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Three-step purification of lipopolysaccharide-free, polyhistidine-tagged recombinant antigens of *Mycobacterium tuberculosis*

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Abstract

Previous work has shown that the study of host immune responses against *Mycobacterium tuberculosis*, the causative agent of tuberculosis, requires the availability of multiple mycobacterial antigens. Since purification of protein from *M. tuberculosis* cells is extremely cumbersome, we developed a protocol for purifying milligram amounts of ten recombinant antigens of *M. tuberculosis* from *E. coli* cells. Purified proteins were immunologically active and free of contaminants that confound interpretation of cell-based immunological assays. The method utilizes a three-step purification protocol consisting of immobilized metal-chelate affinity chromatography, size exclusion chromatography and anion-exchange chromatography. The first two chromatographic steps yielded recombinant protein free of protein contaminants, while the third step (anion-exchange chromatography) efficiently removed *E. coli* lipopolysaccharide, a potent polyclonal activator of lymphoid cells. The recombinant proteins were immunologically indistinguishable from their native (i.e., purified from *M. tuberculosis*) counterparts. Thus the method provides a way to utilize recombinant proteins for immunological analyses that require highly purified antigens. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: *Mycobacterium tuberculosis*; Purification; Proteins

1. Introduction

Tuberculosis (TB) is the leading cause of death from a single infectious agent. About one-third of the world's population is infected with *Mycobacterium tuberculosis*; each year 8 million cases of disease arise and 3 million people die worldwide [1]. Crucial to developing accurate immunodiagnostic assays and effective vaccines is an understanding of the host immune responses elicited by antigens of *M. tuberculosis*. The immune response to TB involves many different antigens of *M. tuberculosis* [2,3], thus a large number of antigens need to be evaluated in immunological assays. These antigens must be obtained pure and in good yield, especially for cellular immunological assays that are easily compromised by contaminants in antigen preparations. *M. tuberculosis* grows very slowly and must be cultivated in expensive containment facilities; consequently, obtaining pure protein from *M. tuberculosis* cells is a

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lengthy and cumbersome process. However, large amounts of highly purified antigens can be obtained by combining recombinant DNA and chromatographic techniques.

Our work has focused on the extracellular proteins of *M. tuberculosis*, because proteins found in the culture supernatant (operationally referred to as culture filtrate [CF] in the case of *M. tuberculosis*) are known to induce strong immune responses in TB (reviewed in [4,5]). We set out to purify ten recombinant CF antigens of *M. tuberculosis* in mg amounts, in a form that was immunologically active, and free of lipopolysaccharide (LPS) of *Escherichia coli*, a polyclonal activator of lymphoid cells that confounds interpretation of most cell-based immunological in vitro assays. We cloned genes coding for CF proteins of *M. tuberculosis* in plasmid vectors of *E. coli* that express recombinant proteins as polyhistidine-tagged fusions for simple purification by immobilized metal-affinity chromatography (IMAC) [6,7]. For protein purification, we adopted a three-step protocol involving immobilized metal-chelate affinity chromatography, size exclusion chromatography and anion-exchange chromatography. Three recombinant proteins that were expressed as insoluble inclusion bodies in *E. coli* were solubilized with urea, which was subsequently removed by a gradient-based technique while protein was bound to the IMAC column. Recombinant proteins purified by these methods were immunologically indistinguishable from the corresponding proteins purified from the culture filtrate of *M. tuberculosis*.

2. Experimental

2.1. Cloning of genes encoding *M. tuberculosis* secreted proteins

Genes coding for secreted proteins of *M. tuberculosis* were cloned by either of two methods, polymerase chain reaction (PCR) and immunoscreening of a bacteriophage-lambda-based expression library of *M. tuberculosis* $H_{37}Rv$ DNA with rabbit polyclonal antibodies against *M. tuberculosis* culture filtrate proteins [8,9].

2.2. Expression of recombinant *M. tuberculosis* proteins

Overnight cultures of the *E. coli* strain XL1-Blue MRF’ harbouring recombinant plasmids expressing *M. tuberculosis* proteins as poly-histidine tagged fusions were diluted 1:50 in fresh LB broth containing 100 μg/ml ampicillin and grown at 37°C with vigorous shaking to an OD_{600} of 0.7. Isopropyl-thio-β-D-galactoside (IPTG) was added to the cultures to a final concentration of 1 m*M*, and the induced cultures were grown for an additional 4 h. Culture volumes ranged from 1–9 l depending on the expression level of the recombinant proteins determined in small-scale (100 ml of cell culture) pilot experiments. Cells were washed in 10 m*M* sodium phosphate buffer, pH 7.4 and collected by centrifugation at 20 000 *g* for 30 min. Cell pellets were weighed and stored at −70°C.

2.3. Purification of soluble recombinant proteins

Cells containing soluble recombinant proteins were lysed by either enzymatic or mechanical methods. For enzymatic lysis, cells were resuspended in cold 50 m*M* sodium phosphate buffer, 0.3 *M* NaCl, pH 8.0 at 4°C to a concentration of 5–20 mg of cell paste per ml. Freshly prepared lysozyme (Sigma) was added to the cell suspension to a final concentration of 1 mg/ml. The cell suspension was incubated for 45 min at 4°C with stirring. To reduce viscosity of the lysate prior to chromatography, the lytic mixture was twice expelled from a syringe through a 18-gauge needle and once through a 25-gauge needle prior to centrifugation at 15 000 *g* for 20 min at 4°C. For mechanical lysis, cells were resuspended in 10 m*M* sodium phosphate buffer, pH 7.4 to a density of 50 mg of cell paste per ml and subjected to two 1300-psgi cycles of French press (American Instrument Company). Cell lysates were clarified by centrifugation at 20 000×*g* for 30 min.

The NH_2-terminal poly-histidine sequence common to all of the recombinant antigens was the target for the initial capture using IMAC. HiTrap Chelating columns (Amersham Pharmacia Biotech) were charged with nickel ions by adding 0.5 column volume (V_c) of 0.1 *M* $NiSO_4$ followed by equilibra-

tion with 5 V_c of 20 m*M* sodium phosphate buffer, 0.5 *M* NaCl, 20 m*M* imidazole, pH 7.4. Subsequent chromatography was performed at room temperature with FPLC (Amersham Pharmacia Biotech). A nominal binding capacity of 8–10 mg poly-histidine-tagged protein per ml of charged gel was used to select column size (1,5 or 10 ml) based on the expression levels determined in small-scale cultures. The clarified cell lysate was applied to the column at 150 cm/h. After sample loading the column was washed with 10 V_c of 20 m*M* sodium phosphate buffer, 0.5 *M* NaCl, 20 m*M* imidazole, pH 7.4. Protein was eluted by applying a 10 V_c linear gradient with imidazole (20 m*M*–0.5 *M*) in 20 m*M* sodium phosphate buffer, 0.5 *M* NaCl, pH 7.4. Protein purification was monitored by apparent molecular weight on SDS–PAGE followed by silver staining in PhastGel Gradient 10–15 using the Phast System and the PhastGel Silver Kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Fractions of 1 V_c were collected during the imidazole gradient and those containing the recombinant protein (as judged by apparent M_r during SDS–PAGE relative to the theoretical M_r deduced from the gene sequence) were pooled for further purification. Priority was given to optimizing yield rather than purity in selecting fractions to be pooled.

2.4. Purification of insoluble recombinant proteins

Insoluble recombinant proteins were solubilized by adding cold 8 *M* urea in 20 m*M* sodium phosphate buffer, 0.5 *M* NaCl, pH 7.4 to cell pellets to a final density of 5–10 mg of cell paste per ml. The mixture was stirred for 1 h at 4°C, subjected to additional mechanical lysis by French press (as described above) and clarified by centrifugation at 20 000 *g* for 30 min. Clarified cell lysates were applied at 150 cm/h to Ni^{2+}-charged HiTrap columns pre-equilibrated with 5 V_c of 20 m*M* sodium phosphate buffer, 0.5 *M* NaCl, 8 *M* urea, pH 7.4. After sample loading, the column was washed with 10 V_c of 20 m*M* sodium phosphate buffer, 0.5 *M* NaCl, 8 *M* urea, pH 7.4. Prior to protein elution, urea was removed using a 10-V_c linear gradient from 8 to 0 *M* urea at 150 cm/h and the column was washed with 10 V_c of 20 m*M* sodium phosphate buffer, 0.5 *M* NaCl, pH 7.4. Protein was eluted using a 10-V_c gradient with imidazole (0–0.5 *M*) in 20 m*M* sodium phosphate buffer, 0.5 *M* NaCl, pH 7.4. Protein purification was monitored according to apparent molecular weight of the recombinant product using SDS–PAGE followed by silver staining. Fractions were pooled for further purification as described above.

2.5. Size exclusion chromatography (SEC)

Pooled fractions containing protein with M_r in the range of 10 to 60 kDa were applied at 60 cm/h to either a HiLoad 26/60 Superdex 75 prep grade column (V_c=300) or HiLoad 16/60 Superdex 75 prep grade column (V_c=120) (Amersham Pharmacia Biotech) equilibrated in 50 m*M* Tris, pH 8.0. Proteins having an M_r greater than 60 kDa were purified using HiLoad 26/60 Superdex 200 prep grade. In all cases, column size was selected to maintain V_s<6% V_t. Sample volumes exceeding 18 ml (6% of V_c=300 ml) were either reduced by ultrafiltration through a DIAFLOW YM5 membrane (Amicon) or sample aliquots were run sequentially on the same column. Fractions were assayed by SDS–PAGE and those containing the protein of interest were pooled. Priority was given to purity rather than yield in the fractions to be pooled. When the total sample volume was in excess of 6% V_t, multiple cycles were required to process the entire sample volume. The column was washed with 2 V_c of 0.1 *M* NaOH between cycles to ensure removal of trace contaminants. Between different protein samples, columns were cleaned with 1.0 *M* NaOH to remove endotoxin and prevent cross-contamination between samples [10].

2.6. Anion-exchange chromatography (AEC)

Fractions collected by size-exclusion chromatography were further purified and concentrated by application to a HiTrap Q column (Pharmacia Biotech) equilibrated with 10 V_c of 50 m*M* Tris, pH 8.0 at 150 cm/h. The column was washed with 10 V_c 50 m*M* Tris, pH 8.0 and protein was eluted by using a 10 V_c linear gradient from 0 to 1 *M* NaCl in 50 m*M* Tris, pH 8.0. Fractions were assayed by SDS–PAGE

and those containing the protein of interest were pooled and stored at −70°C. Protein concentrations were determined using the Bradford assay [11].

2.7. Enzyme-linked immunosorbent assay (ELISA)

Polystyrene microtiter 96-well plates (Costar) were coated with 0.3 mg per well of purified protein in 0.05 *M* carbonate buffer, pH 9.6 at 4°C overnight. One hundred μl of sera appropriately diluted in phosphate-buffered saline plus 0.05% Tween 20 (PBS-T) were added to antigen-coated wells in duplicate. After 90 min incubation at room temperature, plates were extensively washed with PBS-T, and incubated for 1 h with 100 ml of the appropriate alkaline-phosphatase-conjugated anti-IgG antibody (Bio-Rad or Sigma) diluted 1:1000 in PBS-T. Plates were washed with PBS-T, and alkaline phosphatase activity was assayed with *p*-nitrophenylphosphate (Bio-Rad). Optical density was measured at 405 nm (OD_{405}) using an automatic microplate reader (Spectra Shell, Tecan).

2.8. Guinea pig skin test

Guinea pigs weighing approximately 300 g were immunized with 2×10^7 cells of *M. bovis* BCG by intradermal injection. Five weeks after immunization, animals were shaven in the back and given intradermal injections of 2 μg of each purified *M. tuberculosis* antigen in 0.1 ml of PBS, pH 7.4. Skin reactions (diameter of erythema and induration) at the sites of antigen injection were measured after 24 h.

2.9. Lipopolysaccharide (LPS) assay

LPS content of purified recombinant protein preparations was measured by using the lucigenin-induced chemiluminescence assay in 1% blood [12] (courtesy of Dr. Jerrold Weiss, New York University Medical Center).

2.10. Catalase activity

Six to 40 mg protein was loaded onto nondenaturing 9% polyacrylamide gel (30:8 acrylamide:bisacrylamide) in Tris-glycine buffer pH 8.3 and elecrophoresed at 100 volts for 2 h at 4°C. After washing in water for 30 min, the gel was soaked in 5 m*M* H_2O_2 for 2 min, and transferred to a 1:1 mixture of 2% $FeCl_3$ and 2% potassium ferrocyanide to reveal catalase activity [13]. Catalase activity was visualized as a clear area on a dark-green background.

3. Results

3.1. Design of protein purification protocol

Ten genes encoding culture filtrate proteins of *M. tuberculosis* were cloned in the pQE30 (Qiagen) plasmid vector of *E. coli* and expressed as *lacZ* fusions with polyhystidine purification tags at their NH_2 termini. To assess solubility of the recombinant products prior to large-scale purification, parallel 100 ml cultures for each gene were lysed under nondenaturing and denaturing (8 *M* urea) conditions. Lysates were clarified by centrifugation and subjected to IMAC. Comparison of relative protein yield from denaturing and nondenaturing lysis was used to determine solubility of the recombinant product, and it was found that high-level expression of four of ten recombinant proteins resulted in the formation of insoluble inclusion bodies (Table 1).

The criteria used to design a large-scale protein purification protocol were as follows. First, the protein should be >99% pure, as judged by densitometry/image analysis of optical density-in-lane of silver stained SDS–PAGE gel. Second, the protein should be immunologically active, as judged by antibody-based and cell-based immunoassays. Third, the protein should be free of contamination with LPS of *E. coli*, as judged by a very sensitive lucigenin-based assay (see below). Finally, lytic procedures and volumes of cultures were chosen on the basis of protein solubility and expression levels evaluated in small-scale pilot experiments described above.

Since four of our recombinant proteins were produced in an insoluble form in *E. coli*, we adopted as first chromatographic step IMAC, a capture procedure for poly-histidine-tagged fusion proteins from crude *E. coli* cell lysates that can also be used in the presence of 8 *M* urea [7]. We chose urea as denaturing agent over mild detergents such as Tween 80 or sarcosyl, for these detergents are difficult to remove [14] and interfere with plate coating in

Table 1
Purification of polybistidine-tagged antigens of *M. tuberculosis*

Protein	Expression state	Purification conditions	Method of cell lysis	Yield (mg protein per g of cell paste)
KatG	Soluble	Nondenaturing	French press	54.0
MPT51	Soluble	Nondenaturing	Lysozyme	7.0
MPT64	Soluble	Nondenaturing	Lysozyme	0.6
45/47 kDa	Soluble	Nondenaturing	Lysozyme	0.2
MTC28	Soluble	Nondenaturing	French press	0.6
14 kDa	Soluble	Nondenaturing	French press	6.0
ESAT-6	Insoluble	Denaturing	Urea	5.0
Ag85B	Insoluble	Denaturing	French press+urea	0.1
19 kDa	Insoluble	Denaturing	French press+urea	0.4
38 kDa	Insoluble	Non denaturing	French press	0.1

ELISA and with cellular assays. IMAC alone is insufficient to give >99% purity because low M_r contaminants often copurify with the poly-histidine-tagged recombinant protein. It is unclear whether low M_r contaminants are histidine-containing host proteins, NH_2-terminal fragments of the fusion protein, or host proteins that associate with the recombinant product. While the presence of 20 m*M* imidazole in the sample and binding buffer has been used to reduce the level of contaminants binding to the column [6], even the use of high-resolution gradient elution conditions with competitive elution agents typically fails to achieve >90% purity [15]. Further, IMAC is inadequate to reduce the LPS content of the sample to a level acceptable for cell-based immunological assays.

Due to the limitations of single-step IMAC purification, a three-step purification strategy was developed. Knowledge of theoretical isoelectric points and molecular weights of recombinant proteins (calculated from the deduced amino acid sequences) made it possible to design two additional steps (size exclusion chromatography and anion-exchange chromatography). Anion-exchange chromatography was chosen as a final purification step because it is very effective in removing LPS and usually concentrates the sample [16]. Size exclusion chromatography was selected as the intermediate purification step between IMAC and anion-exchange to provide the highest resolution from contaminants, and to achieve buffer exchange prior to anion-exchange chromatography.

3.2. Immobilized metal affinity chromatography

Three of four insoluble proteins were maintained in 8 *M* urea during cell lysis and loading of clarified cell lysates onto the IMAC column. Urea was gradually removed prior to elution with imidazole, as described in Section 2. Examples of the purification of one soluble antigen, MTC28, and one insoluble antigen, ESAT-6, are shown in Figs. 1 and 2. Initial purification of either soluble or insoluble polyhistidine-tagged fusion proteins by IMAC typically resulted in greater than 90% purity as judged by image analysis of silver stained SDS–PAGE gels (Panel A in Figs. 1 and 2).

It is worth noting that gradient-based removal of urea resulted in effective solubilization without significant precipitation of protein in the column. In contrast, attempts to remove urea by step dilution or by dialysis of eluted protein resulted in protein precipitation and low yield (data not shown). In the case of one insoluble protein, the 38 kDa antigen, the culture was lysed only by French press, a technique that does not disrupt inclusion bodies, and affinity chromatography was carried out under nondenaturing conditions. Using this procedure, we were able to purify small amounts of the soluble antigen present in *E. coli* cells (Table 1). These results suggest that, in the case of insoluble proteins that do not readily refold after removal of urea, purification of the μg amounts of soluble cytoplasmic proteins is feasible using this procedure.

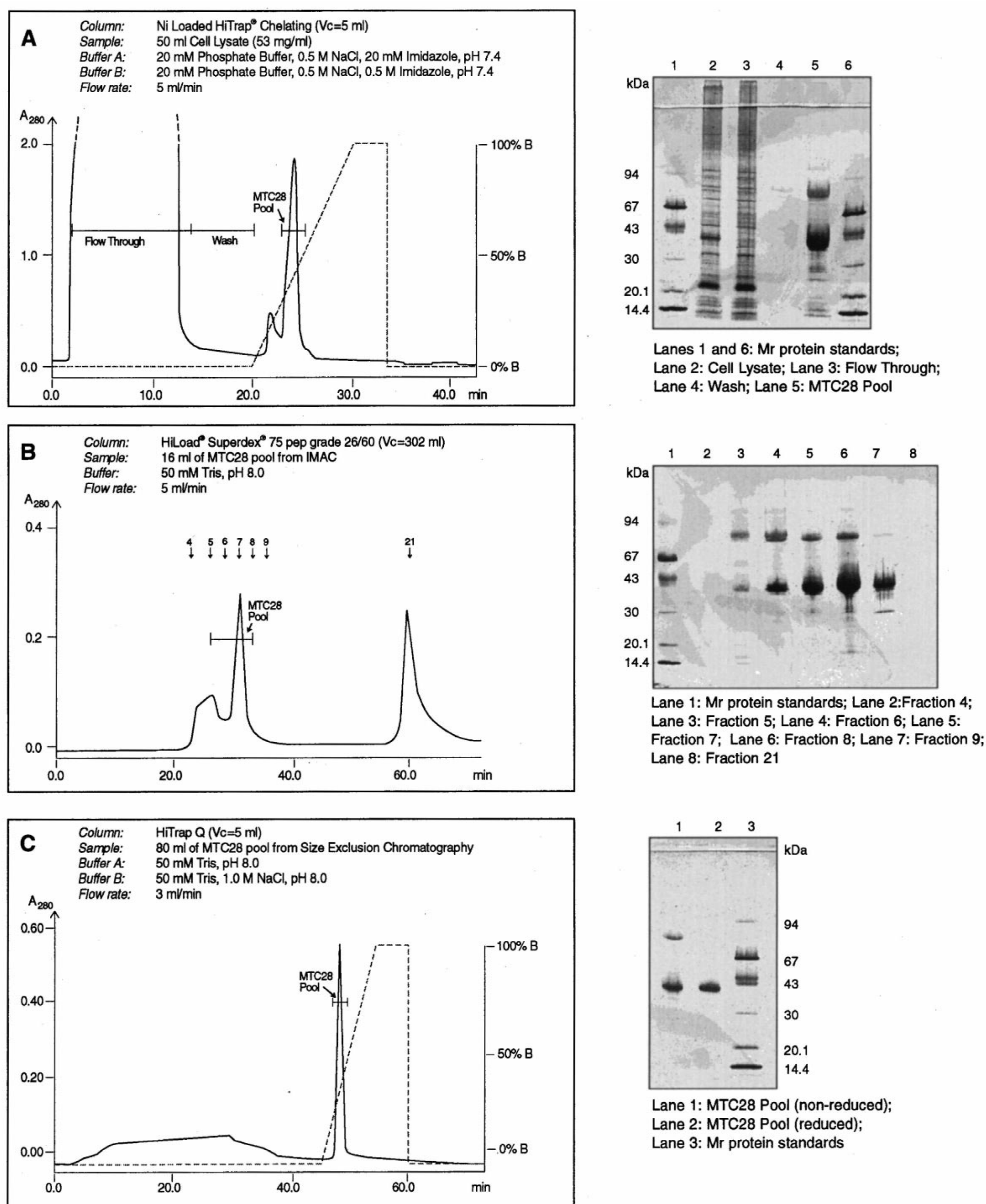

Fig. 1. Three-step purification of MTC28 polyhistidine-tagged recombinant antigen. Panel A: Chromatogram of the initial capture step by IMAC and fraction analysis by SDS–PAGE with silver staining. Panel B: Second-step purification by size exclusion chromatography and fraction analysis by SDS–PAGE with silver staining (nonreduced). Panel C: Third-step polishing by anion-exchange chromatography and fraction analysis by SDS–PAGE with silver staining.

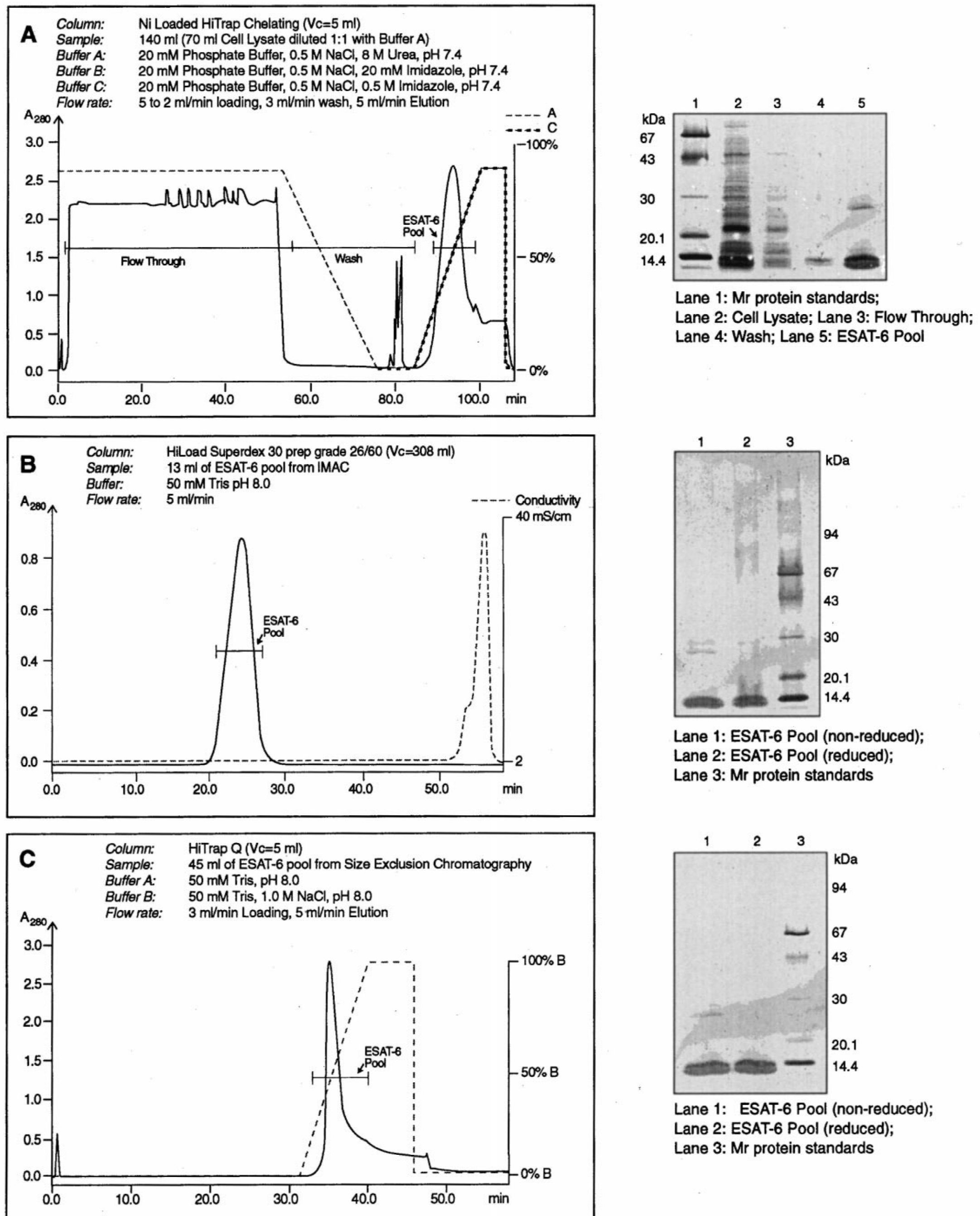

Fig. 2. Three-step purification of ESAT-6 polyhistidine-tagged recombinant antigen. Panel A: Chromatogram of the initial capture step by IMAC and fraction analysis by SDS–PAGE with silver staining. Panel B: Second-step purification by size exclusion chromatography and fraction analysis by SDS–PAGE with silver staining. Panel C: Third-step polishing by anion-exchange chromatography and fraction analysis by SDS–PAGE with silver staining.

3.3. Size exclusion chromatography

Three SEC media (Superdex 30, Superdex 75 and Superdex 200) were chosen to provide selectivities adequate to the range of molecular weights displayed by the recombinant proteins (6 to 80 kDa). All proteins eluted between V_0 and V_t. The SEC step effectively removed low-molecular-weight contaminants and high-molecular-weight aggregates present in the sample after IMAC (Panel B in Figs. 1 and 2). The protein of interest was recovered in 50 m*M* Tris, pH 8.0, ready for application to an anion-exchange column.

3.4. Anion-exchange chromatography

Fractions containing recombinant proteins were further purified and concentrated using AEC. Initial binding conditions for anion-exchange were selected based on isoelectric points calculated using the deduced amino acid sequences and the Wisconsin sequence analysis package by the Genetics Computer Group. Since the range of calculated isoelectric points was between 4.1 and 7.5, a binding pH of 8.0 was chosen to allow a 0.5 pH unit difference with the highest calculated p*I* of the recombinant proteins. Accordingly, 50 m*M* Tris pH 8.0 was chosen as the binding buffer. A single, inexpensive disposable column (HiTrapQ 1 or 5 ml) was used for each protein to prevent LPS carryover or cross-sample contamination.

All of the antigens bound to the HiTrap Q column at pH 8.0. However, the dynamic binding capacity observed for the MTC28 recombinant antigen was less than 1 mg/ml and no significant improvement was observed by raising the binding pH to 9.7, reducing the ionic strength of the binding buffer, or lowering the flow-rate. The causes of this behavior are presently being investigated. All recombinant antigens were eluted during the gradient in less than 500 m*M* NaCl and were stable in the eluant when stored at −70°C for at least one year. The anion-exchange step yielded highly concentrated proteins that, in nine out of ten cases, migrated as single bands when analyzed by SDS–PAGE and silver staining under reducing conditions (Fig. 3). Migration of ESAT-6 as a doublet (Fig. 3, lane 6) may be due to partial proteolysis or to the presence of a prematurely terminated polypeptide. Similar observations have been made with other recombinant proteins expressed at high levels in *E. coli* [8].

SDS–PAGE analysis of the anion-exchange fractions revealed the presence of high-molecular-weight forms in samples treated with 1.5 to 5 m*M* 2-mercaptoethanol, a procedure routinely used for reducing disulfide bridges between cystine/cystine residues. Western blot analysis of selected recombinant proteins with monospecific rabbit polyclonal antibodies indicated that both monomeric and high-molecular-weight forms were immunoreactive (data not shown). High-molecular-weight forms only disappeared upon treatment with 7.5 m*M* 2-mercap-

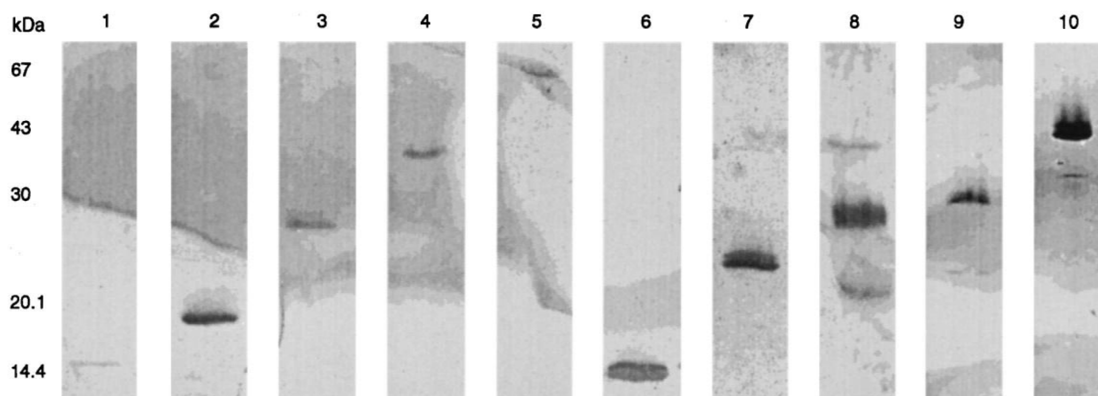

Fig. 3. SDS–PAGE analysis of three-step purified *M. tuberculosis* antigens. Lane 1: 14 kDa; Lane 2: MPT64; Lane 3: MTC28; Lane 4: 45/47 kDa; Lane 5: KatG; Lane 6: ESAT-6; Lane 7: 19 kDa; Lane 8: MPT51; Lane 9: Ag85B; Lane 10: 38 kDa. Lanes 1–10 were obtained from various gels and graphically positioned with respect to M_r.

toethanol prior to SDS–PAGE (examples are presented in panel C, Figs. 1 and 2). This behavior suggests an alternative aggregation mechanism, possibly involving bridging between NH_2-terminal 6-histidine helices by divalent metal ions. We are currently examining this possibility.

3.5. LPS content

A problem often encountered with recombinant antigens purified from *E. coli* cells is contamination with LPS, a potent polyclonal activator that interferes with cellular immunological assays [17]. LPS concentration in antigens purified by the three-step protocol described above was measured by using lucigenin-induced chemiluminescence in 1% blood [12]. This assay can measure LPS concentrations as low as <10 pg/ml (J. Weiss, personal communication). Using this assay, the specific activity of fractions obtained throughout the three-step purification of the MTC28 protein (refer to Fig. 1) was measured and converted to ng LPS per mg of protein using an LPS standard curve. While IMAC reduced LPS content of the protein preparations by 10- to 100-fold, the final anion-exchange step was most effective in reducing any residual LPS in the samples to a level below the sensitivity threshold of the assay (Fig. 4). LPS content of the highly purified preparations of MTC28 and seven other proteins (data not shown) was much lower than 10 pg/μg of protein. Such levels of LPS contamination of recombinant antigen preparations are below sensitivity of cellular assays in vitro (data not shown).

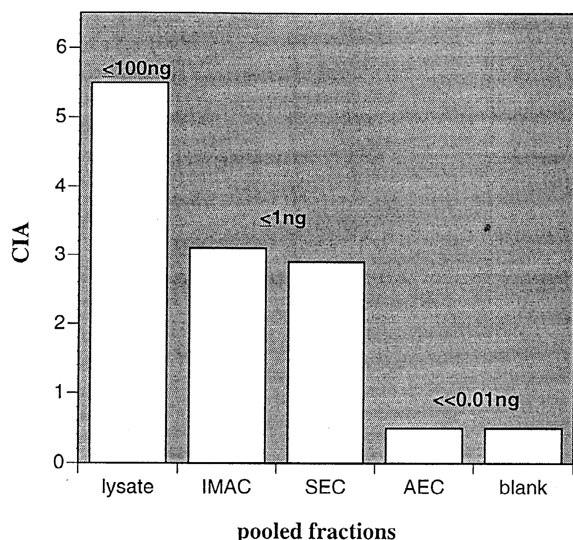

Fig. 4. Lucigenin-induced chemiluminescence of 1% blood in response to fractions obtained during purification of MTC28. CIA represents the peak of chemiluminescence-inducing activity in arbitrary units. Corresponding amounts of LPS (ng LPS per mg protein), shown above each bar, were calculated using an LPS standard curve. Lysate=*E. coli* cell lysate; IMAC=immobilized metal affinity chromatography; SEC=size-exclusion chromatography; AEC=anion-exchange chromatography; blank=buffer only.

In contrast, the 14-kDa and the 19-kDa proteins showed appreciable chemiluminescence activity (corresponding to ≤50 ng LPS per μg protein) even after several attempts at further purification. Whether this residual activity is due to contaminating LPS or other lipids tightly associated with these proteins, or is intrinsic to the proteins (the lucigenin-based assay is not entirely LPS-specific) is yet to be determined. Since the 14 kDa is a membrane-associated protein and the 19 kDa is a lipoprotein in *M. tuberculosis* [4], it is possible that these proteins are associating with lipids in *E. coli* that may induce the chemiluminescence signal.

3.6. Immunological activity

Culture filtrate proteins of *M. tuberculosis* are involved in both humoral and cell-mediated immune responses to TB. Thus we set out to measure antigenic activity of the purified proteins in both types of immune responses. First, we determined whether our ten purified proteins were antigenically active by measuring by ELISA, IgG antibodies against each of these proteins in rabbit hyperimmune sera, in sera from guinea pigs experimentally infected with *M. tuberculosis*, and in sera from cattle experimentally infected with *M. bovis* (genes encoding all of the proteins purified in this studies are present in both *M. tuberculosis* and *M. bovis* genomes). All highly purified proteins reacted with at least one of the immune sera (Table 2), but not with normal sera. These results indicated that all purified antigens were serologically active.

We next measured the activity of the purified proteins in delayed-type hypersensitivity (DTH) responses in guinea pigs immunized with *M. bovis*

Table 2
Reactivity of animal sera to purified recombinant antigens of *M. tuberculosis*

Proteins	Rabbit		Guinea pig		Bovine	
	Normal	Immunized with *M. tuberculosis* CF	Normal	Infected with *M. tuberculosis*	Normal	Infected with *M. bovis*
ESAT-6	0.02	0.03	0.04	0.19[a]	0.02	0.60[a]
14 kDa	0.07	0.22[b]	0.12	0.35	0.04	0.72[a]
19 kDa	0.03	0.53[a]	0.04	0.66[a]	0.05	0.13
MPT64	0.06	0.46[a]	0.04	0.59[a]	0.04	0.45[a]
MPT51	0.07	0.99[a]	0.03	0.42[a]	0.05	0.12
Ag85B	0.04	0.52[a]	0.04	0.72[a]	0.05	0.19
38 kDa	0.09	0.55[a]	0.13	1.07[a]	0.05	0.07
MTC28	0.10	0.43[a]	0.08	0.89[a]	0.04	0.06
45/47 kDa	0.04	0.43[a]	0.03	0.47[a]	0.03	0.56[a]
KatG	0.05	0.22[a]	0.07	0.76[a]	0.04	0.06
M. tuberculosis CF	0.04	0.80[a]	0.06	1.03[a]		
M. bovis CF					0.09	1.45[a]

Figures represent OD_{405} readings in ELISA.
[a] Values $\geq 4\times OD_{405}$ in normal sera taken as indicative of strong antibody reactivity.
[b] Measured using an anti-*M. bovis* culture filtrate antiserum.
CF=culture filtrate.

BCG as described in the Section 2. Skin test experiments indicated that the recombinant antigens (with the exception of ESAT-6, the gene of which is absent in *M. bovis* BCG [18]) evoked DTH responses in the immunized animals. These results, which are part of a larger study to be published elsewhere, indicated that the recombinant antigens purified in this work can elicit cell-mediated immune responses during mycobacterial infection.

3.7. Comparative analysis of recombinant and native antigens

The use of recombinant antigens often raises the question of whether a recombinant product from *E. coli* is as active as the corresponding antigen produced by *M. tuberculosis*. To address this point, we compared immunological activities of two pairs of recombinant and native proteins (Ag85B, and MPT51) by using skin test and antibody-based assays. Native proteins used in these experiments were purified from *M. tuberculosis* $H_{37}Rv$ culture filtrates and kindly given to us by H. Wiker (University of Oslo, Norway). For both proteins, we found no differences between the recombinant products and their native counterparts (Fig. 5). We also monitored the immunological activity of one recombinant protein, the 38 kDa protein, throughout the purification protocol, and we found that, expectedly, the activity of the protein measured by ELISA increased with purity (approximately 8-fold between IMAC and anion-exchange chromatography) (data not shown).

Taken together, these observations indicate that poor antigenic activity of recombinant proteins can often be attributed to insufficient protein purification, unless post-translational modification of the protein, which does not occur in *E. coli*, is specifically required for optimal antigen activity.

3.8. Enzymatic activity

The physiological role of the majority of the *M. tuberculosis* culture filtrate proteins is still unknown. Enzymatic activities have been defined for three of the proteins purified in this work, the 38 kDa antigen [19], the 30 kDa (Ag85B) antigen [20], and the 80 kDa KatG protein that is a catalase [21]. Since catalase activity is amenable to a simple enzymatic assay, we tested catalase activity of the purified 80 kDa recombinant protein. In this assay, the protein was found to be enzymatically active (data not shown).

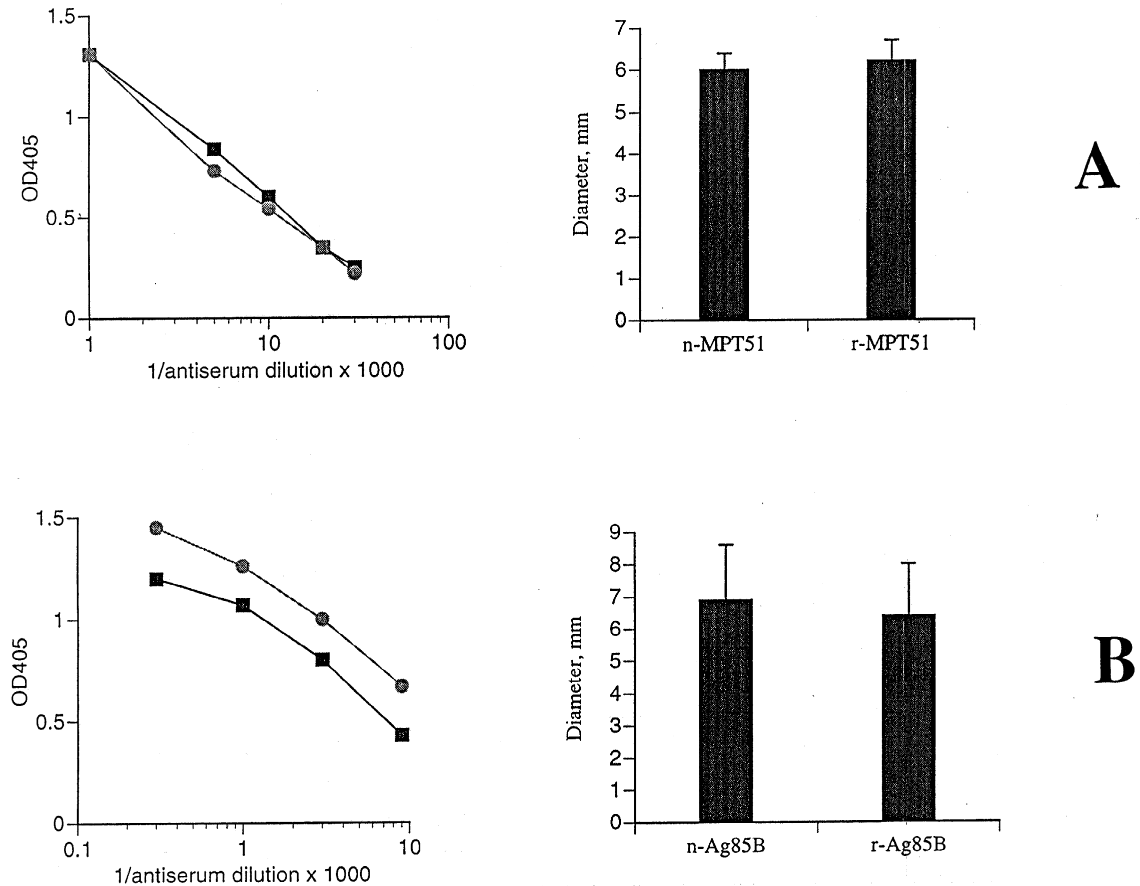

Fig. 5. Comparative immunological analyses of recombinant and native antigens of *M. tuberculosis*. For two antigens of *M. tuberculosis*, MPT51 (panel A) and Ag85B (panel B), immunological activities of native and recombinant proteins were compared by using ELISA (left panel) and guinea pig skin testing (right). For ELISA, microtiter plates were coated with 0.3 μg per well of recombinant (●) and native (■) proteins. Rabbit hyperimmune sera were used at the indicated dilutions. For guinea pig skin testing, 2 μg of purified proteins were used to elicit skin reactions (diameter of erythema, in mm), as described in Section 2. *n*=native protein, *r*=recombinant protein.

4. Discussion

In this report, we describe purification of ten recombinant proteins of *M. tuberculosis* for immunological analyses of TB. We showed that, using gene cloning techniques and protein purification protocols, several recombinant proteins can be obtained in mg amounts, pure and immunologically active. For insoluble recombinant proteins, removal of the solubilizing agent was achieved by a simple gradient technique while protein was still bound to an IMAC column. We also showed that removal of LPS, a major contaminant of recombinant proteins purified from *E. coli*, requires multiple chromatographic steps. Finally, immunological activities of selected recombinant proteins and their counterparts purified from *M. tuberculosis* cells were indistinguishable. The protocols described here should provide guidance to the many laboratories in need of purifying proteins produced by pathogenic microorganisms in a recombinant form to carry out immunological investigations.

The three-step purification protocol adopted in this paper is robust and of general applicability. Only minimal knowledge of the protein is required prior to purification: molecular weight (to monitor purification steps and to choose the sizing column) and p*I* (to choose ion-exchange conditions) can be deduced

from the gene sequence. Care should be taken, however, because deduced parameters may differ significantly from those determined elecrophoretically. Protein solubility can be easily determined from small-scale cultures. Finally, the three-step protocol should be applicable to fusion proteins carrying other purification tags (such as glutatione *S*-transferase, maltose binding protein) simply by changing the capture step.

Two important issues are raised by the present work concerning protein refolding. First, for all insoluble proteins in this study, protein was refolded into an immunologically active state simply by using protein solubilization with urea followed by gradient-based removal of the solubilizing agent while protein was still bound to the IMAC column. This is presumably because removal of urea from column-bound protein minimizes concentration-dependent aggregation or oligomerization of protein as the solubilizing agent diminishes, while gradient elution of protein from the IMAC column increases peak volume of the eluted material, thereby reducing protein concentration below levels required for protein aggregation/oligomerization. A second aspect of proper protein refolding after solubilization is formation of disulfide interactions. Intra- or inter-chain disulfide bonds are usually removed by reduction prior to protein refolding [22], but we made no attempt to reduce potential disulfide interactions while protein was bound to the IMAC column because chelated metal ions in the charged IMAC column are readily displaced by thiol-containing reducing agents [23]. It is clear from our immunological analyses that all proteins purified in this study were recovered in an active form. However, a lack of knowledge on the potential role of disulfide interactions in epitope structure and antigenicity of the proteins purified in this study allows no further evaluation of this aspect of protein refolding. Our recent work on epitope analysis of the *M. tuberculosis* antigen MPT70 strongly suggests that a conformational epitope is conserved on the recombinant protein after solubilization and urea removal (KL, RC and MLG, unpublished results). This finding supports the possibility that proper reoxidation and disulfide bond formation occur during protein refolding. Further experiments using antigens purified by the methodology described in the present work are needed to more clearly address some of the aspects of protein refolding after urea solubilization of recombinant proteins.

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