Purification of native HBHA from Mycobacterium avium subsp. paratuberculosis.
Louise Lefrancois, Christelle Bodier, Sophie Lecher, Florence Gilbert, Thierry Cochard, Grégoire Harichaux, Valérie Labas, Ana Paula Teixeira-Gomes, Dominique Raze, Camille Locht, et al.

To cite this version:
Louise Lefrancois, Christelle Bodier, Sophie Lecher, Florence Gilbert, Thierry Cochard, et al.. Purification of native HBHA from Mycobacterium avium subsp. paratuberculosis.. BMC Research Notes, BioMed Central, 2013, 6 (1), pp.55. 10.1186/1756-0500-6-55. inserm-00796280

HAL Id: inserm-00796280
https://www.hal.inserm.fr/inserm-00796280
Submitted on 2 Mar 2013

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.
Purification of native HBHA from *Mycobacterium avium* subsp. *paratuberculosis*

Louise H Lefrançois¹, Christelle C Bodier¹, Sophie Lecher²³⁴⁵, Florence B Gilbert¹, Thierry Cochard¹, Grégoire Harichaux⁶⁷⁸, Valérie Labas⁶⁷⁸, Ana Paula Teixeira-Gomes¹⁶, Dominique Raze²³⁴⁵, Camille Locht²³⁴⁵ and Franck Biet¹*

**Abstract**

**Background:** Paratuberculosis remains today a major global problem in animal health, especially for dairy cattle. However, the diagnosis of its etiologic agent, *Mycobacterium avium* subsp. *paratuberculosis* (Map), still lacks sensitivity because of the lack of available antigens. Little is known about the virulence factors for this pathogen. In this study we have developed a method to produce and purify the heparin-binding hemagglutinin (HBHA), a major adhesin of *Mycobacterium*, from a culture of Map.

**Findings:** For this extremely slow-growing *Mycobacterium*, a culture was established in a 3-liter bioreactor. Using the bioreactor the amount of the Map biomass was increased 5-fold compared to a classical culture in flasks. The map-HBHA was purified from a Map lysate by heparin-Sepharose chromatography on HiTrap columns. Binding of map-HBHA onto heparin-Sepharose can be reduced in the presence of salt. Consequently, all steps of sample preparation and column equilibration were carried out in 20 mM Tris–HCl (pH 7.2). The map-HBHA was eluted by a linear NaCl gradient. High resolution mass spectrometry analyses revealed that the native form of map-HBHA has posttranslational modifications, including the removal of the initiation methionine, acetylation of the alanine residue at the N-terminal extremity and the presence of methylated lysines in the C-terminal domain of the protein.

**Conclusions:** An optimized culture of Map in a bioreactor was established to purify the native map-HBHA from a Map lysate by heparin-Sepharose chromatography. The availability of this antigen offers the possibility to study the structure of the protein and to examine its role in pathogenicity, in particular to better understand the specific interactions of Map with the intestinal tissue. The map-HBHA obtained in its native immunogenic form may also be useful to improve the diagnostic test, especially for the development of a new T-cell-based interferon gamma release assays.

**Keywords:** *Mycobacterium avium* subsp. *paratuberculosis*, HBHA, heparin-Sepharose chromatography

**Findings**

**Background**

*Mycobacterium avium* subsp. *paratuberculosis* (Map) is the etiologic agent of a severe granulomatous inflammatory bowel disease in ruminants, known as Johne’s disease or paratuberculosis [1,2]. This enzootic remains today a major animal health problem with a high global prevalence causing significant economic losses in the cattle industry [2-6]. One characteristic feature of Map is its adaptation to the gastrointestinal tract of ruminants. However, the molecular and cellular mechanisms of its interactions with M cells [7] and epithelial cells [8] are not yet fully elucidated. *Mycobacterium*-epithelium interactions, much better studied with *Mycobacterium tuberculosis*, involve an adhesin called heparin-binding hemagglutinin (HBHA), which plays a crucial role in the binding of the mycobacteria to epithelial cells and other non-phagocytic cells [9,10]. HBHA is an adhesin that binds sulphated carbohydrates, such as heparin and heparan sulfate, present on the surface of various...
lysine-rich motifs [11]. Native HBHA from *M. tuberculosis* carries methyl groups added post-translationally, which are important for its antigenicity [12]. Several investigations have demonstrated that a HBHA-specific IFN-γ response, indicative of latent *M. tuberculosis* infection and protective immunogenicity depends on the methylation of the antigen [13-16]. Comparative genomics suggests that HBHA is also present in Map. In order to study the biochemical characteristics of the HBHA from Map (map-HBHA) and to evaluate its potential in diagnostics and vaccine development, it is essential to have access to native map-HBHA. Our first attempts to purify map-HBHA by heparin-Sepharose chromatography under conditions used to purify HBHA from *M. tuberculosis* failed to isolate the map-HBHA. In addition, the purification was hampered by the difficulty to obtain sufficient biomass of Map due to the extremely slow growth of this microorganism.

In this study we established a new method of Map culture, using a bioreactor, which substantially increased the biomass yield and shortened the time of cultivation. In addition, we describe a new purification procedure, which led to the isolation of native map-HBHA.

**Results and discussion**

**Optimization of Map biomass production in a bioreactor**

Map is one of the slowest-growing *Mycobacteria* with a mean generation time of 1.38 days [17]. Map cultures thus require long time periods with limited opportunities for recording and correcting parameters. Furthermore, the final product of these cultures was shown to contain high proportions of dead bacilli that may reduce the quality of the proteins harvested [18]. To circumvent these difficulties, we chose to investigate the growth of Map in a bioreactor to increase the biomass, standardize the culture conditions and control potential contaminations. Previous studies using bioreactors have described the standardization of *M. bovis* BCG cultures for vaccine production [19,20]. Growth of Map in a bioreactor was compared to conventional culture flasks inoculated in parallel with the same Map pre-culture at an OD 

\[ \text{OD}_{600nm} = 0.06 \]

After 40 days, the cultures were centrifuged and the harvested biomasses were weighed. As shown in Figure 1, the biomass harvested from the bioreactor was significantly more important in comparison to the conventional culture in flasks. Using the bioreactor we obtained, as an average of three independent experiments, five times more bacteria than with flask cultures (4 versus 20 g L\(^{-1}\)).

**Protein purification**

Our first attempts to purify map-HBHA by heparin-Sepharose chromatography under conditions used to purify HBHA from *M. tuberculosis* have been unsuccessful. This may be due, at least partly, to the nature of the protein. Although the alignment of the HBHA amino acid sequences from different mycobacterial species indicates that these molecules are highly conserved, the C-terminal lysine-rich domain is rather divergent (Figure 2A). These differences may modify the affinity of the proteins for the heparin-rich domain, due to the differences in the distribution of the positively charged lysines involved in the cation- and anion-exchange chromatography (Figure 2B). In a previous study, we have shown that the isolation of the *M. smegmatis* HBHA requires a modification of the elution parameters, as its C-terminal end is less positively charged, compared to that of the *M. tuberculosis* HBHA [21]. As indicated in Figure 2B, the C-terminal sequence of the map-HBHA contains one acidic residue and 9 positively charged lysines compared to 14 positively charged lysines in HBHA from *M. tuberculosis*. The predicted isoelectric point (7) is lower than that calculated for HBHA of *M. tuberculosis* (9.6) or *M. smegmatis* (8.8), suggesting that the binding and elution parameters have to be adjusted. Because of the slow growth of Map, the chromatography conditions were first established with a recombinant form of map-HBHA producing in *E. coli*.

**Heparin-Sepharose column**

First, a lysate of recombinant *E. coli* producing map-HBHA was applied onto two types of heparin-Sepharose columns in 0.5 × PBS: the prepacked HiTrap column or heparin-Sepharose CL6B poured into a Pharmacia column. As shown in Figure 3, the recombinant map-

![Figure 1 Increasing biomass production using a bioreactor. Cultures of Map K-10 (ATCC BAA-968) in flask or bioreactor were inoculated with a pre-culture at an OD\(_{600}\) of 0.06. Both cultures were maintained at 37°C during 40 days under agitation and air in the bioreactor and under static conditions in the flask. The bacteria were harvested by centrifugation and the biomasses were weighed. The figure represents the average of three independent experiments (+/- standard deviations).](Image)
HBHA was successfully purified from a lysate of *E. coli*. It was eluted from the heparin-Sepharose column at low salt concentrations, starting from 100 mM NaCl. Although both columns showed similar performances with a lysate containing large amounts of recombinant map-HBHA, the HiTrap column, more reproducible in our hands, was preferred.

**Buffer conditions**
Surprisingly, when the supernatant of a whole-cell lysate of Map was subjected to purification under the conditions established for recombinant map-HBHA, the native map-HBHA was not isolated. As shown in Figure 4 in late elution fractions only one protein corresponding to Lbp/Hlp,
previously described to bind heparin with strong affinity [22], was eluted at 500 mM NaCl in this study. These results suggest that binding and elution parameters need to be changed to isolate native map-HBHA. The 0.5 × PBS loading buffer was thus replaced by a 20 mM Tris–HCl (pH 7.2) buffer to reduce the presence of salt that can inhibit the binding of the protein to the matrix. The map-HBHA prepared in 20 mM Tris–HCl (pH 7.2) was able to efficiently bind the heparin-Sepharose matrix and was eluted at approximately 200 mM NaCl (Figure 5). The early elution of map-HBHA in the NaCl gradient suggests a relationship between the number of positive charges in the protein and the binding force to the sulfated sugars. As indicated in Figure 5, Lbp/Hlp eluted later, at 500 mM NaCl.

Mass spectrometry analysis
To ascertain the identities of the proteins eluted from heparin-Sepharose, High Resolution tandem Mass Spectrometry (HR-MS/MS) analyses using a bottom-up approach GelC-MS/MS were carried out. These analyses confirmed that the 25-kDa protein eluted at 200 mM NaCl corresponds to map-HBHA and that the 31-kDa protein eluted at 500 mM NaCl corresponds to map-Lbp/Hlp. In addition, HR-MS/MS also revealed important characteristics of the native proteins purified from the Map culture (Table 1). Similar to the M. tuberculosis HBHA [23], native map-HBHA contains no initiation methionine at its N-terminus and that the first amino acid is thus an alanine residue. The cleavage of the initiation methionine is a post-translational common process in Mycobacteria [24,25]. The HR-MS/MS analyses also indicated that the amino-terminal alanine is acetylated. This post-translational modification already reported for other mycobacterial proteins may be an important regulator of protein functions [26,27].

Another feature of native map-HBHA, revealed by HR-MS/MS, is the presence of methylated amino acids as indicated in Figure 6 and Table 1. Among the peptides analyzed in this study, the lysine residues 154 and 168 were mono-methylated. Other proteomic analyses are necessary to determine the complete methylation profile of the HBHA produced by Map. Although the exact role of the methylation remains to be elucidated, this post-translational modification was shown to be crucial for the immunogenicity of the M. tuberculosis HBHA. Only the native, methylated protein is able to induce strong IFN-γ secretion by peripheral blood mononuclear cells from latently infected human [14] and when administered in combination with strong Th1-promoting adjuvants only the methylated form was able to confer protection against M. tuberculosis in mouse models [16].

Altogether these data highlight the importance of having access to proteins in their native form with their specific post-translational modifications to understand their function, their location and to envisage their use as antigens for diagnostics or vaccine development.

Conclusions
The objective of this study was to develop the conditions that allow us to purify native HBHA from a lysate of Map. To obtain sufficient biomass of Map, for which the culture is slow and tedious, a bioreactor culture method was developed. Under these conditions it was possible to obtain a biomass of 20 g per liter of culture, a 5-fold higher yield than in traditional culture conditions. Native map-HBHA was purified by heparin-Sepharose chromatography. HiTrap columns gave the best and most reproducible results. Due to the specific chemical characteristics of native map-HBHA, in particular the charges present in the C-terminal part of the protein, the Map lysate had to be prepared in 20 mM Tris–HCl (pH 7.2) to allow for optimal binding of map-HBHA to the heparin-Sepharose matrix.
HR-MS/MS analyses revealed post-translational modifications, such as presence of an acetyl alanine residue at the N-terminus and methyl-lysines in the C-terminal part of the protein. In this study, we have thus optimized the Map culture conditions and the purification of native map-HBHA, which is therefore now available for structure-function studies and investigations for use in diagnosis or vaccine development against Map.

### Methods

**Bacterial strains, growth conditions and DNA manipulations**

*Mycobacterium avium* subsp. *paratuberculosis* (Map) strain K-10 (ATCC BAA-968) was grown at 37°C in Sauton medium supplemented with 2 mg L\(^{-1}\) of mycobactin J (IDVET, Montpellier, France). *E. coli* TOP10 (Invitrogen Carsbad, CA) and *E. coli* BL21(DE3) (Novagen, Darmstadt, DE) were grown in LB medium supplemented with 50 μg L\(^{-1}\) kanamycin as appropriate. Restriction enzymes, T4 DNA ligase and other molecular biology reagents were purchased from New England Biolabs, Roche or Promega. PCRs were performed using a Bio-Rad thermal cycler model iCycler and the PCR products were sequenced by GenomExpress (Grenoble, France).

**Cultivation of Map K-10 in bioreactor**

A Map K-10 pre-culture was prepared in Sauton medium to obtain an initial optical density at 600 nm of 0.06. This was then inoculated either in 175 cm\(^2\)-Greiner Bio-one Flasks maintained at 37°C in static conditions or in a 3-liter bioreactor (Seric Génie Industriel, Toulouse, France) constantly kept at 37°C with a stirring rate at 110 rpm and 12 L h\(^{-1}\) of air. During growth, serial time samplings were performed to determine absorbance at 600 nm. Once the stationary phase was reached, the bacteria were harvested by centrifugation at 7,000 × g for 15 min at 4°C and stored at −20°C until use.

**Protein purification by heparin-Sepharose chromatography**

A frozen Map pellet corresponding to 5 g was thawed, washed twice with 0.5 × PBS or 20 mM Tris–HCl (pH 7.2) and heated at 80°C during 30 min. Heat-killed bacteria were centrifuged at 10,000 × g for 20 min. The Map pellet was resuspended in 45 mL 0.5 × PBS or 20 mM Tris–HCl (pH 7.2) was poured into a 1.5 × 10 cm column (Pharmacia), according to the recommendations of the supplier. The columns were washed with 100 mL of 0.5 × PBS or

### Table 1

| Peptide identified by HR-MS/MS | Peptide position start - end | Peptide modification |
|-------------------------------|-----------------------------|---------------------|
| A\(_9\)ENPNIDDLR\(_11\) | 2-11 | Acetylated |
| FQEDLPEQTEL\(_{72}\) | 60-72 | |
| DKFTTEELR\(_{81}\) | 73-81 | |
| KAAEGYLEATNR\(_{94}\) | 82-94 | |
| AEEGYLEATNR\(_{94}\) | 83-94 | |
| LRSQTAFEDASAR\(_{121}\) | 109-121 | |
| SQTAFA\(_{111}\) | 111-121 | |
| SQTAFA\(_{111}\) | 111-145 | |
| AEGYDV\(_{122}\) | 122-145 | |
| AA\(_{151}\) | 151-162 | Methylated |
| LGIELPGK\(_{162}\) | 154-162 | |
| LGIELPGKAA\(_{164}\) | 154-168 | Methylated |
| LGIELPGKAAG\(_{170}\) | 154-169 | |

Peptides highlighted onto sequence:

\(\text{MAA}_{9}\text{ENPNIDDLR}\text{APLLAALGAADLALATVNDLIANLRERAEEAETRTTRVEERRARTLKY/QEDLPEQTELRDKFTTEELRKAEGYLEATNRNYELVEREAALQLRLR}

\(\text{SQTAFA}\text{AARAEEGYDV}\text{AELTQEALGTVA}\text{SAGR}\text{AVGERAAK}_{151}\text{LVGIELPGKAEAGK}_{160}\text{KAIAKAPAKAPAKRVTQK.}

*The 13 peptides identified by HR-MS/MS, text in italic, correspond to 52% of sequence coverage.*
20 mM Tris–HCl (pH 7.2) until the absorbance at 280 nm was close to 0. The bound material was eluted by a 0-1 M NaCl linear gradient using a flow rate maintained at 0.6 mL min\(^{-1}\) and automatically collected in 1 mL fractions. Whole cell lysates, flow-through material and eluted fractions were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed according to Laemmli [28].

**Cloning, sequencing and production of recombinant map-HBHA in *E. coli***

The map-HBHA-encoding gene (MAP3968) was amplified by PCR from chromosomal DNA using the DNA polymerase (Promega) and two synthetic oligonucleotides (Sigma) with the following sequences: 5'\-TATACATGACCATGGCCGAAAACCCGAACATCG -3', and 5'\-ATATAAGCGTGGTACCACGAGGTTGTTCCAGGCC -3', containing *NdeI* and *HindIII* sites (underlined). The fragment was amplified after a short denaturation cycle of 3 min at 95°C by using 35 cycles as follows: 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s with a final elongation cycle at 72°C for 10 min. The PCR product containing the map-HBHA-coding sequence was digested by *NdeI* and *HindIII* and then inserted into pET-24a(+) (Novagen), generating pET::map-HBHA. This plasmid was used to transform *E. coli* TOP 10 for sequencing and *E. coli* BL21(DE3) for protein expression. After transformation, *E. coli* BL21(DE3) cells were grown at 37°C in 250 mL LB broth supplemented with 50 μg mL\(^{-1}\) kanamycin. At an OD\(_{600}\) of 0.5, IPTG was added to a final concentration of 1 mM, and growth was continued for 4 h. The culture was then centrifuged at 7,000 \(\times\) g for 15 min at 4°C. The pellet obtained was stored at –20°C until applied onto heparin Sepharose as described above.

The GenBank accession numbers for the map-HBHA (strains K-10 and ATCC19698) sequences are...
Identification of the purified Map proteins by high resolution mass spectrometry analysis

Proteins were digested in-gel with trypsin as previously described [29]. Peptides were analyzed by nanoLC-MS/MS using an Ultimate®3000 RSLC coupled to a LTQ Velos Orbitrap Mass Spectrometer. Samples were loaded onto a trap column (Acclaim PepMap 100 C18, 100 μm inner diameter × 2 cm) at a flow rate of at 5 μL min⁻¹ with 4% solvent B (0.1% formic acid, 15.9% water, 84% acetonitrile) and 96% solvent A (0.1% formic acid, 97.9% water, 2% acetonitrile). Peptides were fractionated using a nano-column (Acclaim PepMap C18, 75 μm inner diameter × 15 cm), with a nanoflow rate of 300 nl min⁻¹ by applying a gradient of 4-55% B for 60 min. The LTQ Velos Orbitrap instrument was operated in a data-dependent mode with R = 30,000. In the scan range of m/z 300–1800, the 10 most intense peptide ions with charge states ≥2 were isolated for HCD fragmentation. Raw data files were converted to MGF. The data were searched against nr NCBI database (Eubacteria) using MASCOT search engine (version 2.3). Carbamido-methylcysteine was set as a fixed modification, and oxidation of methionine, N-terminal acetylation and methylation of lysine were set as variable modifications. Two missed cleavages were allowed. The tolerance of the ions was set to 10 ppm for parent and 0.1 Da for fragment ion matches. Mascot results were subjected to the Scaffold 3 software (version 3.2). Peptide identifications were accepted if they could be established at a greater than 95.0% probability as specified by the Peptide Prophet algorithm [30]. Protein identifications were accepted if they could be established at a greater than 99.0% probability and contained at least two identified peptides [31]. Identified post-translational modifications were validated using Scaffold PTM (version 2.0). Localization sites were accepted if they could be established at a greater than 99% probability as specified by the Ascore algorithm [32].

Competing interests
The authors have no competing interests.

Authors' contributions
FB, LL and DR conceived of the study, participated in its design and coordination, collated and analyzed the data. FB, LL, DR and CL drafted the manuscript. CB, SL, TC, LL, FG, APTG, GH and VL participated in the laboratory and field work. All authors read, criticized and approved the final manuscript.

Acknowledgements
FB, LL, CB, TC, FG and APTG were supported by the Institut National de la Recherche Agronomique (INRA).

The high resolution mass spectrometer was financed (SMHART project) by the European Regional Development Fund (ERDF), the Conseil Régional du Centre, the French National Institute for Agricultural Research (INRA) and the French National Institute of Health and Medical Research.

Author details
1INRA, UMR ISP 1282 Infectiologie et Santé Publique, Nouzilly F-37380, France.
2CNRS UMR, Lille 8204, France.
3INSERM, Lille U1019, France.
4Institut Pasteur de Lille, Lille, France.
5Univ Lille Nord de France, Lille F-59000, France.
6INRA, UMR85 Physiologie de la Reproduction et des Comportements Plate-forme d’Analyse Intégrative des Biomarqueurs, Nouzilly, France.
7CNRS, UMR, Nouzilly 7247, France.
8IFCE, Nouzilly F-37380, France.

Received: 11 October 2012 Accepted: 30 January 2013
Published: 7 February 2013

References
1. Clarke CJ: The pathology and pathogenesis of paratuberculosis in ruminants and other species. J Comp Pathol 1997, 116(3):217–261.
2. Harris NB, Barletta RG: Mycobacterium avium subsp. Paratuberculosis in veterinary medicine. Clin Microbiol Rev 2001, 14(3):489–512.
3. Nielsen SS, Toft N: A review of prevalences of paratuberculosis in farmed animals in europe. Prev Vet Med 2009, 88(1):1–14.
4. Ott SL, Wells SJ, Wagner BA: Herd-level economic losses associated with Johnes's disease on US dairy operations. Prev Vet Med 1999, 40(3–4):179–192.
5. Smith RL, Grohn YT, Pradhan AK, Whitlock RH, Van Kessel JS, Smith JM, Wolfgang DR, Schukken YH: A longitudinal study on the impact of Johnes' disease status on milk production in individual cows. J Dairy Sci 2009, 92(6):2653–2661.
6. Wilson DJ, Rood K, Biswas P, Byrem TM: Herd-level prevalence of Johnes' disease in Utah and adjacent areas of the intermountain west as detected by a bulk-tank milk surveillance project. J Dairy Sci 2010, 93(12):5972–5977.
7. Momotani E, Whipple DL, Thiermann AB, Cheville NF: Role of M cells and macrophages in the entrance of mycobacterium paratuberculosis into domes of ileal Peyer's patches in calves. Vet Pathol 1988, 25(2):131–137.
8. Pott J, Basler T, Duer CU, Roehde M, Goethe R, Hornef MW: Internalization-dependent recognition of mycobacterium avium ssp. Paratuberculosis by intestinal epithelial cells. Cell Microbio 2009, 11(2):1802–1815.
9. Menozzi FD, Bischoff R, Fort E, Brennan MJ, Locht C: Molecular characterization of the mycobacterial heparin-binding hemagglutinin, a mycobacterial adhesin. Proc Natl Acad Sci U S A 1998, 95(21):12625–12630.
10. Pethe K, Alonso S, Biet F, Delogu G, Brennan MJ, Locht C, Menozzi FD: The heparin-binding haemagglutinin of M. Tuberculosis Is required for extrapulmonary dissemination. Nature 2001, 412(6843):190–194.
11. Pethe K, Aumercier M, Fort E, Gator C, Locht C, Menozzi FD: Characterization of the heparin-binding site of the mycobacterial heparin-binding hemagglutinin adhesin. J Biol Chem 2000, 275(19):14237–14240.
12. Pethe K, Bifani P, Drobecq H, Sergeraert C, Debrée AS, Locht C, Menozzi FD: Mycobacterial heparin-binding hemagglutinin and laminin-binding protein share antigenic methyllysines that confer resistance to proteolysis. Proc Natl Acad Sci U S A 2002, 99(16):10759–10764.
13. Mansuri C, Temmerman S, Van Vooren JP, Drowart A, Pethe K, Menozzi FD, Locht C, Mascart F: Differential T and B cell responses against mycobacterium tuberculosis heparin-binding hemagglutinin adhesin in infected healthy individuals and patients with tuberculosis. J Infect Dis 2002, 185(4):513–520.
14. Temmerman S, Pethe K, Parra M, Alonso S, Rouanet C, Pickett T, Drowart A, Debrée AS, Delogu G, Menozzi FD, et al: Methylation-dependent T cell immunity to mycobacterium tuberculosis heparin-binding hemagglutinin. Nat Med 2004, 10(9):935–941.
15. Guerrero GG, Locht C: Recombinant HBHA boosting effect on BCG-induced immunity against mycobacterium tuberculosis infection. Clin Dev Immunol 2011, 2011:30702.
16. Parra M, Pickett T, Delogu G, Omprakash, de C, Debrée AS, Locht C, Brennan MJ: The mycobacterial heparin-binding hemagglutinin is a protective antigen in the mouse aerosol challenge model of tuberculosis. Infect Immun 2004, 72(12):6799–6805.
17. Lambrecht RS, Carriere JF, Collins MT: A model for analyzing growth kinetics of a slowly growing mycobacterium sp. Appl Environ Microbial 1988, 54(4):910–916.
18. Milstien JB, Gibson JJ: Quality control of BCG vaccine by WHO: a review of factors that may influence vaccine effectiveness and safety. Bull World Health Organ 1990, 68(1):93–108.

19. Dietrich G, Mollenkopf HJ, Weber H, Knopp B, Diehl KD, Hess J, Blackkolb F, Broker M, Kaufmann SH, Hundt E: Cultivation of mycobacterium bovis BCG in bioreactors. J Biotechnol 2002, 96(3):259–270.

20. Nyabenda J, Bautens E, Borremans M, Verhoestadt R, Weckx M, Marchal A: The production of mycobacterial antigens by homogeneous culture in a fermentor. J Biol Stand 1988, 16(4):259–267.

21. Biet F, Angela de Melo Marques M, Grayon M, Xavier da Silveira EK, Brennan PJ, Drobecq H, Raze D, Vidal-Pessolani MC, Locht C, Menozzi FD: Mycobacterium smegmatis produces an HBHA homologue which is not involved in epithelial adherence. Microbes Infect 2007, 9(2):175–182.

22. Lefrancois LH, Pujol C, Bodier CC, Teixeira-Gomez AP, Drobecq H, Rosso ML, Raze D, Dias AA, Hugot JP, Chacon O, et al: Characterization of the mycobacterium avium subsp. paratuberculosis laminin-binding/histone-like protein (Lbp/Hlp) which reacts with sera from patients with Crohn’s disease. Microbes Infect 2011, 13(6):585–594.

23. Menozzi FD, Rouse JH, Alavi M, Laude-Sharp M, Muller J, Bischoff R, Brennan MJ, Locht C: Identification of a heparin-binding hemagglutinin present in mycobacteria. J Exp Med 1996, 184(3):993–1001.

24. Kanudia P, Mittal M, Kumaran S, Chakraborti PK: Amino-terminal extension present in the methionine aminopeptidase type 1c of mycobacterium tuberculosis is indispensable for its activity. BMC Biochem 2011, 12:35.

25. Zhang X, Chen S, Hu Z, Zhang L, Wang H: Expression and characterization of two functional methionine aminopeptidases from mycobacterium tuberculosis H37Rv. Curr Microbiol 2009, 59(5):520–525.

26. Hwang CS, Shemory A, Varshavskiy A: N-terminal acetylation of cellular proteins creates specific degradation signals. Science 2010, 327(5968):973–977.

27. Okkels LM, Murer EC, Schmid M, Rosenkrandt I, Kaufmann SH, Andersen P, Jungblut PR: CFP10 Discriminates between nonacetylated and acetylated ESAT-6 of mycobacterium tuberculosis by differential interaction. Proteomics 2004, 4(10):2954–2960.

28. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 227(5259):680–685.

29. Bourin M, Gautron J, Berges M, Attucci S, Le Blay G, Labas Y, Nys Y, Reyholt-Godbert S: Antimicrobial potential of egg yolk ovoinhibitor, a multidomain kazal-like inhibitor of chicken egg. J Agric Food Chem 2011, 59(23):12368–12374.

30. Keller A, Nesvizhskii AI, Kolker E, Aebersold R: Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal Chem 2002, 74(20):5383–5392.

31. Nesvizhskii AI, Keller A, Kohler E, Aebersold R: A statistical model for identifying proteins by tandem mass spectrometry. Anal Chem 2003, 75(17):4646–4658.

32. Beausoleil SA, Villien J, Gerber SA, Rush J, Gygi SP: A probability-based approach for high-throughput protein phosphorylation analysis and site localization. Nat Biotechnol 2006, 24(10):1285–1292.