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Purification of Native Proteins from the Cytoplasm and Periplasm of *Escherichia coli* Using IMAC and Histidine Tails: A Comparison of Proteins and Protocols

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Several proteins were purified in a single step from *Escherichia coli* in native form using fused histidine tails and immobilized metal affinity chromatography (IMAC). The procedures reported and compared are those for antibody fragments (single-chain Fv fragments and V_L domains) carrying a C-terminal $(His)_5$ tail purified from the periplasm, the *E. coli* disulfide isomerase carrying a C-terminal $(His)_6$ tail purified from the periplasm, and yeast citrate synthase carrying both a N-terminal and a C-terminal $(His)_5$ tail purified from the cytoplasm. Because of blocks in the periplasmic folding process, the investigated murine single-chain T-cell receptor could be purified only in denatured form, despite being soluble, thereby demonstrating the sensitivity of the native purification to the folding state. A comparison of columns and metal ions showed that Zn^{2+}–iminodiacetic acid and Ni^{2+}–nitrilotriacetic acid gave equally good one-step purifications of V_L domains and Fv fragments, but only in this combination of metal and chelator. The buffer is of secondary importance, and the preferred method of elution depends on the stability of the protein. In a crystal structure determination of the V_L domain carrying the (metal-free) histidine tail, no significant electron density beyond the first two histidine residues was detected, implying a disordered structure of the unliganded affinity tail. When IMAC is used under denaturing conditions, an *E. coli* protease can be co-purified and refolded or co-purified under native conditions, and sensitive proteins can be digested during their *in vitro* refolding. Plasmid-encoded wild-type RTEM β-lactamase was obtained as a side product in pure form by IMAC in a single step. © 1992 Academic Press, Inc.

The study of proteins and their biotechnological or medical application nearly always requires homogeneous preparations. Usually, a significant part of the effort in studying each protein has thus gone into developing purification schemes individually tailored to each protein. Recombinant DNA technology not only can improve the starting material by the overproduction of the protein of interest, but also can provide an access to "affinity handles," sequence motifs or protein domains that might be used in a general way on all recombinant proteins, and thus lead to general purification procedures no longer dependent on the individuality of the protein.

Currently, three strategies can be distinguished: The first is the use of protein domains or whole proteins with a known affinity for ligands. We refer to this as the affinity domain, and the recombinant protein of interest fused to this as the passenger protein. Popular fusion partners are β-galactosidase, maltose binding protein, and glutathione *S*-transferase (1, 2), but this principle can be used with any protein for which a ligand-affinity purification is available. The advantage of this approach is that the good expression of the car-

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rier protein is often also observed with the hybrid protein. The disadvantage is that the affinity domain must usually be cleaved off and the passenger domain must then be purified again. Furthermore, the binding of the hybrid protein to the immobilized ligand depends only on the correct folding of the affinity domain and in no way implies or guarantees the correct folding of the passenger domain. Such an example of incorrect passenger folding has been found in β-lactamase/T-cell receptor fusions (Wülfing and Plückthun, unpublished data).

The second strategy is the use of peptidic affinity handles. Although in principle peptides with affinities to any protein can be used, peptide epitopes for known antibodies are most convenient because conventional technologies of immunoaffinity detection and purification can be used. For example, a known epitope of the Myc oncoprotein (3) has been used previously (4, 5) for detection purposes and was also employed in some of the studies reported here. The general disadvantage to using this principle in protein purification, however, is that an upscaling is rather costly because an immobilized protein is required on the column in at least stoichiometric amounts.

The third strategy is related to peptide handles and consists of using a short histidine tail as an affinity handle for immobilized metal ions (6–12). Although in principle all surface-bound, accessible histidines can form contacts with the immobilized metal ion, a stretch of consecutive histidines, which can be added by recombinant technology to any protein even in the complete absence of structural information, can serve as an efficient affinity handle for protein purification. We show in this article, however, that correct folding of the protein is a decisive factor in the success of this method.

Only three limitations of this general approach that are intrinsic to the protein domain under study can be seen at this point: The first is a requirement for an exact native sequence at both the N- and the C-terminus for structural or functional reasons, in which case an affinity handle might be incompatible with either. The second is the requirement for accessibility of the N- or C-terminus under the conditions used for purification. The latter limitation might sometimes be overcome by inserting a spacer, e.g., containing one of the commonly used protease cleavage sites to cleave off the extension after purification, between the passenger domain and the affinity handle. The third is the possibility that the affinity handle might change the general properties of some proteins, e.g., if it interfered with the folding pathway, or stabilized a nonnative state. In such a case, cleavage of the handle would be necessary as well.

In addition to the generality of the method, affinity handles have a number of other attractive features: Very often, purification of only the native form is possible because nonnative protein frequently aggregates, sometimes even without giving a precipitate (see the section on the T-cell receptor below). For this reason and/or because of the misfolding itself, the affinity tail would be inaccessible. Furthermore, in studying derivatives of a protein encoded by the host, the host gene would not necessarily have to be inactivated, as the affinity tail would secure an easy separation from the wild-type version encoded in the genome. By the same strategy, mutants of essential proteins, which would by themselves not support growth, can be expressed with an affinity tail and easily separated from the simultaneously expressed wild type protein encoded on the chromosome.

Although a number of chelators have been used for immobilized metal affinity chromatography (13–17), the two most popular resins for protein purification are iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA). In principle, a variety of metals can be bound, but those with a high affinity for histidine ligands are most useful: Ni^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+}. Furthermore, it is not necessarily the highest affinity for the recombinant protein that is desired, but rather the highest selectivity with respect to the bulk of the host proteins. From these considerations, Ni^{2+} and Zn^{2+} seem to be the most promising candidates, as the affinity of most *Escherichia coli* proteins to these immobilized metal ions is only very moderate. A high degree of selectivity for the recombinant protein is thus possible. The columns are usually run with a high salt concentration to eliminate as much as possible the retardation of contaminating proteins by other, nonspecific ionic interactions (i.e., ion-exchange effects) and thereby improve the selectivity of the metal for the histidine ligands. Two different types of elution have been described (13–17): (i) In elution by a competing ligand for the metal, imidazole has been used most frequently. It probably competes with the histidine residues of the protein for the metal ion, and perhaps also somewhat displaces the metal–protein complex from the chelating ligand on the column (see the section on citrate synthase below). In addition to imidazole, glycine and ammonium chloride have also been used. (ii) In elution by pH, protons compete with the metal in binding to the histidine residues, but may also protonate the carboxylates of the chelating ligand on the column and thus elute the protein–metal complex.

GENERAL CONSIDERATIONS IN DESIGNING IMAC AFFINITY HANDLES

The first consideration in designing the affinity handle is its location. Usually, the N-terminus or the C-terminus of the protein is the position of choice. In this regard, maintenance of activity of the recombinant protein is the chief concern. In the case of recombinant antibody fragments, the C-terminus of each domain is pointing away from the binding site and is thus the obvious attachment place. In the case of an antibody single-chain Fv fragment secreted to the periplasm, a location in the linker connecting the V_L and V_H domains was also attempted using the sequence GGGGSHHHHHGGGGS (Pfitzinger and Plückthun, unpublished), but leads to insufficient retardation on a Zn^{2+}–IDA column to be generally useful for purification.

A very important factor for the success of the method is the accessibility of the affinity handle. This requires either that the protein must be folded correctly, that it must not aggregate, and the affinity handle must be accessible in the native structure (native purification) or that any aggregation of the recombinant protein with itself or other proteins must be prevented using strong denaturants (denaturing purification). While in the case of proteins with known structure, the choice of the N- or C-terminus can of course be made rationally, indirect arguments can often be used, e.g., if the N- or C-terminus of the protein is interacting with a receptor or connecting to another domain. An example is the purification of the periplasmically expressed human protein–disulfide isomerase (PDI) from *E. coli* using IMAC (data not shown). As a protein localized in the endoplasmic reticulum (ER), it contains the ER retention sequence Lys–Asp–Glu–Leu (18), which presumably interacts with a receptor localized in the ER. It was thus a reasonable assumption that this ER retention sequence must be accessible and that the $(His)_6$ sequence placed at the C-terminus will thus also be available for interactions with a column-bound metal chelate, and it proved correct.

In the following we present several case studies of different proteins to compare the effects of multivalency, column conditions, and expression conditions on the purification of native recombinant proteins from *E. coli.* We have also compared these to the purification of a denatured protein using 6 M guanidinium chloride by IMAC.

RESULTS AND PROTOCOLS

Materials

The iminodiacetic acid column material used in these studies was Chelating Sepharose Fast Flow, Pharmacia Biosystems GmbH, Freiburg, Germany. The nitrilotriacetic acid column material used in these studies was from Diagen GmbH, Düsseldorf, Germany.

A. Citrate Synthase: The Favorable Case

Eukaryotic citrate synthase (EC 4.1.3.7) is a homodimer with an approximate molecular weight of 100 kDa. The enzyme from yeast mitochondria used in these studies is sufficiently homologous (19) to the one from pig heart that its 3D structure can be used as a guide (20, and citations therein) (Fig. 1).

A tail of five histidine residues was fused to both the N- and the C-termini. The wild-type and the derivative

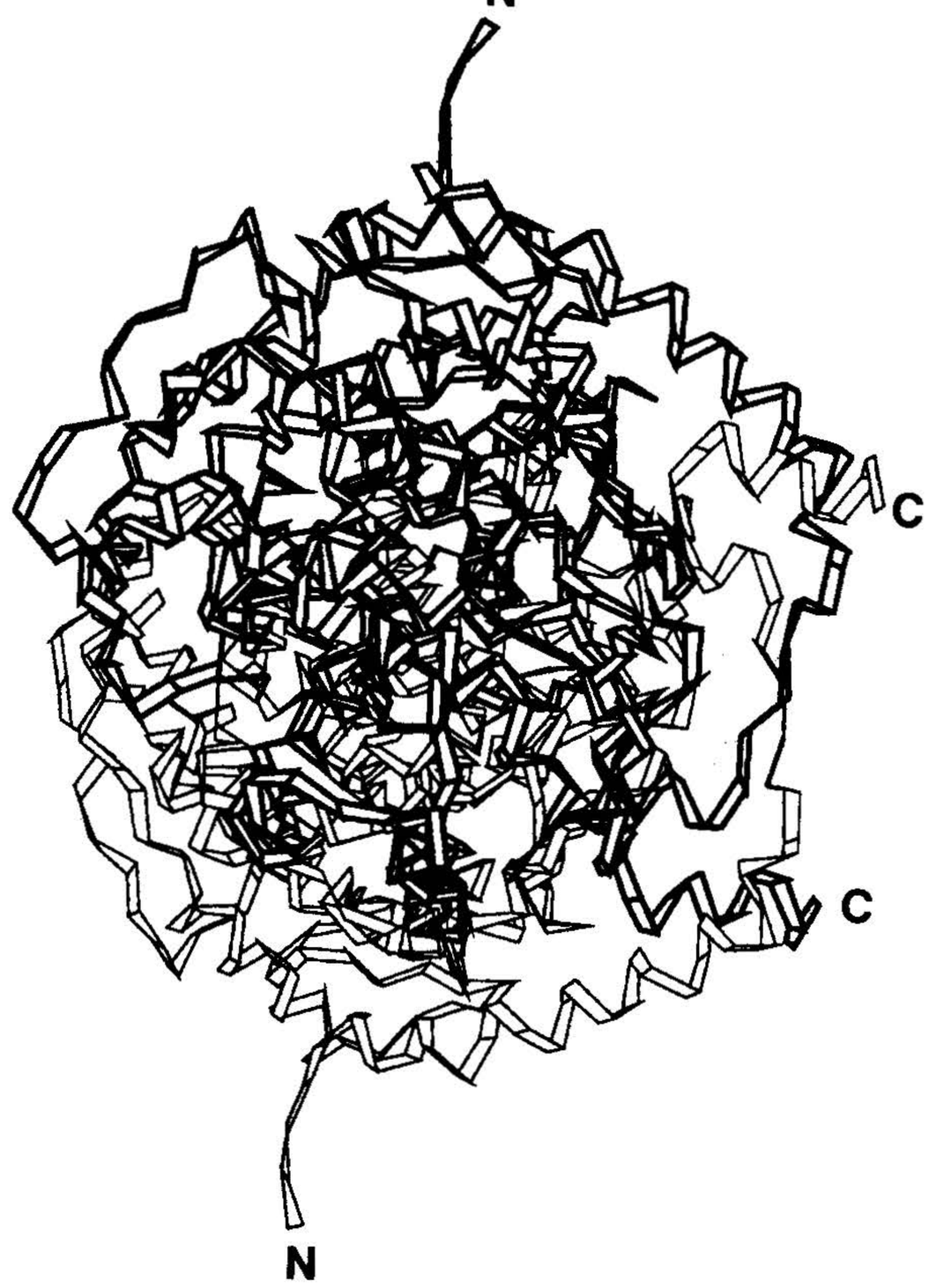

FIG. 1. Ribbon model of pig heart citrate synthase. One of the two identical subunits is shown in bold lines. The natural N- and C-termini are labeled.

could be functionally expressed in the cytoplasm of *E. coli* and purified by a modification of the protocol reported previously (12). The specific activity of the doubly modified enzyme was determined (Lindner and Plückthun, manuscript in preparation) and it was found that the average value of 105 units/mg protein is identical to that of the unmodified protein purified from the yeast *Saccharomyces cerevisiae* directly and lies in the same range as previous literature values (21–23).

Since eukaryotic citrate synthase is a homodimer, the enzyme variant containing both the N-terminal and the C-terminal handle carries a total of four $(His)_5$ tails (Fig. 1). In gradient elution experiments, the affinity to the column was found to be high enough to lead to such a good separation (Fig. 2A) that batch elution could be used (Figs. 2B and 2C). Three factors are believed to be important for this favorable result: (i) The multivalency effect of the multiple peptide tails improves the binding to the column considerably. (ii) The Zn^{2+}–IDA resin was used in order to obtain a rapid elution of the bulk *E. coli* proteins, since the affinity of the recombinant protein with four histidine tails was high enough to be of no concern. (iii) The tails are fully accessible and do not disturb correct folding or interfere with the active site, nor is there any sign of proteolytic degradation.

This has led to the simplified protocol I for the homogeneous preparation of yeast citrate synthase from *E. coli* (Fig. 2C), which can probably be adapted to similar cases. Interestingly, when the protein is dialyzed against buffers containing no EDTA, it precipitates. Possibly some Zn^{2+} that is washed from the column is bound to the histidine tails, and when imidazole from the elution buffer is removed by dialysis, some remaining Zn^{2+} may reversibly crosslink the protein via the histidine tails. When the protein is then dialyzed against buffers containing EDTA (2 mM), however, the precipitate dissolves again and full activity is restored. No precipitation is observed when the protein is dialyzed against EDTA directly after purification.

PROTOCOL I: PURIFICATION OF YEAST CITRATE SYNTHASE FROM THE CYTOPLASM OF *E. coli*

E. coli strain W620 (CGSC 4278), deficient in the endogenous citrate synthase gene (24, and citations therein), is transformed with an expression plasmid carrying the gene for the major yeast citrate synthase, CIT1 (19, 25), with its mitochondrial signal sequence deleted, under the control of the *lac* promoter (Lindner and Plückthun, manuscript in preparation). The gene was extended by 5 histidine codons each at the N-terminus and C-terminus of the protein. Yeast citrate synthase is expressed in fully active form in the *E. coli* cytoplasm.

The cells are grown at room temperature to an OD_{550} of 0.5 in LB medium containing the appropriate antibiotics, and the *lac* promoter is induced by adding IPTG to a final concentration of 1 mM. After overnight growth the cells are harvested by centrifugation and resuspended in 1/150 vol of ice-cold loading buffer (50 mM Tris–HCl, 1 M NaCl, pH 8.0). The following steps are performed at 4°C. The cells are disrupted twice in a French press (SLM Instruments Inc., Urbana, IL) at 18,000 psi (=1.24 kbar) and centrifuged for 30 min at 47,000*g*.

Ten milliliters of the IDA column material, suitable for working up 4 liters of *E. coli* culture at an OD_{550} of 2 to 3, is packed into a glass column (1-cm diameter) and washed with at least 100 ml of cold distilled water. This results in approximately 5.5 ml of gel bed. The gel is loaded with Zn^{2+} ions by applying a 1 mg/ml solution of $ZnCl_2$ to about 100 mol%, assuming an average capacity of 26 μmol Zn^{2+}/ml of (packed) gel. The column is washed with 100 ml of bidistilled water and then with the same amount of loading buffer (see above). The French pressure cell supernatant is applied directly onto the Zn^{2+}–IDA column at a flow rate of 0.6 ml/min and the column is washed with loading buffer until no significant decrease in the OD_{280} can be detected (about 100 ml). About 50 ml of preelution buffer (50 mM Tris–HCl, 1 M NaCl, 50 mM imidazole, pH 8.0) is enough to obtain a sharp peak of weakly bound contaminants and reach the baseline level again.

Pure citrate synthase can be eluted with 50 mM Tris–HCl, 1 M NaCl, 200 mM imidazole, pH 8.0. The elution peak is collected in separate fractions. Thus, relatively high concentrations of citrate synthase (about 1–1.5 mg/ml) can be obtained. The overall yield of the wild-type enzyme is at least 5 mg/liter of *E. coli* culture.

The IDA column is regenerated by applying 100 ml of regeneration buffer (50 mM EDTA, 1 M NaCl, pH 8.0) and stored in 20% ethanol at 4°C. The column material can be used several times without a detectable loss of purification capacity.

B. Recombinant Antibody Domains: Histidine Tails Are Compatible with Membrane Transport and Folding

General Considerations

Previously, we have demonstrated the feasibility of the purification of native antibody single-chain Fv fragments (scFv fragments) (26–28) using a tail of histidines fused to the C-terminus (12). These experiments

showed that the histidines at the C-terminus are compatible with transport through the *E. coli* membrane. This stands in marked contrast to a cluster of 5 arginines, which leads to severe problems of plasmid stability, probably because the transport becomes blocked and the protein is thus toxic for the *E. coli* cell (12). The length of the histidine affinity tail has also been investigated. While a longer tail of 9 histidines leads to stronger affinity to the column, it also was found to decrease the total amount of recombinant protein. Antibody fragments that are retained in the reducing environment of the cytoplasm, and thus cannot fold to their native state, are efficiently degraded (29, 30). Thus we may see here a limitation to the length of the histidine tail which results from an interference of large clusters of positively charged residues with the normal transport processes of the cell.

Since the N-terminus is generally close to the combining site of the antibody, we have concentrated on using a single handle at the C-terminus of a variable domain or a scFv fragment. This approach should therefore be general. It is of course possible that some antigens would bind in the presence of an N-terminal affinity handle as well. To minimize the perturbation of the antibody structure, the last two basic residues of V_L (Lys 107, Arg 108; numbering according to (31))

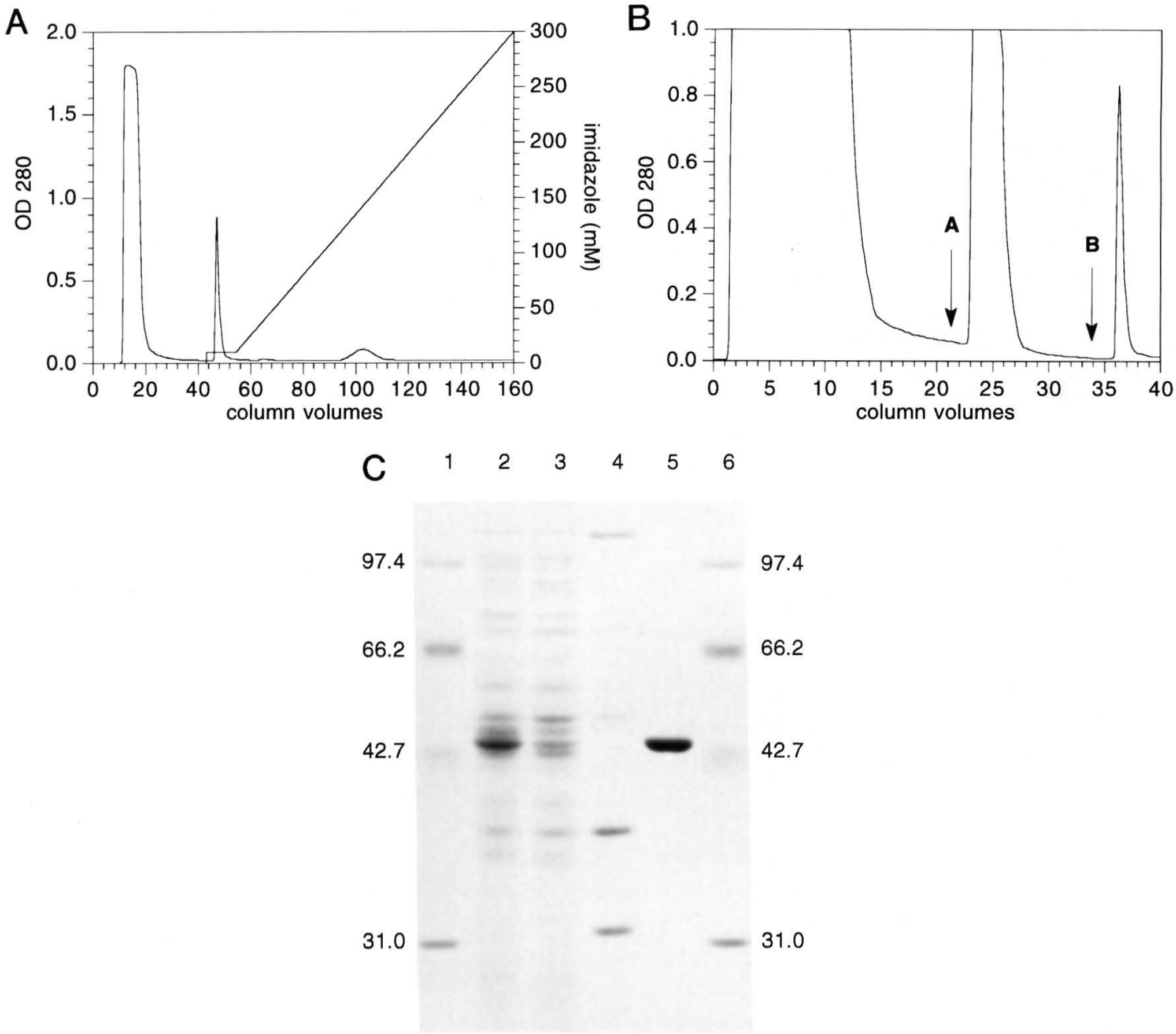

FIG. 2. (A) Gradient elution of citrate synthase with an imidazole gradient of 10–300 mM in loading buffer. (B) Batch elution of citrate synthase: at arrow A washing with 50 mM imidazole in loading buffer was started; at arrow B elution with 200 mM imidazole in loading buffer was started. (C) SDS–PAGE (Coomassie stained) of batch-eluted citrate synthase: lane 1, MW marker; lane 2, crude extract after French press; lane 3, column flow-through; lane 4, pre-elution with 50 mM imidazole; lane 5 elution with 200 mM imidazole; lane 6, MW marker.

were replaced by histidines in V_L or scF_v, and 3 histidine residues were added to the C-terminus. Judging from the structure of the recombinant wild-type domain (32) as well as from the structure of the Fab fragment (33), all 5 histidines should be freely accessible (Fig. 3).

The Histidine Tail Has No Effect on the 3D Structure of the Antibody Domain

A mutated form of the V_L domain of the antibody McPC603 (32, 33) containing the histidine extension (Steipe *et al.*, in preparation) was purified by IMAC as described in protocols II and III. The purified material was dialyzed against 5 mM Mops, pH 7.0, to provide a basis buffer for adding the appropriate precipitant. It was then concentrated to about 10 mg/ml, and crystallization was achieved at pH 4.0 using 1.8 M $(NH_4)_2SO_4$ (32, 34).

The histidine extension has no effect on the structure compared to the same V_L domain purified from the Fv fragment after chain separation (32) (Fig. 3). Both histidines that replace residues from the wild-type sequence have visible electron density for backbone and side-chain atoms. The 3 histidines extending beyond these are mobile and/or disordered and no electron density is visible. At pH 4.0, the histidines will be protonated, and even if Zn^{2+} were still present during the crystallization, it would not be expected to be bound under these conditions. More important, it demonstrates that the tail does not prevent crystallization of this protein; on the contrary, it considerably facilitates the purification of sufficient amounts of protein of a quality suitable for crystallization. A variety of mutants have now been purified and crystallized with this procedure.

Chromatographic Parameters

To test the influence of various purification protocols on the affinity chromatography, the V_L domain of the phosphorylcholine binding antibody MOPC167 (35) was used as a model system. Five histidines were incorporated into the C-terminus by site-directed mutagenesis, as described above. The V_L domain is secreted into the periplasmic space and is thus accessible for purification either from a total cell extract (using French press lysis, analogous to protocol V) or from a periplasmic extract (protocol II). The periplasmic extract is a significant first purification step. EDTA at 1 mM concentration in the periplasmic extract seems to cause no problem in the purification and no run-through of recombinant protein after direct loading of the periplasmic extract on the column is observed. The soluble periplasmic extract from 20 liters of an *E. coli* JM83 culture was split into aliquots, each containing about 1 mg of the protein to be purified. Thus, we can directly compare results from the various elution profiles in the presence of a typical *E. coli* periplasmic protein background.

First, the influence of the column ligand and the metal ion was examined. The iminodiacetic acid chelator and the nitrilotriacetic acid chelator were compared, each with Ni^{2+} or Zn^{2+} as the complexed metal. With the IDA chelator, similar amounts and degrees of purity were obtained for Ni^{2+} and Zn^{2+}, but the bulk of the host protein apparently had a higher affinity to the Ni^{2+}-loaded resin, requiring substantially (3 times) longer wash steps before the gradient elution could be started (Figs. 4A–4D). With the NTA resin, however, Zn^{2+} does not lead to sufficient retention, and the V_L domain is eluted at much lower imidazole concentrations (Figs. 4A–4D). The choice of the optimal metal thus strongly depends on the chelating structure. The behavior of Ni^{2+}–NTA and Zn^{2+}–IDA was comparable for the V_L domain, consistent with our earlier preliminary findings on the scFv fragment (12).

We then tested the influence of the elution method by employing a pH gradient (from 8.0 to 5.0) or an imidazole gradient (from 8 to 150 mM) on V_L domains bound on a Ni^{2+}–NTA column (Fig. 4G). The resolution of the pH gradient elution is very high and the eluted V_L domains are well separated from contaminating proteins. Elution of this protein requires a pH as low as 5.0, and the eluted fractions appeared slightly turbid, suggesting that this protein may not be stable at acidic pH values. In the soluble portion, only half as much V_L was obtained as in the imidazole elutions. Elution using the imidazole gradient also resulted in a well-resolved peak of pure protein, but no turbidity. Imidazole elution appears to be the more general procedure for reasons of protein stability. The major peak eluting at pH 7.5 was identified by SDS–PAGE and N-terminal sequencing as plasmid-encoded pure RTEM β-lactamase.

Finally, the influence of the buffer system on chromatography was investigated (Figs. 4C, 4E, and 4F). This is of interest, since the phosphate buffer originally described by Hochuli *et al.* (8) (50 mM potassium phosphate, 1 M NaCl, pH 8.0) begins to precipitate at temperatures below 8°C. Borate buffer is a good alternative (50 mM sodium borate, 1 M NaCl, pH 8.0) and gives an almost identical elution profile. The amino group in Tris buffer (50 mM Tris–HCl, 1 M NaCl, pH 8.0) is expected to compete with histidine for binding to the

affinity matrix. Indeed, we observe a significantly reduced peak of contaminating proteins in the initial elution step, but the elution of the specifically adsorbed target protein is practically unaffected.

From the comparison of these results, the protocols given in this report were developed. Since the preparation of V_L is a purification of a periplasmic protein, in principle a total cell extract [using French press lysis, see protocol V and Ref. (12)] or a periplasmic extract (protocol II) can be used as the first step. However, the periplasmic extract itself constitutes a partial purification.

Application of the Method to scFv Antibodies

The IMAC purification of a scFv fragment with different affinity tails has been described before (12), and a different loop grafted fragment, carrying a specificity

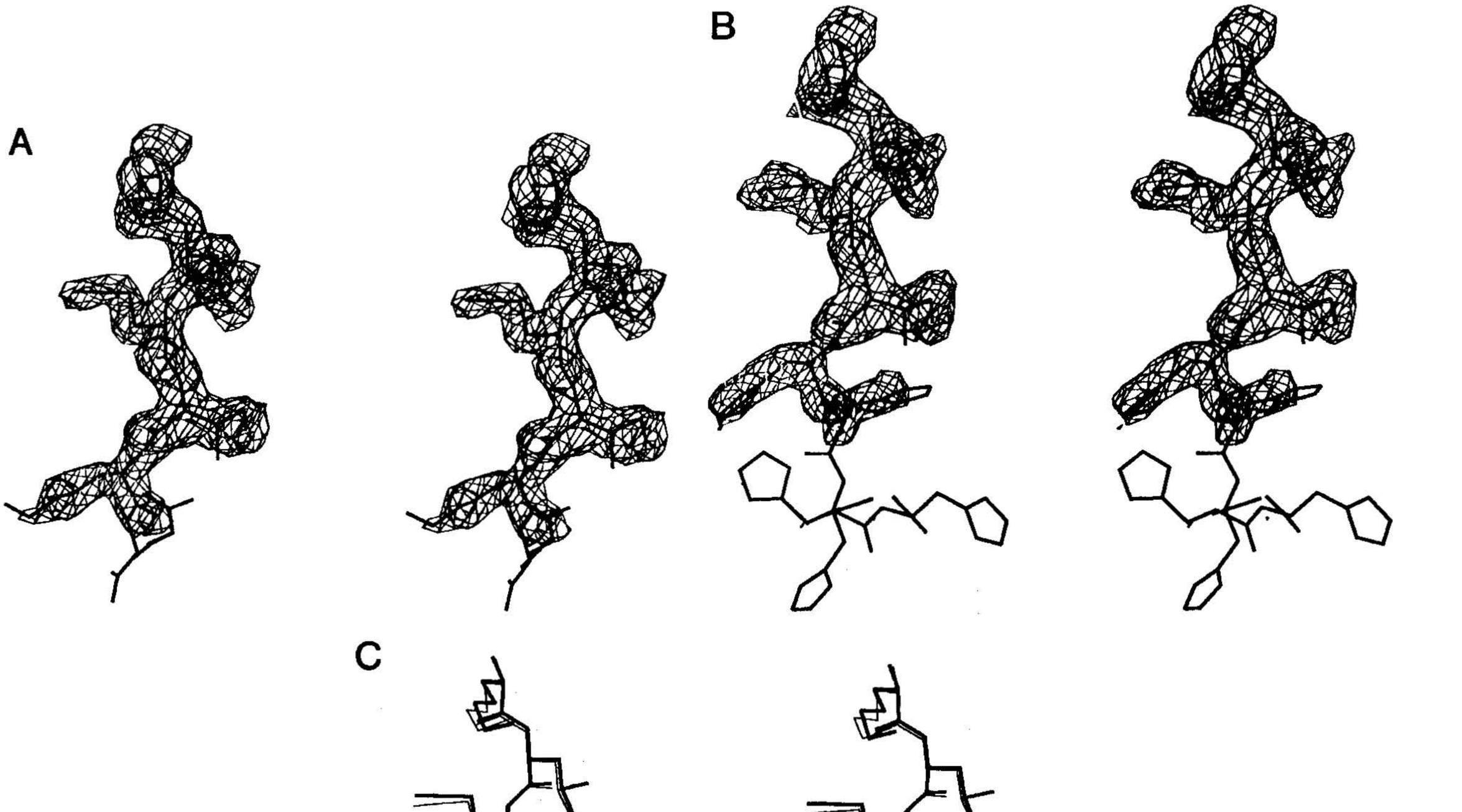

FIG. 3. (A) Stereoview of the C-terminus of the McPC603 V_L domain. Residues from 103 to 108 are shown in electron density contoured at 0.33 $e/Å^3$ (1.0 s). Arg 108 is not defined by electron density. (B) Stereoview of the C-terminus of the McPC603 V_L mutant with a pentahistidine affinity tail. Residues from 103 to 111 are shown in electron density contoured at 0.27 $e/Å^3$ (1.0 s). His 107 and His 108 are still defined in electron density, but the rest of the chain is not visible. It has been modeled along the hinge region coordinates of the McPC603 Fab fragment to illustrate a possible conformation of the affinity handle. (C) Stereoview of a superposition of the C-termini of the McPC603 V_L domain (bold) and the histidine tail mutant (thin). The local structure is not perturbed by the mutation.

for lysozyme (Müller and Plückthun, in preparation), was now also subjected to the modified procedure. The results show that for the scFv fragment as well, the Zn^{2+}–IDA and the Ni^{2+}–NTA give comparable results.

Protocol II: Periplasmic Expression of Antibody Fragments: Growth of Bacteria and Preparation of Periplasmic Extract

E. coli strain JM83 containing the appropriate plasmid encoding the recombinant antibody fragment under the control of a *lac* promoter is grown in 20 liters of LB medium at room temperature to an OD_{550} of about 0.6. The induction is carried out with 1 mM IPTG for 3 h. The bacteria are centrifuged at 4300*g* at 4°C. Pellets are transferred to a precooled weighed glass beaker with a spatula, taking care not to contaminate the pellets with the medium. Per 1 g of bacteria, 2 ml of column buffer [buffer 1: 100 mM Tris–HCl, 1 M NaCl, pH 8.0 (used in the comparative experiments described in Fig. 4); or buffer 2: 50 mM potassium phosphate,

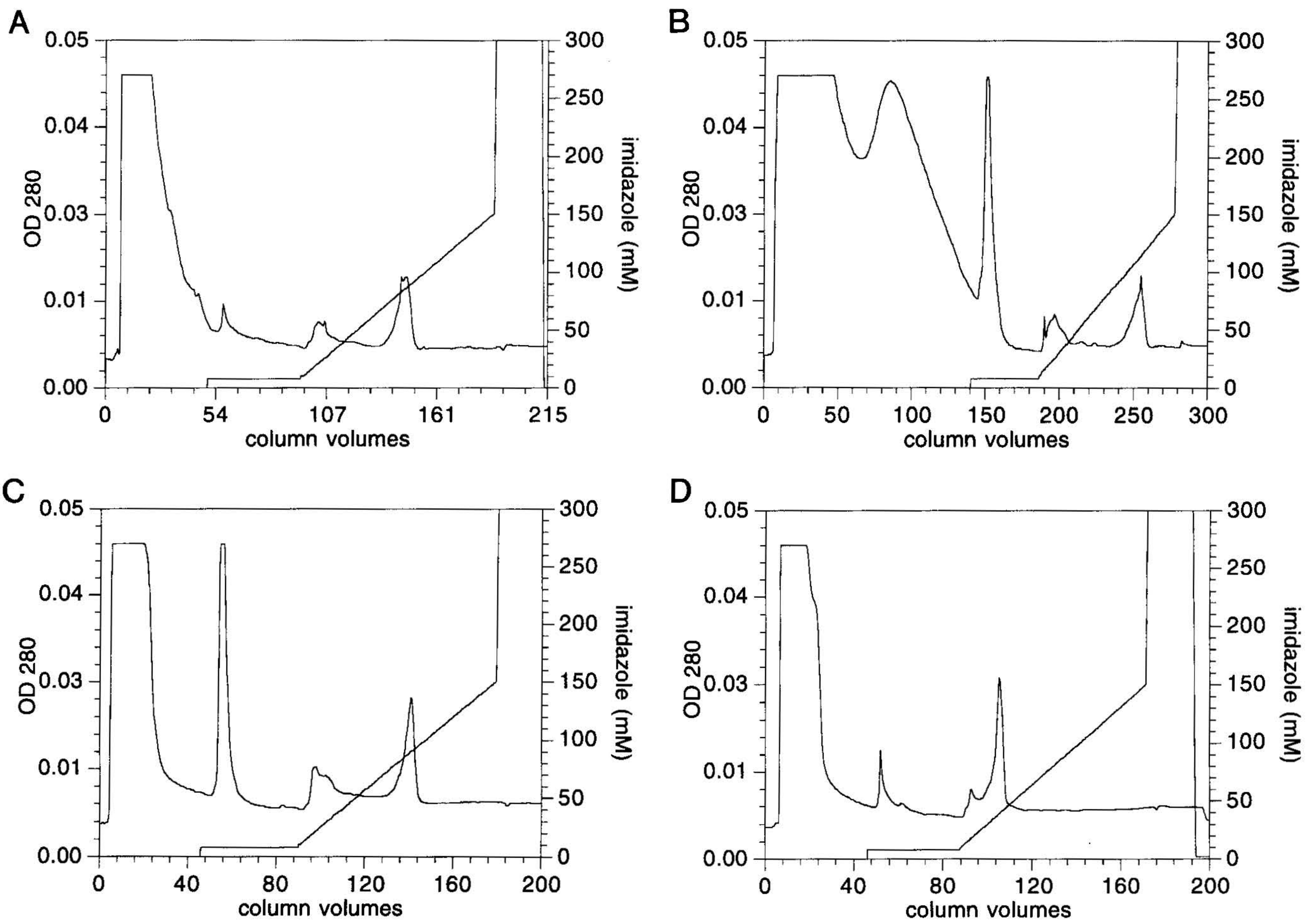

FIG. 4. IMAC of aliquots of the same preparation of the V_L domain of the antibody MOPC167 under different chromatography conditions: (A) Zn^{2+}–IDA, buffer: 50 mM sodium phosphate, 1 M NaCl, pH 8.0; elution by imidazole gradient. (B) Ni^{2+}–IDA, buffer: 50 mM sodium phosphate, 1 M NaCl, pH 8.0; elution by imidazole gradient. (C) Ni^{2+}–NTA, buffer: 50 mM sodium phosphate, 1 M NaCl, pH 8.0; elution by imidazole gradient; (D) Zn^{2+}–NTA, buffer: 50 mM sodium phosphate, 1 M NaCl, pH 8.0; elution by imidazole gradient. (E) Ni^{2+}–NTA, buffer: 50 mM Tris–HCl, 1 M NaCl, pH 8.0; elution by imidazole gradient. (F) Ni^{2+}–NTA, buffer: 50 mM sodium borate, 1M NaCl, pH 8.0; elution by imidazole gradient. (G) Ni^{2+}–NTA, buffer: 50 mM sodium phosphate, 1 M NaCl, pH 8.0; elution by pH gradient. The peak eluting at pH 7.5 is pure plasmid-encoded RTEM β-lactamase, as determined by SDS–PAGE and N-terminal sequencing. (H) SDS–PAGE (Coomassie stain) of the V_L domain purified by the procedures from (A–G): lane M, MW standard; lane 1, periplasmic extract; lane 2, purified from (A); lane 3, purified from (B); lane 4, purified from (D); lane 5, purified from (C); lane 6, purified from (E); lane 7, purified from (F); lane 8, purified from (G).

1 M NaCl, pH 8.0; or buffer 3: 150 mM sodium borate, 1 M NaCl, pH 8.0; each containing 1 mM EDTA for the spheroplast formation] is added and the pellet carefully resuspended with a spatula. Stirring is continued for at least 30 min at 4°C using a large magnetic stir bar at the maximum speed at which the stir bar is still covered. The suspension is centrifuged (48,000*g*, 20 min at 4°C) and the supernatant is aliquoted and frozen or directly loaded onto an IMAC column.

Upon addition of $ZnCl_2$ to a final concentration of 1 mM to the extract to complex EDTA and prevent metal elution from the IMAC column, a precipitate forms. We have found that this addition of metal is an unnecessary step, as recombinant protein from as much as 150 ml of periplasmic extract loaded on a column of 6 ml bed volume is still adsorbed quantitatively. We have therefore not added metal ions to the extract.

Protocol III: IMAC of V_L Domains

Column chromatography experiments described here were carried out with a Pharmacia Hi-Load system. The column (3 ml/1.7 liters of bacterial culture) is packed, washed with H_2O, and loaded with metal ions as described in protocol I. Metal ions are loaded as $ZnCl_2$ or $NiCl_2$ (1 mg/ml) in H_2O. The column is washed with H_2O and equilibrated with the corresponding loading buffer (borate, Tris–HCl, or phosphate buffer, see protocol II). In most of the experiments in Fig. 4, 50 mM potassium phosphate, pH 8.0, 1 M NaCl was used.

Thirteen milliliters of a periplasmic extract prepared according to protocol II (corresponding to 1.7 liters of bacterial culture) is loaded at 1 ml/min and washed with 120 ml of column buffer (e.g., 50 mM potassium

FIG. 4—*Continued*

phosphate, pH 8.0, 1 M NaCl). Washing is continued with 120 ml of the same buffer containing 8 mM imidazole to elute the greater part of the contaminating protein. A gradient (300 ml) of 8 to 150 mM imidazole is used with a flow rate of 0.25 ml/min to elute the protein with the histidine tail. The best results are obtained with a Zn^{2+}–IDA column or a Ni^{2+}–NTA column as discussed above.

PROTOCOL IV: PURIFICATION OF scFv FRAGMENT USING IMAC WITH Ni^{2+}–NTA OR Zn^{2+}-IDA

E. coli JM83 carrying a vector (12) encoding a recombinant scFv fragment engineered to bind lysozyme (Müller and Plückthun, manuscript in preparation) are grown as described for the V_L domains above (protocol II). A periplasmic extract is prepared according to protocol II, using as the buffer 200 mM sodium borate, 1 M NaCl, 1 mM EDTA, pH 8.0. An ammonium sulfate precipitation is carried out, and the scFv precipitates in the fraction of 40–80% saturation. The protein is resuspended and dialyzed against the sodium borate buffer given above. This step is not necessary for all scFv fragments and depends on their particular expression yield.

Ni^{2+}–NTA–agarose and Zn^{2+}–IDA–Sepharose columns are prepared (2 ml/liter of bacterial culture) and run as described in protocol III above. The dialyzed protein is applied to the column (flow rate of 0.5 ml/min). The column is washed with 100 ml of column buffer, followed by 50 ml of the same buffer containing 10 mM imidazole. The protein is eluted with a gradient (250 ml; flow rate, 0.35 ml/min) of 10–150 mM imidazole. In both cases the protein is obtained in pure form as judged on SDS–PAGE. The amounts obtained from both columns were comparable, being about 10–20% higher for Ni^{2+}–NTA than for Zn^{2+}–IDA (Fig. 5).

C. Single-Chain T-Cell Receptor: Sensitivity to Folding and Proteolysis

Description of scTCR Fragment

The genes of the murine T-cell receptor used are from the clone cr15 (36). The known sequence homology of the T-cell receptor to antibody variable domains (37) and a similar genomic arrangement of the genes (38) have led to the hypothesis of a similar three-dimensional topology (37–39). Thus, a gene for a single-chain T-cell receptor fragment was constructed in complete analogy to the scFv fragments reported previously (26–28) (Fig. 7). For ease of detecting the recombinant protein, an epitope of the Myc oncoprotein (myctag) (3) was added to the C-terminus of the scTCR. The 5 histidine residues for IMAC were placed in either of three locations: in the interchain linker, at the C-terminus in front of the myctag, or at the C-terminus behind the myctag (Fig. 6). The two genes of the variable domains were also coexpressed as for the unlinked antibody Fv fragment (40, 41).

Native Purification of scTCR

Unexpectedly, however, in the standard expression system (28) the periplasmically produced T-cell receptor fragment investigated did not in any way behave like the scFv fragments of antibodies previously studied. When a French press lysate of whole *E. coli* cells was loaded onto a Zn^{2+}–IDA column, more than 95% of the scTCR protein was found in the run-through fraction or eluted with the bulk of the *E. coli* protein in 50 mM phosphate buffer, pH 7.0, using a gradient of 25 to 250 mM imidazole. Similar results were obtained with unlinked, but coexpressed variable domains of the TCR: Less than 5% of the variable domains bound to a Zn^{2+}–IDA column. Furthermore, some proteolysis was seen in every case, but immunodetection of the myctag (in this case it was located behind the His tail) (Fig. 6) suggested that it was not simple removal of the His tail that prevented binding to the column. It thus appears that the recombinant TCR, whether in the form of unlinked variable domains or linked scTCR fragment, is not correctly folded, although it is obtained as a soluble protein from the *E. coli* periplasm. Misfolded scTCR cannot bind to IMAC columns.

In later experiments an expression system with enhanced *in vivo* folding capability, based on co-expression of several other proteins, was used (Wülfing and Plückthun, in preparation). It could be shown that the recombinant TCR fragment bound to an IMAC column as expected, though proteolysis still was a problem. After cell rupture in a French pressure cell in column loading buffer including EDTA and PMSF, recombinant scTCR was purified following the protocol of Janknecht *et al.* (11).

Purification of scTCR under Denaturing Conditions

To obtain pure recombinant TCR irrespective of the folding state, denaturing purification was attempted with material from both expression systems.

The scTCR could be purified using IMAC in Ni^{2+}–NTA–agarose in denatured form in the presence of 6 M guanidinium chloride (GdmCl) following the method of Hochuli *et al.* (42). All TCR protein bound to the column and could be eluted in the presence of one fur-

ther contaminating protein. The TCR-containing fractions were then concentrated by $(NH_4)_2SO_4$ precipitation. However, refolding (43) proved difficult because of concomitant proteolytic degradation (Fig. 7), even in the presence of PMSF and EDTA as protease inhibitors. It appears that a protease is copurified on IMAC columns, especially on Ni^{2+}–NTA agarose under these conditions.

To further investigate whether a protease is co-purified, the following set of experiments was performed. With material expressed in the folding-enhanced system, recombinant TCR was purified under denaturing conditions on an IMAC column as described above and refolded in the presence of EDTA and a large molar excess of BSA as a competitive protease substrate (Fig. 7A). This was compared to a different purification scheme under denaturing conditions: recombinant scTCR was loaded onto a DEAE-Sepharose column in the presence of 8 M urea after the cells were disrupted in a French pressure cell. After elution (0–500 mM NaCl

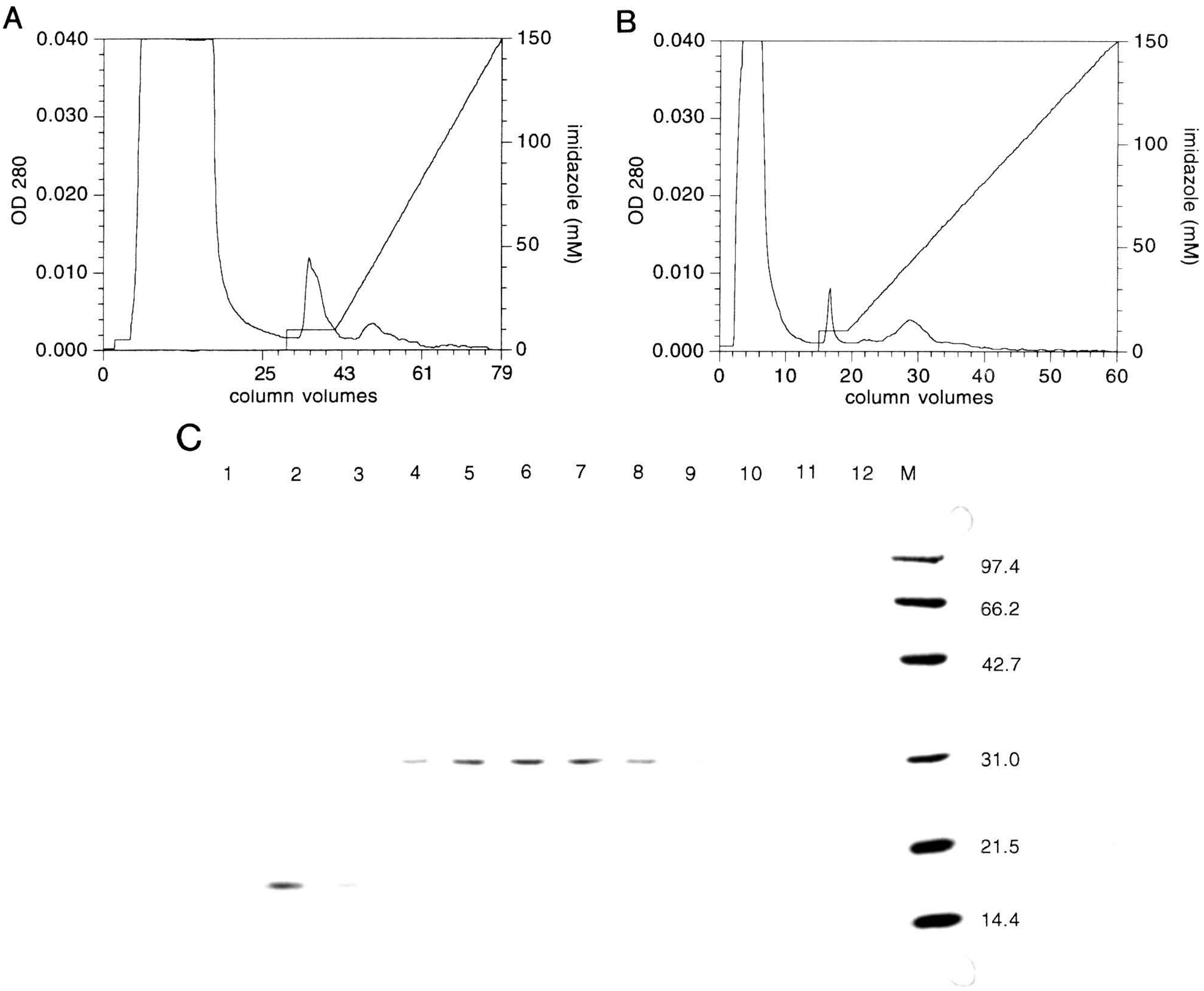

FIG. 5. IMAC of aliquots of the same preparation of a scFv of an engineered antibody. (A) Zn^{2+}–IDA, buffer: 200 mM sodium borate, 1 M NaCl, pH 8.0; elution by imidazole gradient. (B) Ni^{2+}–NTA, buffer: 200 mM sodium borate, 1 M NaCl, pH 8.0; elution by imidazole gradient. (C) SDS–PAGE (Coomassie stain) of the scFv fragment prepared by protocol IV using the Ni^{2+}–NTA column. Lanes 1–3, preelution with 10 mM imidazole; lanes 4–12, imidazole gradient with the scFv band.

gradient) the fractions containing the scTCR were concentrated by ultrafiltration and refolded as described above. Only slight proteolysis—already occurring in the cell—could be seen (Fig. 7B). This material could be purified to homogeneity by IMAC under native conditions using the protocol of Janknecht *et al.* (11). During the purification on a DEAE column in the presence of urea and during subsequent refolding the scTCR could be identified only by blotting with an anti-myctag antibody. Purification of the scTCR to homogeneity showed that separation from the putative protease is the basis of the results shown in Fig. 7B and that the Western blots of the myctag reflect the true amount of scTCR, as the myctag is not selectively removed. As a further control experiment recombinant TCR purified by IMAC under denaturing conditions was passed over a DEAE-Sepharose column in the presence of 8 M urea after a change of buffers on a PD10 column (Pharmacia). After refolding as described above, only slight proteolysis was seen.

In summary, two conclusions can be drawn from the experiments above: (i) The capability of recombinant material to bind to IMAC columns under native conditions is strongly dependent on the folding state. (ii) One or several proteases not inhibited by EDTA or PMSF are co-purified on IMAC columns under denaturing and probably native conditions as well.

D. Disulfide Isomerase from *E. coli* (DsbA), a Periplasmic Protein

The general applicability of IMAC for periplasmic proteins carrying a histidine tail was also demonstrated by purifying *E. coli* disulfide isomerase (DsbA) (44). The expression of the DsbA protein tagged with a C-terminal $(His)_6$ peptide led to substantial amounts (1 mg purified protein/liter of *E. coli* culture) without optimizing the procedure or the vector. Minor impurities could be removed by a DEAE ion-exchange chromatography in a second step. DEAE ion-exchange chromatography was found to be very useful for this purpose. This shows that ion-exchange effects play only a minor role in the IMAC purification under these conditions.

Protocol V: French Press Lysis as the First Step in the Purification of a Periplasmic Protein (DsbA).

DsbA is purified using an expression plasmid constructed by inserting the *DsbA* gene including its own promoter (44) into the antibody Fab expression plasmid pASK29-L220 (45). The $(His)_6$ tail was introduced by site-directed mutagenesis to give the plasmid pCKTh.

E. coli strain JM83 harboring the plasmid pCKTh is grown in 10 liters of LB medium at room temperature for 24 h. The cells are harvested by centrifugation, resuspended in 100 ml of 50 mM Tris–HCl, 1 M NaCl, 0.8 mM imidazole, pH 8.0, and lysed in a French pressure cell at 18,000 psi (=1.24 kbar). The lysate is centrifuged at 48,000*g* and filtered through a 0.45-μm-pore filter.

Protcol VI: Purification of Periplasmic DsbA by IMAC

The lysate from the above step is applied directly to a Ni^{2+}–NTA column (20 ml bed volume). After a washing with 300 ml of the same buffer, a further washing with 100 ml of the same buffer containing 8 mM imidazole is carried out. A gradient (200 ml) of imidazole ranging from 8 to 70 mM is applied in the same buffer. DsbA is eluted at about 60 mM imidazole (Fig. 8). To remove minor impurities, the DsbA-containing fractions are pooled (90 ml), concentrated to 10 ml, dialyzed against 10 mM Mops, pH 7.0, and applied to a DEAE–Sepharose CL6B column (60 ml). A gradient of NaCl (0–200 mM, 400 ml) in 10 mM Mops, pH 7.0, is applied and DsbA elutes at about 140 mM NaCl. It was judged pure by SDS–PAGE.

DISCUSSION

Native versus Nonnative Purification

The nonnative purification using IMAC appears to be the most general purification procedure and is least dependent on the nature of the protein (42). However,

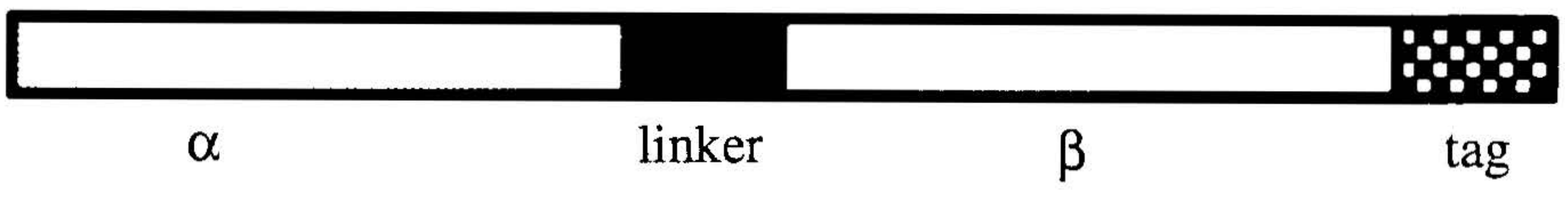

FIG. 6. Schematic arrangement of the TCR genes in the scTCR constructs: α and β are the genes for the variable domains of the respective TCR chains. Three linker/tag combinations have been realized: (i) linker, GGGGSGGGGSGGGGS; tag-mycpeptideHHHHH; (ii) linker, GGGGSGGGGSGGGGS; tag, HHHHHmycpeptide; and (iii) linker, GGGGSHHHHHSGGGS; tag, mycpeptide.

this method has its drawbacks. The generality of the purification procedure is somewhat counterbalanced by each protein's idiosyncratic *in vitro* refolding procedure, again requiring extensive optimization and no net time gain. All in all, the purification of proteins by IMAC in the native state combines most of the advantages of the method and should be attempted whenever possible.

Co-purified Protease

The results from the proteolysis-sensitive scTCR have shown that one (or more) protease(s) is apparently co-purified on Ni^{2+}–NTA and probably Zn^{2+}–IDA, irrespective of whether the procedure is carried out under nondenaturing or denaturing conditions. This suggests that this protease can itself be easily refolded. Its identity is not yet clear, and it cannot be inhibited by the protease inhibitors EDTA and PMSF.

Influence of the Protein Structure and Its Native State

The above comparison of various proteins shows that the accessibility of the tail is the major determinant in the purification results. This implies that the accessibility of the N-terminus and the C-terminus in the native structure must be a crucial feature. Another equally important condition is the absence of misfolding and/or aggregation, which might lead to a masking of the affinity tail as observed for the scTCR described above. Proteins of somewhat similar folding topology (such as the scFv fragment and the scTCR fragment) can thus behave completely differently in the same expression system and under the same chromatographic conditions. A nonnative folding state may lead to an aggregation of the protein, even while it is still soluble, with itself or other proteins of the host. We have observed that even though it is soluble, less than 5% of the recombinant scTCR binds to the column; it does bind if it is first completely denatured by a strong denaturant like 6 M GdmCl. We believe that aggregation of partially denatured soluble protein might be a problem in this case and could conceivably lead to the histidine tails becoming inaccessible to the affinity matrix.

Another example illustrating the same point is that the same antibody scFv fragment, which can be purified

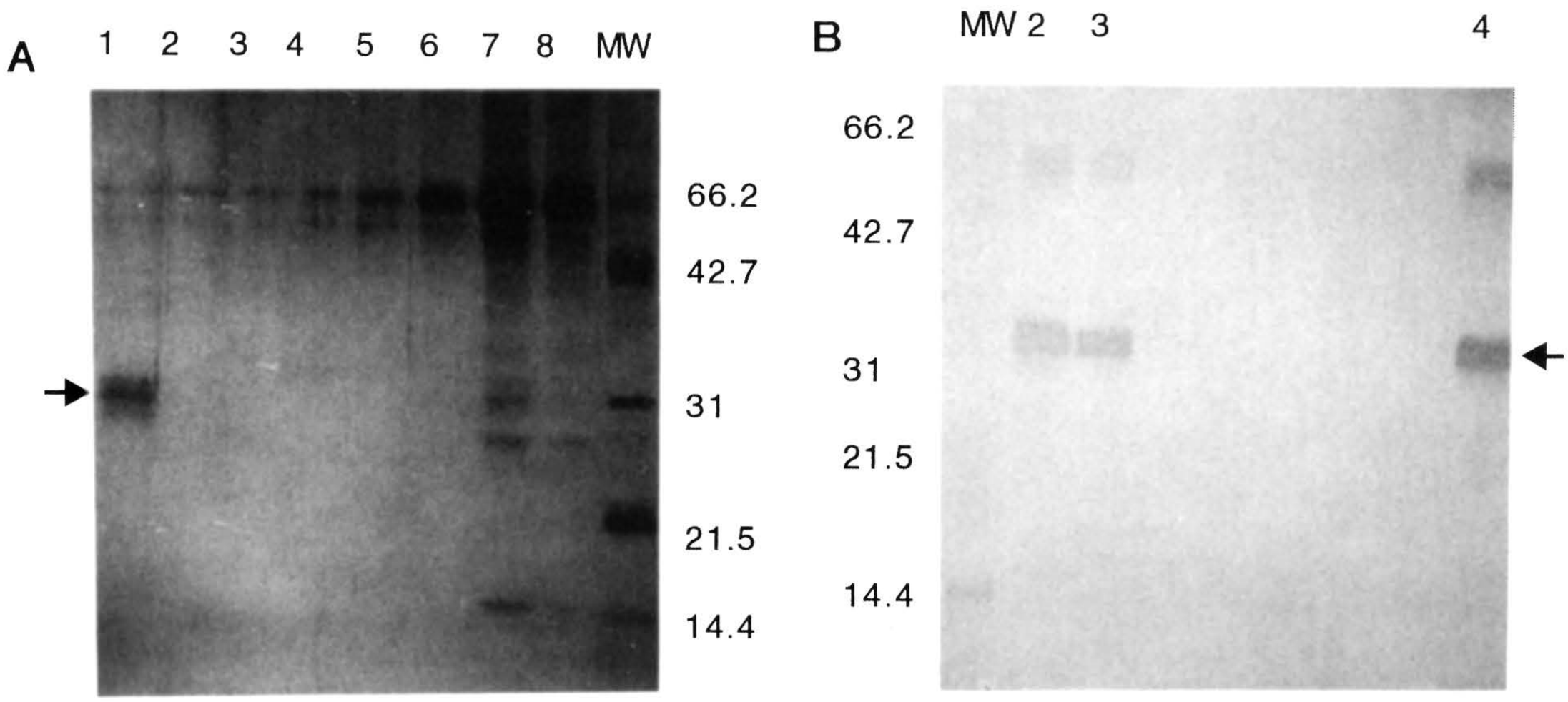

FIG. 7. Degradation of recombinant TCR by a protease co-purified by IMAC. (A) Recombinant scTCR has been purified on Ni^{2+}–NTA–agarose in the presence of 6 M GdmCl (42) and has been refolded (43) after concentration by ammonium sulfate precipitation in 0.1 M Tris, pH 8.0. To add a competing protease substrate in excess during refolding, BSA was added at a concentration of 0.4 mg/ml. The identity of the recombinant scTCR (arrow) has been confirmed by blotting with an anti-myctag antibody (3). A silver-stained SDS–PAGE gel is shown: lane 1, aliquot of scTCR before refolding; lane 2, buffer lane; lanes 3–8, column elution fractions, MW molecular weight marker. In lane 1, 80% of the amount in lanes 3–8 was applied. (B) Recombinant scTCR has been purified from *E. coli* on DEAE-Sepharose in the presence of 8 M urea using a NaCl gradient elution and has been refolded (43). scTCR was detected with an anti-myctag antibody and was shown to be able to bind to Ni^{2+}–NTA agarose. A Western blot is shown: MW, molecular weight marker (Rainbow marker, Amersham); lanes 2 and 3, elution fractions from the DEAE column; lane 4, aliquot of scTCR before refolding. In lane 4, 160% of the amount in lanes 2 and 3 was applied.

by IMAC after secretion to the periplasm, cannot be purified as cytoplasmic material (29). In this case, vectors, strains, and purification schemes were identical, and the experiments differed only by the presence of the signal sequence. In the cytoplasm, disulfide bonds of the scFv fragment cannot form (29, 30), and the soluble, but presumably nonnative, material neither binds to a metal ion affinity column nor to a hapten affinity column. In contrast, the same protein does bind to both columns, if it is purified from the periplasm. Therefore, at least in the cases investigated here, successful IMAC purification correlates with the correct folding of the recombinant protein.

Accessible tails allowing multivalent binding as in the case of citrate synthase lead to such powerful interactions that even batch elution suffices for obtaining completely homogeneous product. This is an almost ideal case of protein purification in the native form, and the IMAC purification is optimally tailored for such proteins.

Folded antibody domains (V_L or scFv) are an intermediate case. The tail is accessible and does not interfere with antigen binding or folding, but its interaction energy derived from monovalent association requires an optimization of column conditions to allow a one-step purification. This has been achieved, and the protocols reported here should be useful as a general method.

Influence of the Histidine Tail Sequence

Previously, tails other than $(His)_5$ have been investigated with antibody domains (12). Their sequences (alternating His–Trp or His–Gly) were inspired by observations (6) that the tripeptides His–Trp–His and His–Gly–His bind particularly well to metal ion affinity columns. The interaction energy in the context of a protein, however, was considerably worse than that with $(His)_5$. Longer tails may bind better, but appear to lead to lower protein amounts, perhaps because of proteolytic degradation. We cannot rigorously exclude

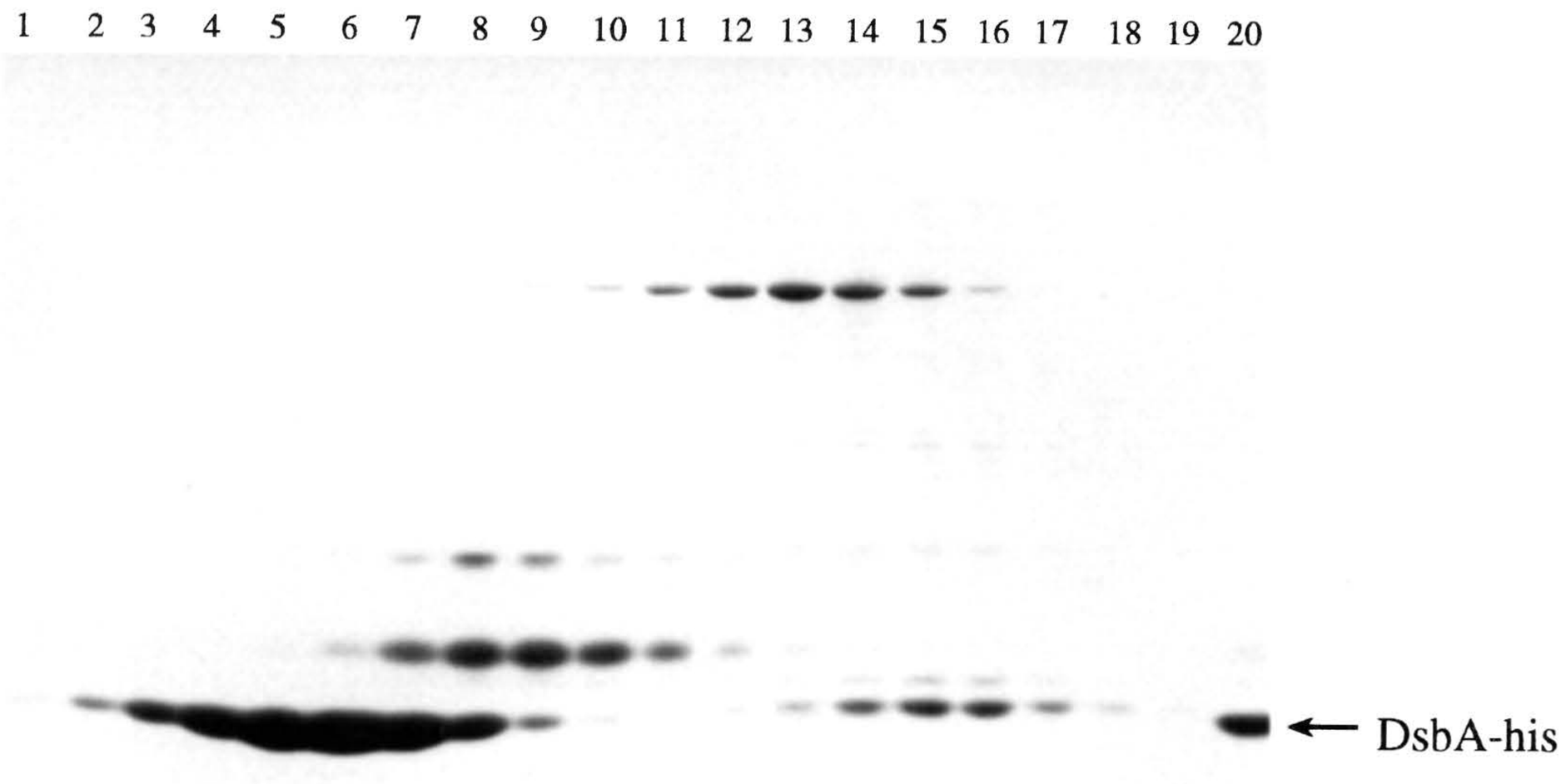

FIG. 8. Elution of DsbA, containing a $(His)_6$ tail, on a Ni^{2+}–NTA column with an imidazole gradient. A Coomassie-stained SDS–PAGE is shown. Lanes 19 to 1 are the fractions of the gradient from 8 to 70 mM imidazole; lane 20, pooled fractions 2–9.

problems with the transport through the membrane using very long histidine tails, but no toxic effects or significant precursor bands were observed with a $(His)_9$ tail in the case of the scFv fragment. In combination, these observations are consistent with the idea that no stable metal complex between any particular histidines is formed, but rather a dynamic equilibrium involving all juxtaposed imidazoles of the tail contributes to the binding (Hochuli, personal communication). Thus, His–X–His–X–His sequences would simply provide fewer ligands than $(His)_5$ sequences. Consistent with this idea, sequences providing fewer histidines sometimes need to be arranged in tandem to provide high-affinity binding during IMAC (10).

Influence of the Subcellular Location of the Protein in *E. coli*

The methods described here are compatible with cytoplasmic or periplasmic expression. This shows that clusters of 5 or 6 histidines pass the membrane without any problem. Since the chromatography seems to work well only with folded, soluble proteins (unless it is carried out in the presence of guanidinium chloride (42)), it may lead to an enrichment of folded, full-length product, also taking advantage of the N-terminal or C-terminal location of the affinity tail.

Influence of the Chromatography Conditions

The results with single V_L domains, for which a variety of chromatographic parameters have been tested from aliquots of the same bacterial culture, demonstrate that the buffer composition itself is of secondary importance and can thus be adapted to other requirements of the procedure. There does not seem to be a significant difference in the yield. Elution can be affected by gradients with imidazole or pH, and the preferred method will depend on the stability of the protein at low pH.

The choice of the ligand and the metal, however, is very important, as they are clearly interrelated. IDA is used most advantageously with Zn^{2+}, as Ni^{2+} leads to a long tailing of host proteins and thus exceedingly long washes. NTA, on the other hand, must be used with Ni^{2+}, as the recombinant protein elutes too early with Zn^{2+}. There does not seem to be a dramatic difference between the yields of both columns for V_L nor for scFv; all experiments in Fig. 4 gave yields within ±10%, with the exception of the pH gradient elution, where only 50% of the recombinant V_L domain remained soluble. It is possible, however, that this will not be true for every protein, as it depends on its stability.

If homogeneity cannot be achieved by a standard protocol, it may often be more economical to simply add a second step of purification than to optimize the method into a single-column procedure, unless a general procedure is to be developed as for the antibody fragments. A fractionated ammonium sulfate precipitation or an ion-exchange step has been found useful.

CONCLUSIONS

In summary, IMAC is a very powerful method for purifying recombinant proteins with histidine affinity tails to homogeneity in their native state. It can be carried out in a rational manner and thus relieves much of the effort involved in protein purification. IMAC may be an important technique in the study of proteins for many purposes, and the small requirements of the handle may make it possible to carry out many experiments with the derivatized protein directly.

In conclusion, we have reported the facile purification of a number of recombinant proteins from *E. coli* using IMAC. These proteins can be purified using different protocols under denaturing and nondenaturing conditions. Given the inherent difficulties in attempting the *in vitro* refolding of proteins, we believe that the purification of proteins by IMAC in the native state combines most of the advantages of the method. It is therefore our method of choice.

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