Available online at www.sciencedirect.com
SCIENCE DIRECT®
Protein Expression and Purification 45 (2006) 132–141
Protein Expression & Purification
www.elsevier.com/locate/yprep

Characterization of recombinant *Francisella tularensis* acid phosphatase A

Thomas J. Reilly [a,b,*], Richard L. Felts [c], Michael T. Henzl [d], Michael J. Calcutt [a], John J. Tanner [c,d]

[a] *Department of Veterinary Pathobiology, University of Missouri, Columbia, MO 65211, USA*
[b] *Veterinary Medicine Diagnostic Laboratory, University of Missouri, Columbia, MO 65211, USA*
[c] *Department of Chemistry, University of Missouri, Columbia, MO 65211, USA*
[d] *Department of Biochemistry, University of Missouri, Columbia, MO 65211, USA*

Received 13 April 2005, and in revised form 5 May 2005
Available online 31 May 2005

Abstract

Francisella tularensis is the etiologic agent of the potentially fatal human disease tularemia and is capable of survival and multiplication within professional phagocytes of the host. While the mechanisms that allow intracellular survival of the bacterium are only now beginning to be elucidated at the molecular level, previous work demonstrated that *F. tularensis* produces copious levels of an acid phosphatase which in crude and purified form affected the dose-dependent abrogation of the respiratory burst of stimulated neutrophils. The work presented here was undertaken to provide a source of recombinant *F. tularensis* acid phosphatase for detailed biochemical, biological, and structural studies. Results from this work are consistent with the ability to generate milligram amounts of recombinant enzyme whose attributes are demonstrably equivalent to those of the native enzyme. Such properties include molecular mass, broad substrate specificity, sensitivity and resistance to various inhibitors, pH optimum, and reactivity with rabbit polyclonal antibody to the native enzyme.

Keywords: *Francisella tularensis*; Acid phosphatase A; TRAP; Burst-inhibiting acid phosphatase; Phosphomonoesterase

Francisella tularensis, the etiologic agent of tularemia, is a facultative intracellular pathogen that causes serious and potentially fatal illness in a number of mammalian hosts including man. The organism grows readily in broth culture and is highly infectious, requiring fewer than 10 organisms to establish infection [1]. It can be spread by aerosol resulting in the pneumonic form of the disease, inhalation tularemia [1]. Because of its pathogenicity, persistence in a multitude of host species including arthropods, ease of isolation, growth, and dissemination, the agent is designated a Category A Priority Pathogen by the Centers for Disease Control and is considered a potential bioterrorism threat. Currently available antimicrobials are effective in treating tularemia; however, the organism can be engineered to carry numerous resistance genes whose presence would impede treatment if such a manipulated strain was employed in an attack [1]. An attenuated tularemia vaccine strain is available [2], although its success in preventing human disease is variable and little is known about vaccine efficacy against an aerosolized agent [1]. Understanding the biology and biochemistry of *F. tularensis* is imperative if we are to better prepare ourselves to prevent or control a deliberate release of *F. tularensis* as a weapon of mass destruction. Thus, a number of investigators have initiated molecular and biochemical studies to elucidate mechanisms for host colonization of *F. tularensis* as well as intra- and extracellular survival.

* Corresponding author.
E-mail address: reillyt@missouri.edu (T.J. Reilly).

1046-5928/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2005.05.001

Results from recent molecular approaches have identified several *F. tularensis* gene products required for intracellular survival. These products include IglC and PdpD both encoded within a *F. tularensis* pathogenicity island [3], MglA, a homolog of stringent starvation protein A (SspA)[1] of *Escherichia coli* and thought to be involved in transcriptional activation [4,5]. In addition, a number of metabolic enzymes including glutamine phosphoribosylpyrophosphate, amidotransferase, alanine racemase, and ClpB protease have been found to be important for intracellular survival of the pathogen [3]. Biochemical approaches to identify potential virulence factors have resulted in the identification of additional important targets and have resulted in purification and characterization of *F. tularensis* proteins. One such molecule is *F. tularensis* AcpA (FtAcpA), originally defined as the respiratory burst-inhibiting acid phosphatase. While acid phosphatases are ubiquitous, some intracellular pathogens including *Yersinia pestis* [6], *Legionella micdadei* [7], *Leishmania donovani* [8], *Coxiella burnetii* [9], and others [10,11] produce acid phosphatases which are believed to be critical virulence factors. The 57-kDa secreted acid phosphatase of *F. tularensis* has broad substrate specificity, a pH optimum of 6.0, and low-level phospholipase C activity for *p*-nitrophenylphosphoryl choline. Biologically, the enzyme was found to affect the diminution of both fMLP (*N*-formyl-methionyl-leucyl-phenylalanine) and PMA (phorbol 12-myristate13-acetate)-stimulated respiratory bursts in neutrophils in a dose-dependent manner. As such this enzyme is thought to be important for intracellular survival within cells of the reticuloendothelial system [12].

This investigation was undertaken as a prelude to in-depth characterization of the protein with respect to its enzymology, its potential as a vaccine candidate, and for production of diffraction quality crystals from which we could elucidate the three-dimensional structure of this important enzyme. To this end we report here, the cloning, expression, purification, and characterization of recombinant *F. tularensis* AcpA (rFtAcpA).

Materials and methods

Materials

The plasmid pGB2 described previously [12] contained the entire *acp*A open reading frame, including endogenous signal sequence of *F. tularensis acp*A and was used as the template for PCR amplification. *E. coli* DH5α competent cells were obtained from Invitrogen (Carlsbad, CA). The overexpression vector pET20b and *E. coli* expression strain BL21 (DE3) were obtained from Novagen (Madison, WI). Plasmid pZERO Blunt and *E. coli* DH10B were obtained from Invitrogen. PCR primers FtAcpANterm (CGACTTTTGCCATGG ATGTGAATAATAGC) and FtAcpACterm (CCGAG TAAATACTCGAGGTTTAATTTATC) were obtained from Integrated DNA Technologies (Coralville, IA). All bacteriological media were from Difco (Detroit, MI) and purchased through Fischer Scientific (Chicago, IL). All other chemicals including secondary antibody, unless otherwise stated, were obtained from Sigma Chemical (St. Louis, MO) and were of the highest purity available. Ion exchange, HiTRAP metal-chelating, and gel filtration chromatography standards were purchased from GE Biosciences (Piscataway, NJ). Protein electrophoresis reagents were obtained from BioRad (Richmond, CA). Perfect protein SDS–PAGE molecular weight standards were purchased from Novagen.

Construction of overexpression vector pFtAcpAExp

PCR primers FtAcpANterm and FtAcpACterm containing *Nco*I and *Xho*I sites, respectively (underlined above), were used to amplify *F. tularensis acp*A contained in plasmid pGB2. Amplification parameters included 1 cycle at 94 °C for 30 s, 25 cycles at 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min followed by a 7 min incubation at 72 °C. The ~1.5-kb Pfu polymerase-generated amplicon was gel-purified and cloned into plasmid pZERO-Blunt. *E. coli* DH10B transformants were grown overnight in Luria–Bertani (LB) containing kanamycin at 40 μg/ml. Plasmids were recovered and digested with *Nco*I and *Xho*I; a ~1.5-kb insert was gel-purified and ligated into similarly digested pET20b. Ligation reactions were initially transformed into *E. coli* DH5α and grown on LB agar containing ampicillin at 50 μg/ml. Plasmid pRAcpAExp was purified, submitted to the University of Missouri DNA core facility for sequencing. The sequence of the insert of pRAcpAExp was compared to that of *acp*A and was found to be free of PCR-induced mutations. However, during sequence confirmation, it became apparent that compressions during manual sequencing of *acp*A had led to sequencing errors at two nucleotides. These nucleotides have been corrected in the GenBank submission.

Enzyme assays

Phosphomonoesterase activity was measured using a discontinuous colorimetric assay. The standard 0.2 ml assay mixture contained 0.2 M sodium acetate, pH 6.0, 2.0 mM *p*-nitrophenylphosphate (*p*NPP), and varying amounts of enzyme. The mixtures were incubated at 37 °C for 15 min at which time the reaction was stopped by addition of 0.2 ml of 0.5 M glycine, pH 10. The

[1] *Abbreviations used:* SspA, stringent starvation protein A; FtAcpA, *F. tularensis* AcpA; rFtAcpA, recombinant *F. tularensis* AcpA; BCA, bichinchoninic acid; *p*NPP, *p*-nitrophenylphosphate; PLC, phospholipase C; BSA, bovine serum albumin.

concentration of *p*-nitrophenol produced in the assay was determined by comparison to linear regression values ($r^2 = 0.97$–0.99) of *p*-nitrophenol standards at known concentrations using a BioRad ELISA Plate Reader Model 680 set to 405 nm. Enzyme fractions were diluted in 50 mM sodium acetate, pH 5.6, containing 150 mM NaCl to a concentration that resulted in a linear response of the plate reader with increasing amounts of enzyme. One unit of activity was defined as the amount of enzyme necessary to catalyze conversion of 1 nmol substrate to product per hour at 37 °C. Preliminary assays to determine the pH optimum of the enzyme for *p*NPP were performed in 0.2 M sodium acetate at several different pHs. Enzyme activity for substrates other than *p*NPP was determined by discontinuous colorimetric assay as described by Lanzetta et al. [13]. Enzyme-catalyzed dephosphorylation of each substrate was tested at 1 mM final substrate concentration in 0.2 M sodium acetate, pH 6.0, and a constant amount of diluted enzyme. The reactions were stopped after 15 min incubation at 37 °C with the malachite green ammonium molybdate detection reagent. Concentration of inorganic phosphate was determined by comparison of each sample to a K_2HPO_4 standard curve subtracting background absorbance for each substrate under similar conditions in absence of enzyme. Standard enzyme assays were conducted as previously described [12] to determine the effect of potential inhibitors on rFtAcpA activity. Prior to use, each inhibitor was tested for its effect on the pH of the assay buffer. Determination of the Michaelis–Menten constants of substrates was performed using the Lanzetta assay and a wide concentration range, at least $K_m/10$ to $5 \times K_m$ of substrate. Each substrate was tested at a minimum of 14 different concentrations using five different volumes of diluted enzyme per substrate concentration. Data were analyzed using a nonlinear least squares regression program [14]. The phospholipase C (PLC) activity of rFtAcpA was conducted according to the method of Kurioka and Matsuda [15]. Briefly, purified enzyme was assayed for PLC activity in a discontinuous colorimetric assay using 20 mM *p*-nitrophenylphosphorylcholine in 0.25 mM Tris, pH 7.4, containing 40% w/w sorbitol. Dephosphorylation of the substrate was measured by the increase in absorbance at 405 nm as previously described.

Overexpression of rFtAcpA

A single colony of *E. coli* BL21 (DE3) containing plasmid pRFtAcpAExp was used to inoculate 5 ml LB containing ampicillin (50 μg/ml) and incubated overnight at 37 °C with constant aeration. A 1:1000 dilution of this overnight culture was then used to inoculate 25 ml of fresh media. The sample was incubated as before until the density of the culture reached an OD A_{600} of 0.6. The sample was then chilled on ice for 10 min and placed at 4 °C for overnight storage. To remove secreted β-lactamases, the *E. coli* starter culture was centrifuged at 3660*g* for 10 min at 4 °C and suspended in fresh LB prior to equal distribution into four 1.8 L flasks containing 500 ml LB supplemented with ampicillin 50 μg/ml and 0.2% (wt/vol) filter-sterilized glucose. The culture was grown with constant aeration at 37 °C to an OD A_{600} of 0.4 at which time IPTG was added to a final concentration of 0.4 mM. To determine time of maximum induction as determined by maximum specific activity, 1 ml aliquots were removed at regular intervals from each of the four flasks, pelleted, and suspended in 50 mM acetate, pH 6.0, containing 150 mM NaCl prior to quantitation of enzyme activity and protein concentration. Three hours post IPTG-induction was found to be the optimum time for expression and used as a stopping point in subsequent inductions.

Protein purification

Four 0.5 L cultures of induced *E. coli* BL21 (DE3) harboring plasmid pRFtAcpAExp were centrifuged at 5000*g* for 10 min and the pellet was suspended in 20–25 ml of 50 mM sodium acetate, pH 6.0 (buffer A). All subsequent purification procedures were conducted at 4 °C unless otherwise noted. The cells were disrupted by two cycles of French Press (Aminco) adjusted to 10,000 psi in a 40 K rapid fill cell and a flow rate of 20 drops/min. French press-treated extract was then stirred at 4 °C in the presence of 1 M NaCl. Unbroken cells and pelletable debris were removed by centrifugation at 31,000*g* for 10 min. Bacterial membranes were pelleted by centrifugation at 192,000*g* for 1 h. The ultracentrifugation supernatant (~20 ml) containing the majority of total acid phosphatase activity was dialyzed against 100 volumes 50 mM sodium acetate, pH 6.0, containing 50 mM NaCl (buffer B) at 4 °C. The dialyzed supernatant was then applied at 1 ml/min to a SP-Sepharose cation exchange resin (25 ml) pre-equilibrated in buffer B. After washing with at least two column volumes with buffer B, the column was developed with a 400 ml linear 0.05–0.5 M NaCl gradient and fractionated into 4 ml fractions. The most active fractions were pooled (27 ml total), dialyzed against 10 mM sodium phosphate, pH 7.0 (buffer C). The sample was then applied (1 ml/min) to a HiTRAP metal-chelate chromatography resin (5 ml) pre-equilibrated with buffer C containing 0.5 M NaCl and charged with one column volume 100 mM $NiCl_2$ prior to sample application as per supplier's recommendation. The applied sample was washed with 3 volumes of buffer C. The phosphatase was eluted from the metal-charged column with a 150 ml linear imidazole gradient (0–0.5 M in buffer C). The eluted sample was dialyzed for 12 h against buffer A at 4 °C.

Molecular mass and oligomeric state

The molecular mass of recombinant FtAcpA was determined using MALDI-TOF mass spectrometry at the University of Missouri Proteomics Center and by SDS–PAGE analysis. Polyacrylamide gels were 3% T stacking and 10% T resolving and Novagen Perfect Protein Standards were used for determination of denatured molecular mass.

The oligomeric state of rFtAcpA was determined by analytical ultracentrifugation, gel filtration chromatography, and dynamic light scattering. Ultracentrifugation was performed in a Beckman XL-I Optima analytical ultracentrifuge, employing an An50Ti rotor. Samples were dialyzed to equilibrium against the reference buffer (buffer A) prior to loading into charcoal-filled Epon six-channel centerpieces. The six-channel format permits simultaneous observation of three sample and reference pairs. Data were collected at a spacing of 0.001 cm, averaging 20 readings at each step. The three absorbance scans from each cell were extracted from the raw data with software supplied with the instrument. Data were acquired at an instrument setting of 20 °C. Global non-linear least-squares minimization of the data was performed with Origin v5.0 (OriginLab). A solvent density of 1.00 g/cm^3 was employed for the calculations [16], and the partial specific volume was assumed to be 0.73 cm^3/g.

Gel filtration chromatography of rFtAcpA under non-denaturing conditions was performed on a Superdex 200 gel filtration chromatography resin with 50 mM sodium acetate, pH 6.0, containing 150 mM NaCl and 0.05% (wt/vol) azide as the mobile phase, with a flow rate of 1 ml/min. The partition coefficient of the phosphatase was compared to those of the following gel filtration standards: alcohol dehydrogenase (150,000 Da, $K_{av} = 0.310$), bovine serum albumin (BSA) (66,000 Da, $K_{av} = 0.394$), carbonic anhydrase (29,000 Da, $K_{av} = 0.487$), and cytochrome *c* (12,400 Da, $K_{av} = 0.569$).

Dynamic light scattering analysis was performed using a Protein Solutions DynaPro 99 Molecular Sizing Instrument. An average of 10–20 measurements were collected for each sample at 20 °C using a laser wavelength of 836.3 nm and a scattering angle of 90°. The measurements were analyzed using Dynamics Version 5.26.38 software.

Protein concentration assay and Western blot

Protein concentrations were determined using bichinchoninic acid (BCA Protein Assay Reagent, Pierce, Rockford, IL) as previously described [17]. BSA was used as the assay standard. For Western blots, proteins separated by SDS–PAGE were transferred to a PVDF membrane in 25 mM Tris, 192 mM glycine, pH 7.3, at 75 mA for 6 h in a Hoeffer Transblot apparatus [18]. Recombinant FtAcpA was detected using primary rabbit antibodies previously described [12] and alkaline phosphatase-conjugated anti-rabbit IgG secondary antibodies.

Results

Overexpression of rFtAcp

The purification and initial physico-chemical and biological properties of native *F. tularensis* AcpA have been described previously [12]. Facile production of the protein from *Escherichia coli* was a necessary step for large scale purification of the antigen for vaccine studies, structural analysis and delineation of eukaryotic substrates that may be integral to dimunition of the respiratory burst. While *acp*A encodes an endogenous signal for secretion, when cloned into an expression vector downstream of a strong promoter and transformed into *E. coli*, the construct was deleterious to cell growth (unpublished results). The construct, pRFtAcpAExp, in which the endogenous *acp*A leader sequence was replaced with the *pel*B-encoded leader sequence of *Erwinia chrysanthemi* [19] provided in pET20b was not detrimental to cell growth and resulted in production of copious amounts of recombinant protein upon IPTG induction (Table 1 and Fig. 1B, lane 3).

Results from five separate induction experiments are shown in Fig. 1A. Approximately 18,000 U/mg protein of phosphomonoesterase activity was detected prior to induction. This activity was heat-labile and linear with increasing time of incubation with substrate at 37 °C or increasing amounts of extract from IPTG-induced *E. coli* bearing the overexpression plasmid. A 10-fold increase in specific phosphomonoesterase activity was observed 3 h after IPTG addition reaching a maximum of

Table 1
Purification table for recombinant *F. tularensis* phosphomonoesterase

Step	Total activity[a] (U × 10^7)	Total protein (mg)	Specific activity (nmol/h/mg × 10^4)	Purification (fold)	Yield (%)
Pre extract	6.9	562.5	12.26	—	100
Post extract	7.9	372.0	21.24	1.7	114
Post SP-Sepharose	2.1	15.0	140.00	11.4	30
Post Ni-chelate	1.0	6.7	149.25	12.2	15

[a] Activity provided in nanomoles *p*NPP hydrolyzed per hour.

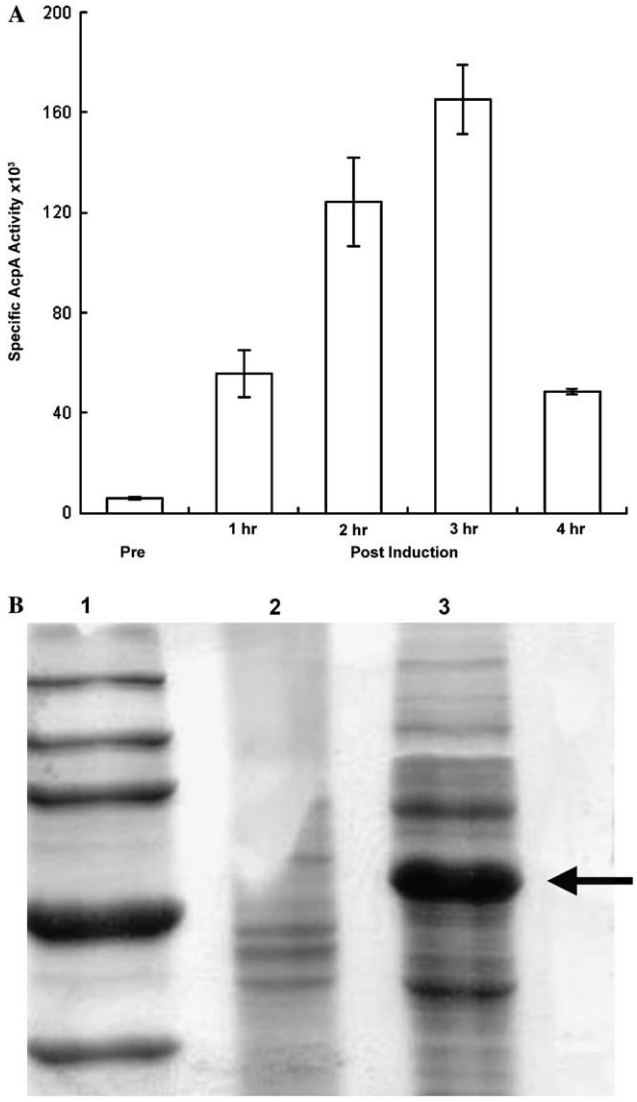

Fig. 1. IPTG induction of rFtAcpA activity in *E. coli* BL21 (DE3) containing overexpression plasmid pRAcpAExp. IPTG-induction was conducted as described under Materials and methods. Samples taken at indicated time points were analyzed for specific phosphomonoesterase activity (A) and for total cell protein (B) at pre-induction (lane 2) and 3 h post-induction (lane 3). Equivalent amounts of sample ~10 μg from indicated each time point were separated on a 10% SDS–PAGE gel and stained with Coomassie R-250. Lane 1: Novagen perfect protein standards (150, 100, 75, 50, 35, 25, and 15 kDa).

180,000 U/mg protein. Based on specific activity and presence of an intense ~57-kDa protein band on Coomassie blue-stained SDS–PAGE gel (Fig. 1B, lane 3 arrow) rFtAcpA represents a significant fraction of the total cell protein after 3-h IPTG exposure. These induction conditions were deemed suitable for use in large-scale purification of rAcpA.

Purification of rFtAcpA

Large-scale preparation of rFtAcpA was carried out as described under Materials and methods. After 3-h induction with IPTG, the *E. coli* cells containing pRFtAcpAExp were found to harbor significant levels of cell-associated phosphomonoesterase activity. The difference in total phosphomonoesterase activity measured in whole cells and French press-treated cells was approximately 13% (Table 1), suggesting the majority of activity was accessible to substrate in intact cells and consistent with its secretion into the periplasmic space. Most of the activity was solubilized in the presence of 1 M NaCl and found in the ultracentrifugation supernatant (Table 1 post extract). After dialysis the phosphatase was retained by SP-Sepharose cation exchange chromatography resin at pH 6.0 consistent with the cationic character of the enzyme. The protein was eluted from the resin in a linear 0–0.5 M NaCl gradient (Fig. 2A). The most active fractions, 46–59, containing 0.24–0.27 M NaCl were pooled, analyzed for enzymatic activity, and processed further. The specific activity of the enzyme after cation exchange chromatography was increased by approximately 7-fold over the previous step with a total yield of 30%. Application of rFtAcpA to a Ni-chelate chromatography resin resulted in no detectable activity in flowthrough or wash eluent. The enzyme

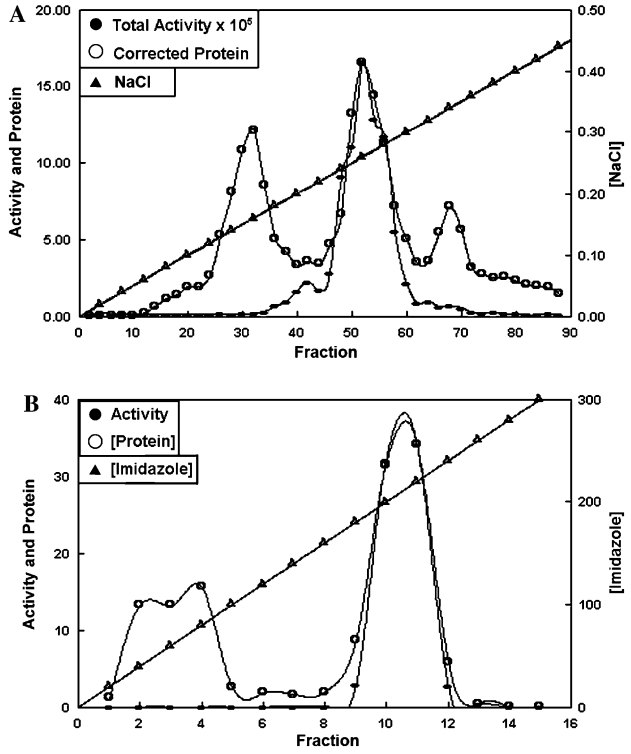

Fig. 2. Chromatography of rFtAcpA. For both chromatograms: phosphomonoesterase activity (○), protein concentration (●), and eluent concentration (NaCl or imidazole, △). (A) SP-Sepharose cation exchange chromatography of the salt-extracted dialyzed supernatant from French press-treated cells using a 0–0.5 M NaCl linear gradient (–) as described under Materials and methods. (B) Ni-chelate chromatography of pooled, phosphatase-containing fractions from SP-Sepharose chromatography using a 0–0.5 M linear imidazole gradient as described under Materials and methods.

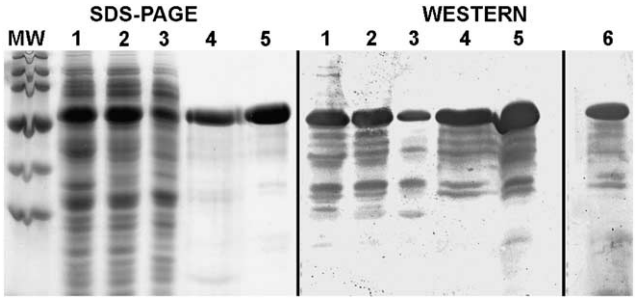

Fig. 3. SDS–PAGE separation and Western blot detection of samples from the rFtAcpA purification procedure. From left to right: lane MW, Novagen perfect protein standards (150, 100, 75, 50, 35, 25, and 15 kDa); lane 1, 25 μg IPTG-induced cells; lane 2, 25 μg protein following French Press; lane 3, 25 μg sample ultracentrifuge supernatant; lane 4, 8 μg sample pooled active fractions from SP-Sepharose cation exchange chromatography; lane 5, 8 μg sample of pooled active fractions following Ni-chelate chromatography. Western blot assessment of rFtAcpA purification fractions. From left to right: lane 1, 25 μg of protein IPTG-induced cells; lane 2, 25 μg protein following French Press; lane 3, 25 μg sample ultracentrifuge supernatant; lane 4, 8 μg sample pooled active fractions from SP-Sepharose cation exchange chromatography; lane 5, 8 μg sample of pooled active fractions following Ni-chelate chromatography. Lanes 1–5 treated with 1:100,000 dilution of rabbit anti-native AcpA and detected with goat anti-rabbit IgG H + L conjugated to alkaline phosphatase. Lane 6 same as lane 5 except 1:1000 anti-hexahistidine primary antibody was used and detected with 1:5000 rabbit anti-mouse H + L conjugated to alkaline phosphatase used to detect hexahistidine tag at C-terminus of recombinant protein.

was eluted from this resin in a linear 0–0.5 M imidazole gradient contained in 10 mM $NaPO_4$, 0.5 M NaCl, pH 7.0 (Fig. 2B). The most active fractions eluted between 0.19 and 0.22 M imidazole. The final dialyzed preparation contained approximately 15% of total starting activity and was purified 150-fold over that of the starting material (Table 1). Assessment of purity was performed by SDS–PAGE. Fig. 3 shows a protein band of 57 kDa whose relative amount increased throughout the purification process (Fig. 3 SDS–PAGE lanes 1–5). This protein comprises >99% total protein in the purified preparation. A protein of similar size and intensity contained epitopes recognized by purified rabbit polyclonal α-native AcpA IgG antibody [12] (Fig. 3 Western blot lanes 1–5) as well as an epitope recognized by mouse α-hexahistidine IgG antibody (Fig. 3 Western blot lane 6). Collectively, these data are consistent with expression and isolation of the recombinant polyhistidine-tagged *F. tularensis* AcpA.

Molecular mass and oligomeric state determination

The molecular mass of the purified recombinant enzyme was determined by mass spectrometry and SDS–PAGE. The observed $M+H^+$ for rFtAcpA was 56,651.2 Da. This value is within 1.0% of the theoretical value as calculated from the deduced amino acid sequence with the ExPASY proteomics tool [20]. A value of approximately 58 kDa was obtained with SDS–PAGE (data not shown) when the protein was denatured and run under reducing conditions.

Analytical ultracentrifugation, gel filtration chromatography, and dynamic light scattering were used to determine the oligomeric state. Samples of the protein, at nominal loading concentrations of 0.14–0.43 mg/ml, were centrifuged in an An50Ti rotor to equilibrium at 7000 and 10,000 rpm. The best fit to a single-species model (not shown) afforded an apparent molecular weight of 136,000 suggesting that the protein was not monomeric. Attempts to fit the data to a monomer–dimer, monomer–dimer–trimer, and monomer–dimer–tetramer models were unsuccessful.

The best agreement was obtained with the following two-species model:

$$c(r) = c_1(r_o)\exp\left[\frac{M_1\omega^2(1-\bar{v}\rho)}{2RT}(r^2-r_o^2)\right] + c_2(r_o) \times \exp\left[\frac{M_2\omega^2(1-\bar{v}\rho)}{2RT}(r^2-r_o^2)\right] + \mathrm{BL}. \quad (1)$$

In this equation, $c(r)$ is the total protein concentration at radial position r; $c_1(r_o)$ and $c_2(r_o)$ are the concentrations of Species 1 and 2, respectively, at an arbitrary reference position, r_o; M_1 and M_2 are the molecular weights of Species 1 and 2, ω is the radial velocity (rad/s); $\bar{v}$ is the partial specific volume of the protein; ρ is the solvent density; R is the gas constant; T is the absolute temperature, and BL is a baseline offset to allow for optical mismatch between reference and sample cells.

M_1 and M_2 were global parameters shared by all 10 data sets. $c_1(r_o)$, $c_2(r_o)$, and BL were local parameters, unique to each data set. Initially, M_1 was fixed at the predicted dimer molecular weight. Then, as convergence was reached, M_1 was allowed to vary. The fit indicated by the solid lines in Fig. 4 afforded M_1 and M_2 values of 115,000 ± 8000 and 258,000 ± 47,000. Species 2 is assumed to represent irreversibly aggregated protein. Its apparent molecular weight is roughly twice the dimer molecular weight, suggesting that it contains, on average, four polypeptide chains. The concentration of Species 2 varied from sample cell to sample cell, comprising between 1 and 5% of the total sample concentration.

Results from other methods including gel filtration and dynamic light scattering analysis are consistent with the observation of an apparent rFtAcpA dimer using ultracentrifugation. Gel filtration chromatography of rFtAcpA through Superdex 200 chromatography resin yielded a partition coefficient of 0.352 (data not shown). This value was compared to the regression line generated from standards, and corresponded to an apparent molecular mass of 100 kDa. Dynamic light scattering experiments (data not shown) performed at a concentration of 1 mg/ml indicated that the rFtAcpA solution was

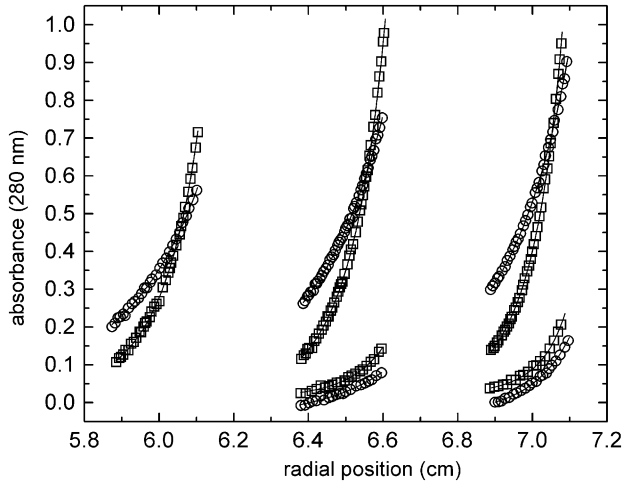

Fig. 4. Global fit of sedimentation equilibrium data to an ideal two-species model. Data were acquired at 7000 (circles) and 10,000 (squares) rpm in an An50Ti centrifuge rotor, at five different loading concentrations. The 10 data sets were subjected to non-linear least squares minimization as described in the text, assuming the presence of two molecular species. The solid lines indicate best fit to the data. To improve visual clarity, a vertical offset was applied to several of the datasets after least-squares analysis.

highly monodisperse with a mean hydrodynamic radius (R_h) of 4.446 nm which corresponds to a molecular mass of 108 kDa. As monomer molecular mass was 56 kDa, determined by mass spectrometry. Results from dynamic light scattering indicate that the prevalent form of rFtAcpA present in solution is a dimer. Collectively these data suggest that the recombinant enzyme is a apparent homodimer under the conditions tested.

Kinetic characterization

In addition to its physical properties, a number of enzymatic properties were determined for recombinant *F. tularensis* phosphomonoesterase. These properties included: pH optimum, substrate specificity, effects of potential phosphomonoesterase inhibitors, and kinetic parameters for possible physiological substrates.

The purified recombinant phosphomonoesterase behaved as an acid phosphatase (acid pH optimum) in all buffers with appropriate pK_as tested. Maximum activity was found in 0.2 M sodium acetate at pH 5.9–6.1 for adenosine 5′ monophosphate and *p*-nitrophenyl phosphate (Fig. 5A). Optimal activity was markedly diminished at 1 pH to either side of this optimum. Activity of the enzyme was appreciably reduced in the presence of Mes, Hepes, and Tris buffers even at pHs compatible with high enzymatic activity observed in sodium acetate (data not shown). The effects of potential acid phosphatase inhibitors are shown in Fig. 5B. Each was tested in the presence of 0.2 M sodium acetate at pH 6.0 and checked for the effect of the inhibitor on pH of the assay medium. Barium, lead, magnesium, and nickel

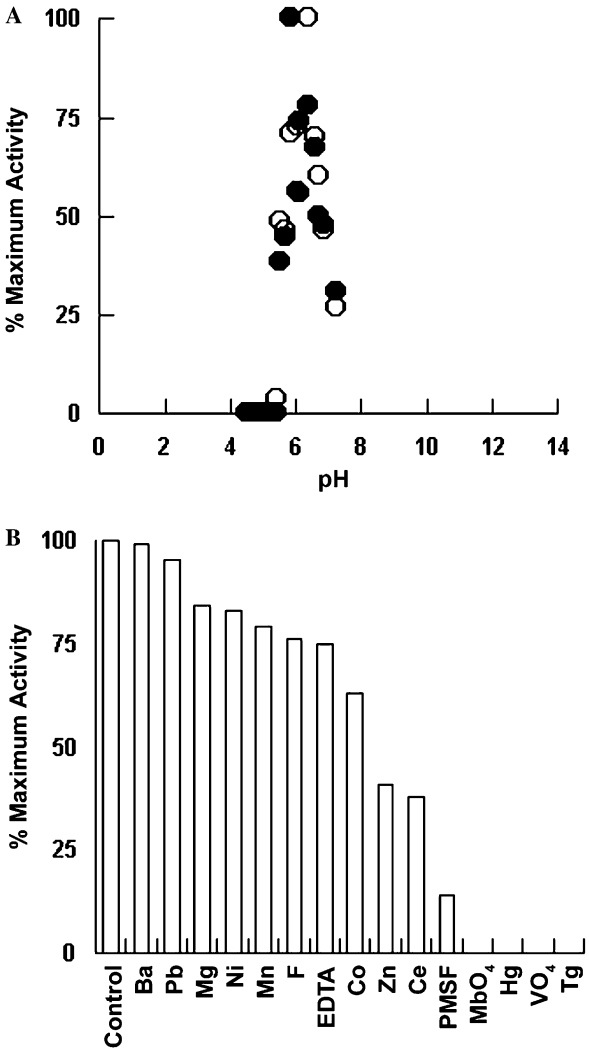

Fig. 5. Effect of pH (A) and potential phosphomonoesterase inhibitors (B) on activity of rFtAcpA. (A) Five hundred units of rFtAcpA incubated with 1 mM adenosine 5′ monophosphate (●) or 1 mM *p*NPP (○) were analyzed for catalytic activity in 0.2 M sodium acetate buffer at the indicated pH in the standard discontinuous assays as described under Materials and methods. The data presented as percent maximum activity for each substrate tested. (B) The enzyme (500) units assayed with *p*NPP in the presence of each indicated inhibitor. Data presented as percent maximum activity achieved in control tested under similar conditions in absence of inhibitor as described under Materials and methods.

salts were the least effective inhibitors, diminishing catalysis of *p*NPP less than 20%. EDTA and fluoride reduced rFtAcpA-catalyzed hydrolysis of *p*NPP by approximately 25%. Significant inhibitors of enzymatic activity include the two metal oxyanions, molybdate and vanadate, as well as mercury, and tungstate. Presence of these inhibitors reduced rFtAcpA-mediated hydrolysis of *p*NPP to less than 5% that of control (Fig. 5B).

The recombinant enzyme had broad in vitro substrate specificity (Fig. 6). All but seven of the 28 substrates tested were hydrolyzed at 50% or greater the rate of the control *p*NPP reaction when tested at 1 mM final

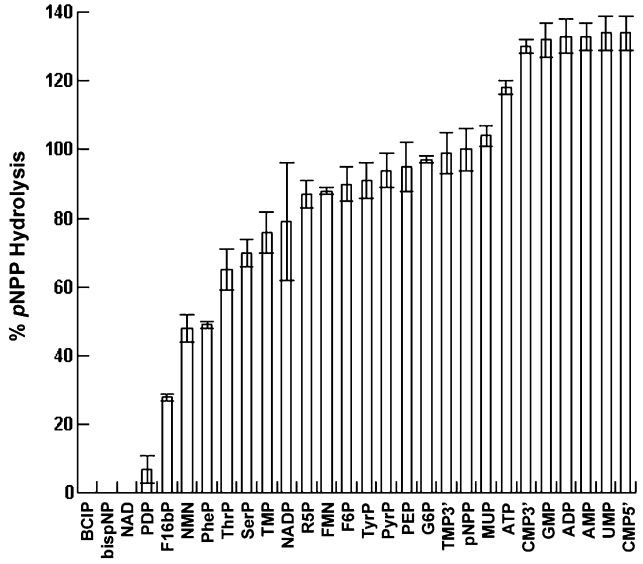

Fig. 6. Substrate specificity of rFtAcpA. Recombinant enzyme was incubated with each the indicated substrates and assessed for phosphatase activity by measuring the amount of inorganic phosphate produced as described under Materials and methods. BCIP, 5-bromo-4-chloro-3-indolyl phosphate; bispNP, bis(*p*-nitrophenylphosphate); NAD, β-nicotinamide adenine dinucleotide; PDP, phenotheilien diphosphate; F16bP, fructose 1,6 bisphosphate; NMN, nicotinamide adenine mononucleotide; PheP, phenylphosphate; ThrP, threonine phosphate; SerP, serine phosphate; TMP, 5′ thymine monophosphate; NADP, nicotinamide adenine diphosphate; R5P, ribose 5 phosphate; FMN, flavin mononucleotide; F6P, fructose 6 phosphate; TyrP, tyrosine phosphate; PyrP, pyruvate phosphate; PEP, phosphoenolpyruvate; G6P, glucose 6 phosphate; TMP3′, thymine 3′ monophosphate; *p*NPP, *p*-nitrophenyl phosphate; MUP, 4-methylumbelliferyl phosphate; ATP, adenine triphosphate; CMP3′, cytosine 3′ monophosphate; ADP, adenine diphosphate; AMP, adenine monophosphate; UMP, uracil monophosphate; CMP5′, cytosine 5′ monophosphate. Results are presented as percent activity relative to the amount of inorganic phosphate released from the phosphomonoesterase-catalyzed hydrolysis of *p*NPP.

concentration. The enzyme was most active with all nucleoside 5′ monophosphates tested, catalyzing their hydrolysis at 1.2–1.4-fold higher than *p*NPP (Fig. 6). Results (Table 2) from the kinetic analysis of the substrates tested for Fig. 6 show a wide range of K_m (15.6–360.8 μM) and a narrow V_{max} range (5.21–13.48 μmol/h). Based on the determined kinetic parameters, the favored rFtAcpA substrate is ATP; the least optimal substrate is $NADP^+$. The K_m and V_{max} for tyrosine phosphate were in the median range for both kinetic parameters and suggests a possible role of protein substrates as targets of respiratory burst inhibition in vivo. In addition to the phosphomonoesterase activity described above, the recombinant enzyme also displayed detectable phospholipase C activity for the synthetic substrate *p*-nitrophenylphosphoryl choline at pH 7.4 in the presence of 40% w/w sorbitol. The activity was heat-labile and linear with increasing amounts of enzyme, time, or substrate concentration.

Table 2
Kinetic parameters of rFtAcpA substrates

Substrate	V_{max} (μmol/h)	K_M (μM)	V_{max}/K_M
ATP	13.48	15.6	0.864
MUP	5.98	23.8	0.251
*p*NPP	6.04	26.5	0.228
ADP	6.66	33.8	0.197
Fruc1,6-bP	6.68	34.3	0.195
AMP	8.41	48.1	0.175
TyrP	10.6	68.6	0.154
GMP5′	9.48	68.6	0.138
GMP3′	8.09	60.6	0.134
PEP	8.84	72.9	0.121
ThrP	8.34	150.5	0.055
UMP5′	6.95	156.4	0.044
TMP3′	9.14	312.5	0.029
$NADP^+$	5.21	360.8	0.014

Discussion

This work was undertaken to provide a source for large quantities of purified recombinant *F. tularensis* AcpA. Facile production of the protein was performed as a prelude to in depth assessment of its enzymology, its potential as part of a recombinant subunit vaccine, its role with regard to intracellular survival of *F. tularensis*, and for structural analysis. A necessary step before these studies could be initiated was to ensure that the recombinant protein was a suitable surrogate for native enzyme. In this work, we have demonstrated high-level production of rFtAcpA from induced *E. coli* and purification of the enzyme to near homogeneity in two chromatography steps. Results from all physico-chemical characterization methods are consistent with identification of the enzyme as recombinant *F. tularensis* acid phosphatase A. This conclusion is based on the enzymatic attributes of the recombinant protein and strong reactivity with polyclonal rabbit anti-*F. tularensis* AcpA antibodies.

The purified recombinant enzyme had a denatured molecular mass of ~57-kDa, similar to that of native enzyme when determined by SDS–PAGE. The recombinant protein was found to be slightly larger than native protein (rFtAcpA = 56,651 Da; native = 55,759 Da) when determined by MALDI-TOF-MS. The observed difference in molecular mass is largely accounted for by differences in the primary structure of the recombinant protein including absence of the N-terminal threonine residue and addition of six histidine residues to the C-terminus of the protein. Potential differences in molecular masses between recombinant and native enzyme which are due to post-translational modification systems as observed in other prokaryotes [21] have been considered but not determined for either recombinant or native enzyme. Significantly, the recombinant enzyme was found to behave as a homodimer when analyzed by analytical ultracentrifugation, gel filtration chromatography, and dynamic light scattering. The availability

of only microgram amounts of purified native enzyme precluded study of its concentration-dependent oligomeric state. Other physical properties of the enzyme, including its cationic nature were similar to that of the native enzyme as evidenced by adsorption to and elution of both forms of the enzyme from SP-Sepharose at the same NaCl concentration.

Results from enzymatic characterization of the recombinant protein suggest that its activity is indistinguishable from that of the native protein. Both proteins displayed a narrow pH optimum in sodium acetate as well as diminished activity in Hepes and Mes buffers. Diminished activity in Mes and Hepes by the wild type and recombinant enzymes may be indicative of the competition of the sulfonate buffer, present at 200 mM for occupancy of the enzyme's active site as demonstrated structurally in other acid phosphatases [22]. Both enzymes displayed broad substrate specificity, catalyzing the dephosphorylation of the vast majority of phosphomonoesters tested, especially the aryl-phosphates. Both enzymes were acutely sensitive to mercury chloride, molybdate, and vanadate. In this work, availability of milligram quantities of enzyme allowed for a more thorough assessment of the kinetic parameters. Results from these experiments are new and significant (Table 2). FtAcpA has been demonstrated to abrogate the respiratory burst of stimulated neutrophils. While the mechanism of inhibition has yet to be elucidated, one hypothesis [12] suggested the enzyme, with its broad substrate specificity, dephosphorylated a number of physiological substrates, perhaps most importantly NADPH, the requisite reducing agent of the superoxide anion generating system, NADPH oxidase [23]. Results shown in Table 2 show that rFtAcpA hydrolyzes the oxidized form of NADPH ($NADP^+$), but not as efficiently as the other substrates tested. Conversely, the enzyme had significantly better kinetic parameters for ATP of which large amounts are utilized in the production of superoxide anion. Tyrosine phosphate had a K_m and V_{max} in the median range and could be considered a viable candidate. Results from these studies have demonstrated successful facile production of the recombinant phosphomonoesterase of *F. tularensis*. This work was done in prelude to determination of protein structure, design of specific enzyme inhibitors, and to test the efficacy of the recombinant molecule as a vaccine. To our knowledge this work represents the first virulence-related protein from this important pathogen to be overexpressed and purified.

Acknowledgments

The authors thank the Riley Laboratory at the University of Missouri Research Animal Diagnostic Laboratory for use of their French Press in preparation of purified enzyme. Partial financial support for this work was awarded to T.J.R. by the Midwest Center of Excellence for Biodefense and Emerging Infectious Diseases Research. The authors acknowledge Beverly Degue and The Proteomics Center at the University of Missouri-Columbia for mass spectrometry services.

References

[1] D.T. Dennis, T.V. Inglesby, D.A. Henderson, J.G. Bartlett, M.S. Ascher, E. Eltzen, A.D. Fine, A.M. Friedlander, J. Hauer, M. Layton, S.R. Lillibridge, J.E. McDade, M.T. Osterholm, T. O'Toole, G. Parker, T.M. Pert, P.K. Russell, K. Tonat, Tularemia as a biological weapon, J. Am. Med. Assoc. 285 (2001) 2763.

[2] G. Sandstom, The tularemia vaccine, J. Chem. Technol. Biotechnol. (2005) 315.

[3] F.E. Nano, N. Zhang, S.C. Cowley, K.E. Klose, K.M. Cheung, M.J. Roberts, J.S. Ludu, G.W. Letendre, A. Meierovics, I.G. Stephens, K.L. Elkins, A *Francisella tularensis* pathogenicity island required for intramacrophage growth, J. Bacteriol. 186 (2004) 6430.

[4] G.S. Baron, F.E. Nano, MglA and MglB are required for intramacrophage growth of *Francisella novicida*, Mol. Microbiol. 29 (1998) 247.

[5] C.M. Lauriano, J.R. Barker, S. Yoon, F.E. Nano, B.P. Arulanandam, D.J. Hassett, K.E. Klose, MglA regulates transcription of virulence factors necessary for *Francisella tularensis* intramoebae and intramacrophage survival, Proc. Natl. Acad. Sci. USA 101 (2004) 4246.

[6] J.A. Bliska, J.C. Clemens, J.E. Dixon, S. Falkow, The Yersinia tyrosine phosphatase: specificity of a bacterial virulence determinant for phosphoproteins in the J774.1 macrophage, J. Exp. Med. 276 (1992) 1625.

[7] A.K. Saha, J.N. Dowling, W.M. Pasculle, R.H. Glew, *Legionella micdadei* phosphatase catalyzes hydrolysis of phosphatidylinositol 4,5-bisphosphate in human neutrophils, Arch. Biochem. Biophys. 265 (1988) 94.

[8] A.T. Remaley, D.B. Kuhns, R.E. Basford, R.H. Glew, *Leishmanial* phosphatase blocks O_2-production, J. Biol. Chem. 259 (1984) 11173.

[9] G. Baca O, M.J. Roman, R.H. Glew, R.F. Christner, J.E. Buhler, A.S. Aragon, Acid phosphatase activity in *Coxiella burnetii*: a possible virulence factor, Infect. Immun. 61 (1993) 4232.

[10] M.N. Burtnick, A.J. Bolton, P.J. Brett, D. Watanabe, D.E. Woods, Identification of the acid phosphatase (acpA) gene homologues in pathogenic and nonpathogenic *Burkholderia* spp. facilitates TnphoA mutagenesis, Microbiology 147 (2001) 111.

[11] M.T. Saleh, J.T. Belisle, Secretion of an acid phosphatase (SapM) by *Mycobacterium tuberculosis* that is similar to eukaryotic acid phosphatases, J. Bacteriol. 182 (2000) 6850.

[12] T.J. Reilly, G.S. Baron, F.E. Nano, M.S. Kuhlenschmidt, Characterization and sequencing of a respiratory burst-inhibiting acid phosphatase from *Francisella tularensis*, J. Biol. Chem. 271 (1996) 10973.

[13] P.A. Lanzetta, L.J. Alvarez, P.S. Reinach, O.A. Candia, An improved assay for nanomole amounts of inorganic phosphate, Anal. Biochem. 100 (1979) 95.

[14] S.P.J. Brooks, A simple computer program with statistical tests for the analysis of enzyme kinetics, Biotechniques 13 (1992) 906.

[15] S. Kurioka, M. Matsuda, Phospholipase C assay using *p*-nitrophenylphosphorylcholine, Anal. Biochem. 75 (1976) 281.

[16] T.M. Laue, B.D. Shan, T.M. Ridgeway, S.L. Pelletier, Computer-aided Interpretation of Analytical Sedimentation Data for Proteins, Analytical Ultrcentrifugation in Biochemistry and Polymer Science, Redwood Press, 1992, pp. 99–125.

[17] P.K. Smith, R.I. Krohn, G.T. Hemanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76.

[18] H. Towbin, T. Staehelin, J. Gordon, Electrophoetic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications, Proc. Natl. Acad. Sci. USA 76 (1979) 4350.

[19] N.T. Keen, S. Tamaki, Structure of two pectate lyase genes from *Erwinia chrysanthemi* EC16 and their high level expression in *Escherichia coli*, J. Bacteriol. 168 (1986) 595.

[20] E. Gasteiger, A. Gattiker, C. Hoogland, I. Ivany, R.D. Appel, A. Bairoch, ExPasy: the proteomics server for in-depth protein knowledge and analysis, Nucleic Acids Res. 31 (2003) 3784.

[21] P.M. Power, M.P. Jennings, The genetics of glycosylation in Gram-negative bacteria, FEMS Micro Lett. 218 (2003) 211.

[22] M. Zhang, C.V. Stauffachert, D. Lin, R.L. Van Etten, Crystal structure of a human low molecular weight phosphotyrosyl phosphatase, J. Biol. Chem. 273 (2005) 21714.

[23] P. Vignais, The superoxide-generating NADPH oxidase: structural aspects and activation mechanisms, Cell. Mol. Life Sci. 59 (2002) 1428.