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ORIGINAL ARTICLE

Expression of *Mycobacterium tuberculosis* proteins MPT63 and MPT83 as a fusion: purification, refolding and immunological characterization

Taras Redchuk[1], Natalia Korotkevich[1], Oksana Gorbatiuk[2], Pavlo Gilchuk[2], Andrii Kaberniuk[1], Olena Oliynyk[1], Denis Kolibo[1], Serhiy Komisarenko[1]

[1]Palladin Institute of Biochemistry, NASU, Kiyv, Ukraine
[2]Institute of Molecular Biology and Genetics, NASU, Kiyv, Ukraine

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Summary
Proteins MPT63 and MPT83 which are common for both *Mycobacterium tuberculosis* and *Mycobacterium bovis*, due to their high immunogenicity, are thought to play a promising role in the development of immunodiagnostic reagents and vaccines. To enhance the antigenic and immunogenic properties of these proteins, fragments of the *mpt83* and *mpt63* genes were fused in tandem. In this article we present an effective method for the MPT63-MPT83 fusion product purification by metal-affinity chromatography and *in vitro* refolding. Our results demonstrate that the antigenic properties of the recombinant proteins obtained are comparable to their native analogues. The anti-rMPT63 and anti-rMPT83 sera were found to be highly reactive against the rMPT63-MPT83 fusion protein, which suggests that the fusion protein retains the antigenic properties of the parent proteins. Our results may potentially contribute to the development of improved diagnostic tools or vaccines against human and/or cattle tuberculosis.

Key words: tuberculosis; *Mycobacterium tuberculosis*; MPT63; MPT83; fusion; antigen

INTRODUCTION

Tuberculosis in humans (TB) still remains one of the most dangerous infectious diseases, killing near two million people every year. This stems partially from the imperfection of the available immunological diagnostic methods, prevention, and treatment of TB, despite the availability of the complete *Mycobacterium tuberculosis* genome sequence, which has opened new ways for the identification and characterisation of new antigenic targets (Fleischmann et al. 2002). The vast majority of contemporary TB serological tests have been designed using antigens specific for *M. tuberculosis*. However, single antigen-based test systems have been shown to have insufficient sensitivity and/or specificity, while combinations of several antigens might essentially improve the properties of such test-systems (Raja et al. 2008). A particularly notable improvement may be expected from combinations of secreted and cell-associated antigens, as both these types of antigen often become targets for immune response and may play an important role in the pathogenesis of infectious deseases.

In TB, there are two mycobacterial proteins that seem to be possible candidates for the development of complex-based test systems and vaccines. The

✉ Taras Redchuk, Palladin Institute of Biochemistry (NASU), 9 Leontovicha Street, Kyiv, 01601, Ukraine
rtakyiv@gmail.com
☎ +(380-97) 9170792
+(380-44) 2296365

conserved MPT63 (Rv1926) and MPT83 (Rv2873) proteins are found in species of the *M. tuberculosis* complex (MTC), including virulent *M. tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis*, and attenuated *M. bovis* BCG (sequences are identical for all these species). MPT63 is one of the most abundant secreted proteins of *M. bovis* BCG strains, as well as pathogenic *M. bovis/tuberculosis* (Nagai et al. 1991). Being uniquely specific to the *M. tuberculosis* complex, MPT63 is a plausible candidate for *M. tuberculosis* complex-specific diagnostics. Like other secreted proteins of *Mycobacterium* (Horwitz et al. 1995), MPT63 is a target for the immune response in the infected host and can be important for TB pathogenesis. Indeed some mycobacterial secreted proteins appear to play an essential role in the inhibition of phagosomal maturation during phagocytosis and, thus, contribute to *M. tuberculosis* survival and proliferation inside host macrophages (Clemens and Horwitz 1995).

The structural similarity of MPT63 with immunoglobulin folds and cell surface-binding proteins (Goulding et al. 2002) hints at its involvement in cell-host interactions and the ability to affect phagocytosis during bacterial internalisation. This could potentially explain the protective properties of anti-MPT63 immune responses. For instance, a DNA vaccine encoding both the MPT63 and ESAT-6 antigens has been demonstrated to induce a strong protective response (McShane et al. 2001). The immunogenic properties of MPT63 could be inferred from the high density of T-epitopes in its N-terminal immunodominant region (Lee and Horwitz 1999). Therefore, MPT63 has been proposed as a target for vaccine design and diagnostic tools development (Horwitz et al. 1995).

Another potential target antigen, MPT83, is a highly immunogenic mycobacterial lipoprotein, which has been identified as a cell surface-associated antigen by electron microscopy and flow-cytometry (Vosloo et al. 1997, Harboe et al. 1998). It has been demonstrated that monoclonal antibodies against the MPB83 surface antigen of *M. bovis* (an MPT83 homolog) increase the survival time of infected mice and change the morphology of granulomas and the distribution of acid-fast bacilli in the lungs (Chambers et al. 2004). Vaccination with DNA or RNA constructs expressing the *M. tuberculosis* MPT83 antigen are also capable of inducing both humoral (Green et al. 2009) and T-cell immune responses (Xue et al. 2004). Besides, the MPT83-encoding DNA vaccine reduces *M. bovis* dissemination in a mouse model and better protects kidneys against infection than does BCG (Chambers et al. 2001).

The abovementioned suggests that a combination of MPT63 and MPT83 proteins may be potentially interesting for the development of TB immunodiagnostic reagents and vaccines. To explore this opportunity, we: (a) fused the antigen fragments of the genes *mpt83* and *mpt63* in tandem and expressed the construct in *Escherichia coli*; (b) isolated all target proteins from bacterial cells in a purified and soluble form, and (c) tested these proteins for their capacity to induce specific polyclonal antibodies that normally recognize their native analogues.

MATERIALS AND METHODS

PCR amplification and cloning

Chromosomal DNA of *M. bovis* (BCG) was isolated by the freeze-boil method and was used as a template for PCR amplification. *mpt83* DNA was amplified with following primers: #1 5'-GGA TCC GAC ACC CTC AAC GGC GGC GAG-3' and #2 5'-CTC GAG CTG TGC CGG TGG CAT CAG TAC C-3'; *mpt63* was amplified by nested PCR. The first set of primers was: #3 5'-AGG GAC CAA TGA AGC TCA-3' and #4 5'-TCT ACG GCT CCC AAA TCA-3'. The nested primers were: #5 5'-CTG AGG ATC CAT GAA GCT CAC CAC AAT GAT-3'and #6 5'-TCA GCT CGA GCG GCT CCC AAA TCA GCA GAT-3'. *BamHI* and *XhoI* restriction sites are underlined. PCR was performed with the following settings: 94 °C/1.5 min followed by 30 cycles (94 °C/30 s, 52 °C/45 s, and 72 °C/45 s), and final elongation 72 °C/7 min.

Splicing by the overlap extension PCR procedure (SOE-PCR) was used to obtain a fused gene containing the *mpt83* and *mpt63* gene fragments. Extensions needed for splicing were added by an additional PCR step with the 5' ATC ATT GTG GTG AGC TTC ATC TGT GCC GGT GGC ATC AGT ACC 3' primer instead of #2 for the *mpt83* gene fragment. The primer 5' GGT ACT GAT GCC ACC GGC ACA GAT GAA GCT CAC CAC AAT GAT 3' was used instead of #5 to extend the *mpt63* gene fragment. The obtained products were used for splicing via 5 elongation cycles 95 °C/15 s, 55 °C/3 min, and 72 °C/40 s.

After the splicing procedure, the 5' GGA TCC GAC ACC CTC AAC GGC GGC GAG 3' and 5' TCA GCT CGA GCG GCT CCC AAA TCA GCA GAT 3' primers were added and a standard PCR scheme was performed (10 cycles 94 °C/30 s, 52 °C/45 s, and 72 °C/45 s).

The *mpt63*, *mpt83*, and *mpt63-mpt83* amplified DNA fragments were ligated with the *E. coli* expression vector pET24a(+) (Novagen) using *BamHI* and *XhoI* sites. The resulting plasmid was used to transform *E. coli* Rosetta (DE3) host cells (Novagen) for expression.

Fermentation and preparation of inclusion bodies

For protein expression, a modified auto-induction protocol originally described by Studier (2005) was applied as follows: *E. coli* BL21(DE3) cells harbouring the required plasmid were incubated at 37 °C overnight in 2 ml of 2xYT medium containing 50 ug/ml kanamycin and 1% glucose. 1:1000 dilutions of the overnight culture were used to inoculate 2 ml (small-scale expression) or 400 ml (large-scale expression) fresh 2xYT medium containing 50 ug/ml kanamycin, 25 mM $(NH_4)_2SO_4$, 50 mM KH_2PO_4, 50 mM Na_2HPO_4, 1 mM $MgSO_4$, 0.05% glucose, 0.2% α-lactose, 0.5% glycerol. The cultures were allowed to express for 18–24 h at 37 °C before the cells were harvested by centrifugation at 3000 g for 20 min. Final optical densities (OD600) were typically within the range 9.0 to 18.0, depending on induction time and expressed protein. Bacteria were lysed on ice with 10 mM Tris-HCl buffer pH 8.0 (10 ml per 1 g w/w cells) containing 1 mg/ml chicken egg lysozyme (Fluka), 5 mM $MgSO_4$, DNase I (50 ug/g cell paste) for 20 min. Cell lysates were sonicated in the presence of 0.3% sodium deoxycholate (SDC) and precipitated by spinning at 10,000 g for 20 min. Insoluble material was washed once via sonication with Tris buffer without SDC, pelleted by centrifugation and stored at –70 °C. Proteins were separated by 12% SDS-PAGE. The aliquots of supernatants and pellets were sampled and mixed with electrophoresis loading buffer consisting of 7% (w/v) SDS, 40% (v/v) glycerol, 0.25 M Tris-HCl (pH 6.8), 0.0005% (w/v) bromophenol blue and 100 mM DTT for SDS-PAGE analysis. Gels were stained with Coomassie Brilliant Blue R250. Protein concentrations were measured by densitometry using BSA of known concentration as protein standard.

On-column purification and refolding of recombinant proteins

Pelleted inclusion bodies were solubilized in 20 mM Tris-HCl (pH 8.0) containing 6 M guanidine-HCl, 100 mM Na_2HPO_4, 10 mM imidazole and 10 mM 2-mercaptoethanol for 1 h at room temperature and filtered through a 0.45 um PVDF membrane filter (Millipore). All solutions were filtered through 0.22 um nitrocellulose membrane filters (Millipore) and degassed prior to chromatography. For small-scale optimization experiments, a 1 ml HiTrap chelating column loaded with Ni2+ ions was connected to an FPLC system (Pharmacia) and equilibrated with PBS buffer (pH 8.0) containing 8 M urea and 10 mM imidazole (pump A) with the flow rate of 0.5 ml/min. A 1 ml sample of solubilized inclusion bodies (2 mg/ml) was applied from the connected P1 pump (GE Helthcare) with the flow rate of 0.2 ml/min, and the column was washed with the equilibration buffer from the FPLC pump A until the UV signal reached the base line. On-column refolding was performed as follows. A 15-ml linear gradient from the urea-containing buffer (pump A) to the refolding buffer (PBS, pH 8.0 with 10 mM imidazole) without urea (pump B) was applied with the flow rate of 0.2 ml/min. The refolded protein was eluted in the refolding buffer containing 0.3 M imidazole. For large-scale refolding experiments, a 26/20 XK column (GE Healthcare) packed with 20 ml of Ni-NTA Superflow medium (Qiagen) was connected to FPLC and refolding was performed using the reagent system described above in a 200-ml linear gradient of urea with the flow rate of 2.7 ml/min. The effect of 50, 300, 500 mM imidazole in the elution buffer on the recovery of refolded protein from the column was investigated. All fractions were collected and separated by SDS-PAGE. The purity of refolded proteins and refolding yields were determined as described above. The refolding yield was calculated as the percentage of soluble target protein recovered from inclusion bodies after refolding.

Production of rabbit antiserum against Mycobacterium antigens

Rabbit polyclonal antisera were prepared by immunizing rabbits with rMPT63 and rMPT83. With the first injection each animal received approximately 300 μg of protein mixed with complete Freund's adjuvant (CFA, Sigma). Incomplete Freund's adjuvant (IFA, Sigma) with the same amount of protein was used for subsequent immunizations. The mix was injected thrice at three intradermal sites and once intramuscularly after a 15 days interval. Blood samples were collected on the 7th day after the last immunization.

Immunoblotting

Approximately 1 ug of each purified protein was used for SDS-PAGE. Protein transfer from acrylamide gels to nitrocellulose membranes Hybond-C Extra (GE Helthcare, USA) was performed using the semi-dry method. The membrane was blocked in PBS containing 5% non-fat dry milk for 1 h at 37 °C before the reaction with antiserum. The reaction with rabbit polyclonal antibodies (dilution 1:100 in PBS with 0.1% Tween) lasted for 1 h at 37 °C. Horse

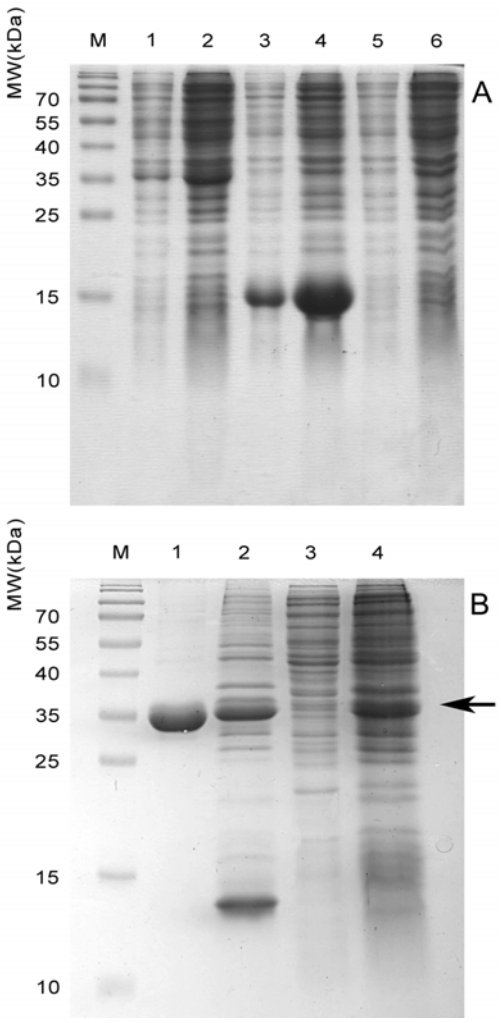

Fig. 1. *(A)* **Small-scale expression of the recombinant proteins rMPT63, rMPT83, and rMPT63-MPT83 fusion on auto-induction medium.** Lanes 1, 2 – soluble and insoluble protein fractions from induced BL21(DE3) cells carrying the plasmid pET-*mpt63-mpt83*. Lanes 3, 4 – soluble and insoluble protein fractions from induced BL21(DE3) cells carrying the plasmid pET-*mpt83*. Lanes 5, 6 – soluble and insoluble protein fractions from induced BL21(DE3) cells carrying the plasmid pET-*mpt63*. The amount of protein corresponding to 45 ul of induced *E. coli* culture was loaded in each lane. *(B)* Expression and purification of the rMPT63-MPT83 fusion protein. Lane 1 – purified and soluble rMPT63-MPT83 recovered from inclusion bodies by on-column refolding. Lane 2 – isolated and solubilized inclusion bodies that were used in refolding. Lane 3 – cytoplasmic soluble fraction. Lane 4 – total proteins of *E. coli* overproducing rMPT63-MPT83 in large-scale experiments. The amount of protein corresponding to 20–40 ul of induced *E. coli* culture was loaded in each lane. M – molecular weight marker. The arrow marks the position of the rMPT63-MPT83 fusion protein.

radish peroxidase conjugated with goat anti-rabbit immunoglobulin G (Sigma Aldrich) was used as a secondary antibody. Identification of target proteins was verified by detecting the same bands with Monoclonal Anti-polyHistidine-Peroxidase antibodies (Sigma Aldrich). The antibody reactivity was visualized with the diaminobenzidine.

RESULTS

Design of recombinant proteins

Fragments of 497 (*mpt63* fragment) and 357 (*mpt83* fragment) base pairs were prepared by PCR amplification from *M. bovis* BCG (Russia) chromosomal DNA. The amplified fragment of *mpt63* corresponded to full-length CDS of the *mpt63* gene. The fragment amplified from mpt83 corresponded to the sequence encoding 115-220 amino acids of the Fasciclin-like domain (94-217aa) of the MPT83 protein. The fragment of 811 bp was obtained using SOE-PCR. Purified amplicons were used to create recombinant plasmids for expression of the target proteins. Thus, the MPT83 sequence was positioned in the gene of the predicted fusion protein sequence after T7 tag residues introduced from the pET24a vector DNA. A PolyHis tag was added from the pET24a vector at the C terminus for the following metal affinity purification. Sequencing of cloned DNA showed that the fragments had been correctly inserted and the sequences were identical to those published (Accession No. NC_008769). Expression was achieved using standard procedures and followed by one-step metal-affinity chromatography purification. The observed molecular masses of the products obtained (16.0 kDa, 17.6 kDa, and 33.55 kDa for rMPT63, rMPT83, and rMPT63-MPT83 fusion protein) corresponded to those predicted (19 kDa, 14.5 kDa and 31 kDa, respectively).

Expression, on-column purification and refolding of recombinant proteins

Initially, expression level optimization was performed following the kit manufacturer's recommendations (Novagen). It was found, however, that the expression strain, induction temperature, time of incubation, and IPTG concentration were not significant for the expression in standard experimental conditions. To enhance the expression, we decided to use the method of protein production by auto-induction in high density shaking cultures (as described in Materials and methods).

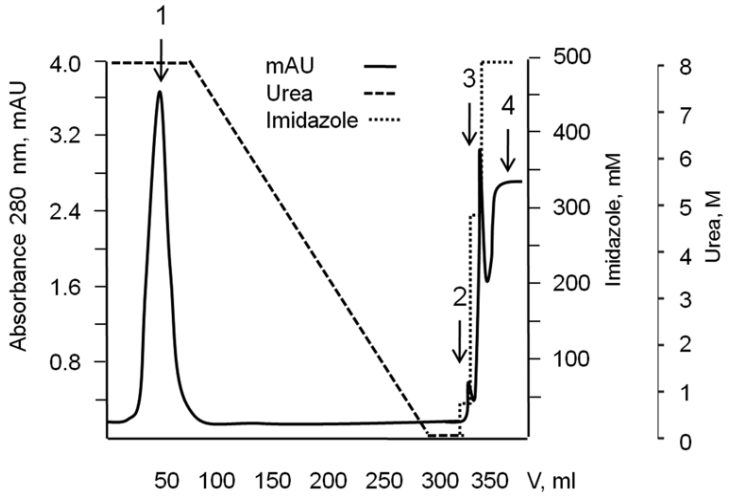

Fig. 2. **Chromatogram of the purification and refolding process on 26/20 XK column packed with 20 ml of Ni-NTA Superflow resin** (the rMPT63-MPT83 fusion protein as an example). Solubilized inclusion bodies were applied to the column loaded with a buffer containing urea, non-specifically bound proteins were washed out with the same buffer, and on-column refolding was carried out as described in Materials and methods. 1 – proteins flowing through the column and washed with the buffer containing urea; 2–4 – refolded protein eluted from the column with 50, 300, and 500 mM imidazole, respectively.

It was shown that most of the expressed rMPT63-MPT83 fusion protein and rMPT83 accumulated as insoluble inclusion bodies (Fig. 1A). We utilized the matrix-assistant refolding method to obtain soluble and purified rMPT63-MPT83 fusion protein. Solubilized under denaturing conditions the protein was loaded into a metal affinity column and refolded in a decreasing urea gradient using an automated FPLC chromatography system as described in "Materials and methods" (Fig. 2). The purity of rMPT63-MPT83 after the refolding was more than 90% based on gel densitometry data (Fig. 1B). All the relevant data on the purification and refolding experiments are summarized in Table 1.

Serological characterization of the obtained rMPT63 and rMPT83

The next stage of our investigation was serological characterization of the obtained proteins: rMPT63, rMPT83, and rMPT63-MPT83 fusion. Purified rMPT63 and rMPT83 were used to obtain polyclonal antiserum in rabbits. The specificity of the obtained sera was tested by a series of Western blots. Their reactivity with the antigen used for immunization was strong in all cases. The same bands were detected with monoclonal anti-polyhistidine-peroxidase antibodies (Fig. 3A).

To compare the antigenic properties of the obtained recombinant analogs with proteins isolated directly from bacterial cells we screened *M. bovis* BCG cell lysates and PPD for the presence of MPT63 and MPT83 (Fig. 3B).

A band of approximately 15 kDa (which corresponds to the molecular weight of native MPT63) was determined in a PPD sample incubated with an anti-rMPT63 serum. This band was absent from the lysate of lyophilized BCG perhaps because of its active secretion outside the bacterial cell. This data corroborates the thesis that MPT63 can be detected in the culture liquid but not in whole-cell extracts (Nagai et al. 1991).

In our experiments anti-MPT83 sera revealed a band with elecrophoretic mobility which corresponded to approx. 25 kDa in BCG cell lysate. According to existing data the observed weight is clearly indicative of native MPT83 (Charlet et al. 2005). The diffused band in PPD filtrate may have been caused by unspecific binding of polyclonal antibodies with degraded proteins from PPD. No antigen recognition was observed with normal animal and control animal (adjuvant administered without protein) sera.

Serological characterization of the obtained rMPT63-rMPT83 fusion

To determine whether the fusion protein possesses the antigenic properties of MPT63 and MPT83, it was tested by both anti-rMPT63 and anti-rMPT83 sera. The anti-polyhistidine monoclonal antibodies were used to identify the recombinant product bands in

Table 1. **Comparison of expression, purification, and refolding of the recombinant proteins rMPT-63, rMPT-83, and rMPT63-MPT83** (data represent one from two/three independent experiments; large-scale expression protocol was used).

	rMPT-63	rMPT-83	rMPT63-MPT83
Production yield (mg/l of *E. coli*)	<20	670	271
Expression level from total *E. coli* proteins, %	not determined	49	13
Final OD600 of induced bacterial culture	10.2	19.2	13.4
Recovery after isolation of inclusion bodies, %	not determined	82	95
Purity in inclusion bodies, %	not determined	83	27
Recovery from inclusion bodies after purification and refolding, %	not determined	96	92
Purity after refolding, %	not determined	95	93
Recovery of refolded protein from induced bacterial culture, %	not determined	79	87

Western blots. In all three assays (with anti-polyhistidine antibodies, anti-rMPT63, and anti-rMPT83 sera) strong bands in the 30 kDa marker area were detected (Fig. 3A).

DISCUSSION

One of the key challenges facing manufacturers of the vaccines and diagnostic tools is the production of pathogen proteins. The manufacturing process can be very complex and even dangerous. Some of these problems were resolved with the invention of pathogen antigen production in non-pathogenic heterologous expression systems. However this powerful approach could be sometimes problematic because some proteins are very difficult to express in *E. coli*, including several proteins of *M. tuberculosis*. Nevertheless, expression as a fusion protein may increase the expression level of the "hard-to-obtain" recombinant protein in the *E. coli* expression system and the overall production yield (Mukherjee et al. 2003).

Comparison of the expression levels of rMPT63, rMPT83, and the rMPT63-MPT83 fusion protein has revealed a dramatically increased expression of MPT63 as part of the rMPT63-MPT83 fusion protein. In particular, the band corresponding rMPT63 protein was undetectable by SDS-PAGE analysis of the rMPT63 overexpressed cell lysates. Based on the sensitivity of SDS-PAGE, we have concluded that protein concentration is less than 20 mg per 1L of *E. coli* culture. At the same time, accumulation of the rMPT63-MPT83 fusion protein was approximately 13% of total *E. coli* proteins under optimal conditions, which corresponded to 271 mg per 1L of *E. coli* culture.

Expression of recombinant proteins as inclusion bodies is a common method because of the high quantity of the target protein in the total extracts, low toxic influence on the host strain, and potential proper protein folding by *in vitro* refolding procedures (Li et al. 2004). A number of approaches for the recovery of soluble proteins from inclusion bodies have been proposed (Clark 2001, Vallejo and Rinas 2004), but to date refolding still remains an empirical process, which needs to be optimized in each individual case to achieve reasonable yields of the protein in its functional form (Tsumoto et al. 2003). In the present study we utilize the matrix-assisted refolding method to obtain a soluble and purified rMPT63-MPT83 fusion protein.

The on-column approach combined the purification and refolding stages and allowed us to obtain the target protein in multi-milligram quantities using a scalable and cost effective procedure. Thus, we developed an efficient method for obtaining a soluble and purified form of the fusion protein rMPT63-MPT83.

Another common problem of the recombinant vaccine production is a misfolding of the proteins, preventing their potential therapeutic use. A growing body of evidence suggests that each novel vaccine and diagnostic tool needs to be thoroughly analysed for its conformational correspondence with the native

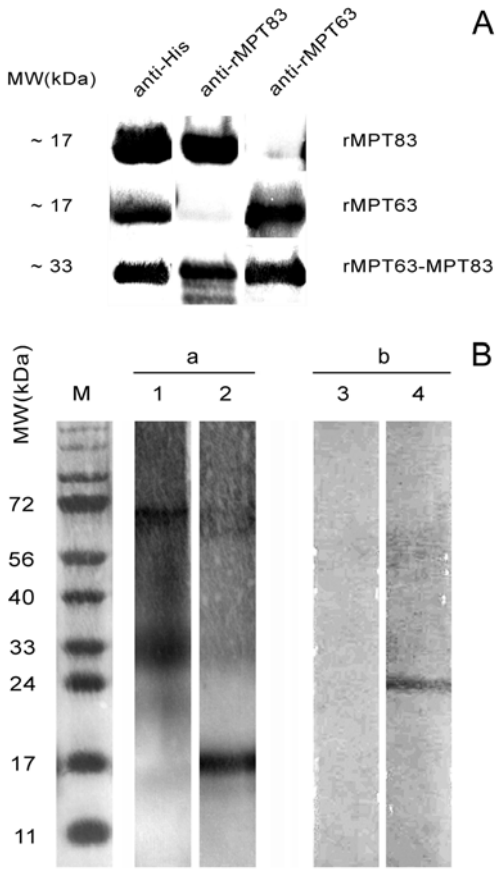

Fig. 3. *(A)* **Western blot analysis of rMPT83, rMPT63, and rMPT63-MPT83.** anti-His – anti-polyhistidine-peroxidase antibodies; anti-rMPT83 – rabbit polyclonal anti-rMPT83 sera. anti-rMPT63 – rabbit polyclonal anti-rMPT63 sera. *(B)* Immunoblotting analysis of PPD *(a)* and BCG Russia cell lysate *(b)*. Lanes 1 and 4 – rabbit polyclonal anti-rMPT83 sera. Lanes 2 and 3 – rabbit polyclonal anti-rMPT63 sera. M – molecular weight marker.

analogs (see for an example Song et al. 2008). While the biological function of MPT63 and MPT83 remains unknown, the only method for predicting native protein folding is immunological characterization.

We have detected a strong reactivity of anti-rMPT63 and anti-rMPT83 antibodies with their parent proteins from BCG lysate (MPT83) and *M. tuberculosis* culture filtrate (MPT63). These results show the ability of recombinant proteins to effectively induce production of polyclonal antibodies that can recognize *M. tuberculosis* antigens. Therefore, our results demonstrate a similarity between the antigenic properties of the obtained recombinant proteins and their native analogues that is the biggest challenge facing the development of vaccines and diagnostic tools.

Moreover, the results obtained with anti-rMPT63 and anti-rMPT83 sera also suggested that antibodies against native MPT63 and MPT83 could be detected using the recombinant fusion protein rMPT63-MPT83.

In summary, our results may potentially create a basis for further development of novel diagnostic tool or subunit vaccines against human and cattle tuberculosis.

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