vector for site-directed mutagenesis. *E. coli* cells were made competent by the RbCl method [9]. *E. coli* was grown in LB medium [15] at 37°C with shaking. DNA sequencing was carried out by the dideoxy-chain termination method, using the Sequenase kit from United States Biochemical (Cleveland, OH, USA). The oligonucleotides used in this work were synthesized in a Pharmacia gene assembler plus DNA synthesizer (Pharmacia, Uppsala, Sweden): SMA-I (5′-GCGCTGGCC GAGCCC GGGCTCGACGCCGCA-GG-3′), HIS-1 (5′-CATCATCACCACCATCACTT) and HIS-2 (5′-AAGTGATGGTGGTGATGATG-3′).

2.3. Preparation of epoxide agarose supports

Epoxide agarose supports were obtained by a modification of the procedures described elsewhere [10,11] using epichlorohydrin or 1,4-butanediol-diglycidyl ether.

2.3.1. Preparation of EPI-10 supports

Activation was performed with epichlorohydrin. Supports with low density of epoxide groups (containing around 10 μmol of epoxide groups/ml gel) (EPI-10) were prepared as follows: 10 ml of crosslinked 6% agarose were washed thoroughly with distilled water and the moist gel was suspended in 30 ml of 1.9 *M* NaOH containing 340 mg of $NaBH_4$, 11.4 ml of acetone and 5.7 ml of epichlorohydrin. The suspension was stirred for 4 h at 4°C and finally washed thoroughly with distilled water. The epoxy groups content was determined by the method described elsewhere [12].

2.3.2. Preparation of EPI-30 supports

Epoxide agarose gel containing 30 μmol of epoxide groups/ml gel was prepared as in the case of EPI-10 but the suspension was stirred in 0.8 *M* NaOH for 8 h at 25°C. Two additions of 5.7 ml of epichlorohydrin were performed after 2 and 4 h of reaction.

2.3.3. Preparation of BDGE-10 supports

Epoxide agarose gel containing 10 μmol of epoxide groups/ml gel was prepared as in the case of EPI-10 but 1,4-butanediol diglycidyl ether (BDGE) substituted the epichlorohydrin and the temperature was 25°C. The concentration of NaOH was decreased to 0.4 M. One addition of 5.7 ml of BDGE was performed after 2 h of reaction.

2.3.4. Preparation of BDGE-30 supports

Epoxide agarose gel containing 30 μmol of epoxide groups/ml gel was prepared as in the case of EPI-10 but BDGE was substituted for epichlorohydrin and the suspension was maintained in

0.4 *M* NaOH at 25°C for 6 h. Two additions of 5.7 ml of BDGE were performed after 2 and 4 h of reaction.

2.4. Preparation of iminodiacetic acid supports

The preparation of iminodiacetic acid (IDA) supports was made according to a modification of the method previously described [10,11]. A 10-ml volume of epoxide–agarose was suspended in 10 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. Then, IDA supports were washed with distilled water.

2.5. Preparation of metal chelate supports

The IDA gels (commercial ones or that prepared as previously described) were incubated in distilled water containing 5 mg/ml of $CuSO_4$ or $ZnCl_2$ or in 50 m*M* sodium phosphate buffer, pH 6.0, containing 1.0 *M* NaCl plus 5 mg/ml of $NiCl_2$ or $CoCl_2$ [13]. Finally, the supports were washed thoroughly with distilled water.

2.6. Standard protein adsorption procedure

A 1-ml volume of chelate–agarose gel was added on 10 ml of protein (maximum concentration used was 5 mg/ml) (crude protein extract from *E. coli* TG1, pure poly-His-tagged glutaryl acylase or crude protein extract from *E. coli* TG1 containing poly-His-tagged glutaryl acylase) dissolved in 10 m*M* sodium phosphate buffer, pH 7.0, containing 0.5 *M* NaCl, at 25°C. When it was indicated, imidazole was also added to the buffer. The amount of adsorbed proteins was analyzed by measuring the absorbance at 280 nm and by Lowry's method [14]. Experiments were carried out in triplicate and the precision was better than 7%.

2.7. Activity and other biochemical analyses

To analyze the proteins adsorbed on the supports, 100 μl of metal chelate support with adsorbed proteins were 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 and the mixture was boiled for 5 min. This treatment released all adsorbed proteins to the supernatant [15]. The soluble samples were treated following the same procedure [15]. Then, electrophoretic analyses were performed using a modification of Laemmli's method [16] and gels were stained with Coomassie blue.

GA activity was determined by the colorimetric method previously described using glutaryl 7-aminocephalosporanic acid as substrate [17]. The amino-terminal sequence of poly-His GA was determined by Edman's degradation with a 477A automated protein sequencer (Applied Biosystem, Foster City, USA).

3. Results

3.1. Construction of poly-His GA

The *gla* gene encoding the enzymatic activity glutaryl acylase from *Acinetobacter* sp. strain YS114 has been cloned using the same procedure developed to clone the *pac* genes encoding the penicillin acylases from *Kluyvera citrophila* and *E. coli* W ATCC11105 [18]. Glutaryl–L-leucine was now used as selective substrate instead of phenylacetyl–L-leucine [5]. The *gla* gene was expressed in *E. coli* under the control of the *tac* promoter in the plasmid pJC200. The sequence of the plasmid pJC200 carrying the *gla* gene revealed that this novel GA enzyme presented the typical structural features that characterize other members of the β-lactam acylase family [8]. That is, GA is a heterodimeric enzyme (α and β subunits) derived from a single precursor polypeptide preceded by a signal peptide to allow its translocation to the periplasmic space, where it is proteolytically processed to become active. The poly-His tag was placed in the N-terminal region of the mature α-subunit. In short, a fragment of the *gla* gene was subcloned into the M13tg131 vector to facilitate the creation, by site-directed mutagenesis, of a new *Sma*I site (oligonucleotide SMA-I) in the region encoding the N-terminal sequence of the α-subunit. It is worth noting that the insertion of the *Sma*I site produced a frame-shifting in the mutant *gla* gene contained in the pJCSM72 derived plasmid. This trick facilitated the selection of plasmid pHG6

by enzymatic analysis of the resulting clones, since the insertion of the histidine linker (His-1/His-2) carrying the six histidine codons into the *Sma*I site of plasmid pJCSM72 abolished the frame-shifting and restored the enzyme production. To ascertain that the sequence encoding the 6-His tail had been correctly inserted, the *H-gla* gene contained in the plasmid pGH6 was sequenced.

Sequence analyses of the signal peptide of the host GA revealed the existence of two putative cleavage sites, AFA/LAEP or AFALA/EP. Interestingly, the α-subunit of the purified poly-His GA (see below), showed an N-terminal amino acid sequence LAEPHHH. This result demonstrated that the signal peptide was processed just after the first putative cleavage site.

Crude extracts of *E. coli* TG1 cells harboring plasmid pHG6, encoding poly-His GA showed a high GA activity on glutaryl-7ACA (58 nmol/min/mg protein) (Fig. 1), which is equivalent to the wild type GA activity found in extracts of *E. coli* cells transformed with plasmid pJC200 [5]. This result suggested that the insertion of the poly-His tag has not significantly altered the enzyme structure.

The next objective of this work was to investigate the possibility of purifying the chimeric GA by selective adsorption on chelate support. Also, this enzyme may be used as a model system to optimize the purification of poly-His tagged proteins, in a single step, by IMAC.

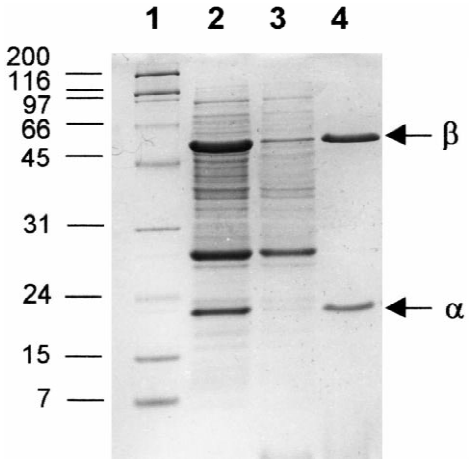

Fig. 1. Over-expression of poly-His GA in *E. coli*. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized with Coomassie blue. Lanes 1=molecular mass markers; 2=crude extract of *E. coli* TG1 (pGH6); 3=crude extract of a control culture of *E. coli* TG1 (pBCKS+); 4=purified poly-His GA following the optimum procedure. Arrows indicate the location of the α- and β-subunits of poly-His GA.

3.2. Metal chelate affinity chromatography

3.2.1. Effect of surface density of the metal chelate on the adsorption of proteins

Table 1 shows that the nature of the metal chelate has a great influence on the percentage of adsorbed proteins. The retention of standard proteins from crude extract from *E. coli* decreased following the sequence Cu>Zn>Ni>Co, using all the commercial supports and our supports BDGE-30. This result agrees with previously published results showing the different requirements and strength of the adsorption of proteins on the different chelates [13,19] and may explain the reason for using Ni^{2+} in purification of His-tagged proteins. The percentage of proteins adsorbed on BDGE-30–IDA–Cu (96%) was 5-fold higher than that adsorbed on BDGE-30–IDA–Co (18%). However, all supports were able to fully adsorb the poly-His tagged GA from pure poly-His-tagged glutaryl acylase preparation (more than 95% of the enzyme activity was removed from the supernatant).

The percentage of natural proteins adsorbed on the supports decreased along with the reduction of the number of chelate groups in the support (10 μmol/ml gel) (conditions where multipoint interactions were more difficult) [4]. For example, the percentage of adsorbed natural proteins from *E. coli* extract decreased from 96 to 78% using BDGE-10–IDA–Cu^{2+} instead of BDGE-30–IDA–Cu^{2+}. Such reduction in the amount of absorbed proteins was larger when the affinity of the chelate for imidazole groups decreased, i. e., using BDGE–IDA–Co as supports the percentage of immobilized protein dropped from 18% in BDGE-30–IDA–Co to 3% in BDGE-10–IDA–Co. This drop in the adsorption yield by a 6-fold factor, when the concentration of IDA groups was only reduced by a 3-fold factor, reinforce the idea that most of the proteins adsorbed to the gel were adsorbed via a multipoint interaction. Thus, the adsorption rate of natural proteins appears to be related to the ligand density on the surface of the

Table 1
Effect of metal chelate on the adsorption of proteins on supports activated with different density of metal chelate supports[a]

Support	$Metal^{2+}$	% Adsorbed natural proteins	% Adsorbed tagged glutaryl acylase
BDGE-30–IDA–Me^{2+}	Cu	96	>95
	Zn	33	>95
	Ni	23	>95
	Co	18	>95
BDGE-10–IDA–Me^{2+}	Cu	78	>95
	Zn	22	>95
	Ni	7	>95
	Co	3	>95
Pharmacia Biotech's support	Cu	93	>95
	Zn	30	>95
	Ni	20	>95
	Co	15	>95
Sigma's support	Cu	97	>95
	Zn	37	>95
	Ni	28	>95
	Co	22	>95

[a] The percentage of adsorbed natural proteins and adsorbed glutaryl acylase were measured by Lowry's method and activity assay, respectively, after 1 h of gently stirring at 25°C

support rather than by the total concentration of groups present in the adsorption media. In fact, the protein adsorption rates observed using three volumes of BDGE-10–IDA–(Co^{2+} or Zn^{2+}) were much lower than that found when using one volume of BDGE-30–IDA–(Co^{2+} or Zn^{2+}) (data not shown).

However, the adsorption of pure poly-His tagged GA was kept almost intact when using the low-density chelate supports. The use of BDGE-30–IDA–Me^{2+} rendered adsorption rates of the poly-His GA around 3-fold higher than that observed using BDGE-10–IDA–Me^{2+} (using the same volume of gel in both cases). Using three volumes of BDGE-10–IDA–Me^{2+} the immobilization rate becomes almost identical to the immobilization rate when using one volume of BDGE-30–IDA–Me (data not shown). This linear dependence between the adsorption rate and the residue concentration in the reaction media but no on the residue surface density suggested that the poly-His GA was adsorbed by a interaction with a single chelate on the support. Thus, low surface density of chelate groups seems to be necessary to prevent the adsorption of natural proteins on the supports, but it keeps the adsorption of the poly-His tagged proteins strong enough. In this sense, commercial supports having a high density of iminodiacetic groups seem to be very adequate for adsorption of most natural proteins, but not so adequate if this adsorption is not desired.

3.3. Influence of the spacer arm length on the adsorption of proteins

It has been found that the spacer arm length has a great effect in the capacity of chelate gels for adsorbing natural proteins from *E. coli* extract (EPI-10–IDA–Zn^{2+} and BDGE-10–IDA–Zn^{2+}). The amount of standard proteins adsorbed on BDGE-10–IDA–Zn^{2+} (long spacer arms) is around twice higher than of the adsorbed protein on EPI-10–IDA–Zn^{2+} (short spacer arms) (Table 2). However, this has a negligible effect on the adsorption of pure poly-His tagged GA.

Thus, the use of metal chelate supports with short spacer arms and low density of chelate groups as EPI-10–IDA–Me^{2+} decreased the non-selective protein adsorption out of crude extract from *E. coli*. This type of tailor-made chelate-supports may be very useful for the selective adsorption of poly-His tagged

Table 2
Effect of the length of spacer arms on adsorption of proteins; the percentage of adsorbed proteins was measured as previously described in Table 1

Support	% Adsorbed natural proteins	% Adsorbed tagged GA
BDGE-10–IDA–Zn^{2+}	22	>95
EPI-10–IDA–Zn^{2+}	9	>95

proteins. Commercial supports having long spacer arms do not seem to be the best option to obtain a selective adsorption of poly-His tagged proteins.

3.4. Selective adsorption of poly His-tagged GA

A crude extract of *E. coli* TG1 (pHG6) containing the poly-His GA was applied to different metal chelate supports. Results agree fully with those obtained in individual experiments using poly-His tagged GA and a crude extract from *E. coli*. Table 3 shows that poly-His GA was almost completely adsorbed on all the lowly activated metal chelate supports (EPI-10–IDA–Me^{2+}), without a significant dependence on the metal chelate used. However, the adsorption of host proteins was strongly dependent on the chelate employed. Copper chelate adsorbed a great amount of the host proteins while nickel chelate only adsorbed a small amount of host proteins together with the poly-His GA. The retention of host proteins was very low on EPI-10–IDA–Zn and EPI-10–IDA–Co, allowing the almost exclusive adsorption of poly-His GA to these supports (>90% of the adsorbed protein was our target protein). These results demonstrate that an adequate design of the support properties (type of chelate, superficial density of chelates and length of the spacer arm) facilitates a very rapid and easy-to-perform purification of poly-His GA by its selective unipunctual adsorption on metal chelates. Natural-GA was not adsorbed on these tailor-made supports.

Table 3
Effect of the metal chelate on the adsorption of host proteins and poly-His GA; the percentage of adsorbed proteins was measured as previously described in Table 1

Support	% Adsorbed host protein	% Adsorbed poly-His GA
EPI-10–IDA–Cu^{2+}	65	>95
EPI-10–IDA–Zn^{2+}	5	>95
EPI-10–IDA–Ni^{2+}	<5	>95
EPI-10–IDA–Co^{2+}	<5	>95

3.5. Single-step purification of poly-His tagged GA

According to the results, it has been possible to design a very simple protocol for a complete purification of His-tagged glutaryl acylase:

A 10-ml volume of EPI-10–IDA–Ni^{2+} or Co^{2+} was added to 5 l of a crude protein preparation (5 mg/ml) from *E. coli* dissolved in 10 m*M* sodium phosphate buffer, pH 7.0, containing 0.5 *M* NaCl. The suspension was very gently stirred for 5 h in order to adsorb all tagged GA. Then, the gel was filtered and washed with buffer. Small traces (<5% of all adsorbed protein) of host proteins were further removed by washing the gel with the same buffer containing 20 m*M* imidazole. Finally, the poly-His GA was desorbed with 50 m*M* imidazole in a completely pure form (Fig. 1, lane 4).

This protocol yields a purification factor of 120 with an activity recovery >80% (see Fig. 1).

3.6. Selective adsorption of poly-his fused proteins on copper chelate supports

At first glance, the protocol developed above for a batch single-step purification of poly-His GA could be extended to any other industrial protein, provided that the poly-His tail is fairly accessible to the support. Nevertheless, since some metal ions may cause inactivation/inhibition effects on certain proteins, the possibility of developing single-step purifications by using any metal chelate (e.g., Ni^{2+}, Co^{2+}, Cu^{2+} or Zn^{2+}) appears to be a very useful tool in protein technology. Thus, taking poly-His GA as a model enzyme we have also tried to design purification protocols by using other metals such as Cu^{2+}.

The adsorption of natural proteins on transition metal chelate supports mainly takes place through the interaction with the imidazole ring of histidine residues [13]. Then, the adsorption of host proteins should decrease when the adsorption process is carried out in the presence of a competitive ligand like imidazole [20]. Thus, the presence of 7 m*M* imidazole reduced the adsorption of host-proteins using most commercial supports or tailor-made

Table 4
Adsorption of host proteins and tagged GA on different iIDA-Cur supports in the presence of imidazole 7 m*M*; the percentage of adsorbed proteins was measured as previously described in Table 1

Support	% Adsorbed host proteins	% Adsorbed tagged GA	Purification factor
EPI-30–IDA–Cu^{2+}	41	>95	10
BDGE-10–IDA–Cu^{2+}	20	>95	50
EPI-10–IDA–Cu^{2+}	<5	>95	100

supports with immobilized Cu^{2+}. Table 4 shows that this reduction of the amount of host-protein adsorption, in agreement with the previous results, reached optimal values using low concentration of Cu^{2+} groups attached to the support via short spacer arms. In all cases, the tagged GA was fully adsorbed to the supports. In this way, the purification of the target protein reached values very close to that achieved by using Co or Ni tailor-made supports. Thus, the combination of tailor-made supports and an adequate design of the adsorption conditions greatly improve the selectivity of the copper chelate supports.

4. Discussion

A number of results discussed in this paper, clearly support the hypothesis, previously suggested by Johnson and Arnold [21] and Todd et al. [4], that the adsorption of natural proteins on metal chelate supports mainly occurs through a multipoint mechanism involving several weak interactions between several residues of each protein and several chelate groups of the support. This mechanism is illustrated by the dramatic effect of the surface density of chelate groups on the rate and extent of adsorption of host proteins from crude extracts. The effect of spacer arms on the protein adsorption is also very interesting. Agarose gels are composed of very thick fibers of ≈200 Å in diameter [22]. When compared to most proteins, these fibers resemble planar surfaces on which proteins become adsorbed. Thus, when using very short spacer arms, only the most external histidine residues placed on the protein surface are able to participate in the adsorption on activated agarose and this short arm is unable to promote any unspecific interaction with the protein [23]. However, using long spacer arms, the immobilized chelate may interact with some residues (e.g. histidines) slightly hidden in protein internal pockets. Also, these long arms may give rise to some unspecific interactions (hydrophobic) with the proteins. Both phenomena could explain the dramatic decrease of the adsorption rate of host proteins when decreasing the length of the spacer arm even at low surface density of chelate groups. Thus, supports having low chelate surface density and short spacer arms might only absorb host proteins that naturally have, by random, two or more His placed at the right distance to interact with a single chelate residue.

In contrast to natural proteins, poly-His tagged proteins seem to be able to become adsorbed on metal chelates through a one-point strong adsorption. Poly-His GA becomes adsorbed even on low activated supports containing low affinity metal ions (e.g. cobalt). Besides, the rate of adsorption does not depend on the density of chelate groups on the support surface but only on the total concentration of groups in the immobilization system. Furthermore, the adsorption rate does not depend on the length of the spacer arm. In this case, the poly-His tail was placed on the amino terminal region which is usually fairly exposed to the medium. A highly exposed poly-His tail should be able to interact, rapidly and strongly, with chelate groups even when they are very close to the large planar surface of agarose gels.

Also, from this different adsorption mechanisms of natural and poly-His tagged proteins, it is possible to assume an additional advantage of the selective adsorption of the poly-His tagged proteins. It may be expected that using standard supports (highly activated supports with long spacer arms), standard proteins with a relative high density of reactive groups on their surfaces may become strongly adsorbed via multipoint interactions. In some cases,

this adsorption may become as strong as that of the poly-His tagged proteins, making very difficult the final separation among these proteins and the tagged proteins. Using the tailor-made supports proposed in this paper, these proteins will not be adsorbed on the supports, because multipoint interaction is now impossible, therefore the preemptive desorption of these proteins may not be such a problem.

Such selective adsorption provides a number of advantages for purification of tagged proteins both on a laboratory and an industrial scale:

1. Much smaller chromatographic columns could be used. Besides, some purification protocols could even be performed in very simple batch reactors
2. Selective desorption of almost-pure fused proteins, selectively adsorbed in a weaker fashion, could be performed under much more gentle experimental conditions (e.g., pH values close to neutrality).

These advantages could represent important technological and economical incentives for the production of pure and inexpensive industrial enzymes.

5. Conclusion

The selective adsorption of poly-His GA may greatly improve the development of chromatographic methods for single-step purification of this interesting industrial enzyme. We assume that this method, here exemplified with poly-His GA, could be applicable to almost any poly-His tagged protein provided that the poly-His tail is placed in the outermost part of the protein structure.

The use of 6% agarose gels containing a low density of metal chelates (5–10 μmol Zn^{2+} or Co^{2+}/ml of gel) secluded from the support through a minimal spacer arm (2 carbon or less if it were possible) is here proposed to get a strong enough but highly selective adsorption of poly-His tagged proteins with minimal undesired adsorption of contaminant proteins. On the contrary, commonly up to 30 or 40 μmol of Me^{2+} per ml of gel are attached to most commercial chelating supports through a longer spacer arm (7 or 12 carbon). Hence they are able to adsorb, in addition to the tagged protein, a very high percentage of contaminant proteins and may require very drastic desorption conditions (very low pH values or very high imidazole concentration).

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References

[1] J. Crowe, H. Döbeli, R. Gentz, E. Hochuli, D. Stüber, K. Henco, Methods Molec. Biol. 31 (1994) 371–387.
[2] S. Piesecki, W.-Y. Teng, E. Hochuli, Biotech. Bioeng. 42 (1993) 178–184.
[3] J. Porath, J. Carlsson, Y. Olsson, G. Belfrage, Nature (Lond.) 258 (1975) 598–599.
[4] R.J. Todd, D. Johnson, F.H. Arnold, J. Chromatogr. A. 662 (1994) 13–26.
[5] C. Croux, J. Costa, J. L. Barredo, F. Salto, Eur. Pat. 91307108.0 (1991).
[6] B.L. Wong, Y-Q. Shen, Int. Appl. Pat. PCT/US90/01696 (1990).
[7] S.W. Queener, Antimicrob. Agents Chem. 34 (1990) 943–948.
[8] B.S. Deshpande, S.S. Ambedkar, V.K. Sudhakaran, J.G. Shewale, World J. Microbiol. Biotechnol. 10 (1994) 129–138.
[9] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[10] H. Hubert, J. Porath, J. Chromatogr. 198 (1980) 247–255.
[11] L. Sundberg, J. Porath, J. Chromatogr. 90 (1974) 87–98.
[12] P. Armisén, Doctoral Dissertation, Complutense University, Madrid, 1997.
[13] E.S. Hemdan, Y.-J. Zhao, E. Sulkowski, J. Porath, Proc. Nat. Acad. Sci. USA 86 (1989) 1811–1815.
[14] O.H. Lowry, M.J. Rosebrough, A.C. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[15] A. Bastida, P. Sabuquillo, P. Armisen, R. Fernandez-Lafuente, J. Huguet, J.M. Guisan, Biotechnol. Bioeng. 58 (1998) 486–493.
[16] U.K. Laemmli, Nature 27 (1970) 680–685.

[17] K. Balasingham, D. Warburton, P. Dunnill, D. Lilly, Biochim. Biophys. Acta 276 (1972) 250–256.
[18] J.L. García, J.M. Buesa, J. Biotechnol. 3 (1986) 187–195.
[19] E. Sulkowski, in: S.K. Sikdar, P.W. Todd, M. Bier (Eds.), Frontiers in Bioprocessing, CRC Press, Boca Raton, FL, 1989.
[20] S. Vunnum, S.R. Gallant, Y.J. Kim, S.M. Cramer, Chem. Eng. Sci. 50 (1995) 1785–1803.
[21] R.D. Johnson, F.H. Arnold, Biotechnol. Bioeng. 48 (1995) 437–443.
[22] A.S. Medin, Doctoral Dissertation Uppsala University, Uppsala, 1995.
[23] F.B. Anspach, J. Chromatogr A 676 (1994) 249–266.