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Purification of recombinant antigen *Bm*SXP used in panLF Rapid kit for lymphatic filariasis

Teng Kew Khoo, Rahmah Noordin & Amutha Santhanam*
Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia (USM), 11800, Pulau Pinang, Malaysia

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A rapid antibody detection test is very useful for the detection of lymphatic filariasis, especially for certification and surveillance of post-mass drug administration. panLF Rapid kit is suitable for this purpose since it can detect all species of lymphatic filaria. It is based on the detection of anti-filarial IgG4 antibodies that react with recombinant *B. malayi* antigens, *Bm*R1 and *Bm*SXP. There is an increase demand for the test due to its attributes of being rapid, sensitive and specific results, as well as its field-applicability. The main aim of this paper is to obtain high recovery and purity of recombinant antigen *Bm*SXP via a modified method of immobilized metal affinity chromatography (IMAC). The highest product yield of 11.82 mg/g dry cell weight (DCW) was obtained when IMAC was performed using the optimized protocol of 10 m*M* imidazole concentration in lysis buffer, 30 m*M* imidazole concentration in wash buffer, and 10 column volume wash buffer containing 300 m*M* salt concentration. This gave a 54% protein recovery improvement over the manufacturer's protocol which recorded a product yield of only 7.68 mg/g DCW. The recovered *Bm*SXP recombinant antigen showed good western blot reactivity, high sensitivity (31/32, 97%) and specificity (32/32, 100%) in ELISA, thus attesting to its good purity and quality.

Keywords*:* BmSXP recombinant antigen, Lymphatic filariasis, PanLF Rapid, Purification optimizations

Lymphatic filariasis (LF) or elephantiasis as commonly known is a parasitic disease caused by thread-like filarial nematodes or round worms that live in the human lymphatic system. LF is mainly caused by three species of filarial nematodes namely *Wuchereria bancrofti, Brugia malayi* and *Brugia timori*. Currently over 120 million people are infected, with more than 40 million incapacitated or disfigured with swollen limbs (lymphoedema) and genitals (hydrocele)[1]. The Global Programme to Eliminate Lymphatic Filariasis (GPELF)[2] has been initiated by World Health Organization (WHO) in 1997 with two major goals, which is to interrupt transmission of the parasite and morbidity control by providing care for those who suffer the devastating clinical manifestations of the disease[1].

Commercialized rapid antibody based detection test kits are now available to detect both kinds of lymphatic filariasis. The commercialized product, panLF Rapid kit (http://reszonics.com/product3.html) developed in our laboratory, is a rapid immuno-chromatographic test strip that utilized *Bm*SXP and *Bm*R1 recombinant antigens for the detection of specific IgG4 antibodies to both bancroftian and brugian filarial worms with high sensitivity and specificity (>95%)[3-4]. For cost-effective production of the tests, there need to be a large scale expression, purification and a high recovery of the purified recombinant antigens.

Generally, the purification of soluble target proteins from cytoplasmic proteins pool is quite difficult since it contains vast majority of total cellular proteins[5]. Even though immobilized metal affinity chromatography (IMAC) is the choice of purification technique to separate biomolecules in the laboratory, sometimes there is co-elution of contaminated proteins during the procedure. This contamination may be due to several reasons[6], one identified contaminant of IMAC purification is SlyD, a metal-binding protein synthesized by the host cell required for the lysis of *E. coli* upon infection with bacteriophage PhiX174[7]. In addition, native host proteins which show affinity towards divalent cations also co-purify along with the target protein[8]. This is because histidine is also present in the native host proteins and enzymes as dispersed residues. Moreover, the over expression of a target protein by the host has been linked to the over expression

*Correspondent author
Telephone: +604-6534802
Fax: +604-6534803
E-mail: amutha_santhanam@yahoo.com

of native bacterial proteins as a response to stress conditions following the high production of foreign materials[9].

With the advancement in recombinant DNA techniques, the *Bm*SXP recombinant antigen was constructed in such a way that 6x-Histidine affinity tag was added to the protein sequence of interest to provide additional means for efficient purification of the target antigen[4]. IMAC purification was performed using Nickel-Sepharose resin matrix which allows the specific ligand to interact reversibly with histidine amino acid residues. Due to the reasons stated above, there is a need to optimize the purification process of the recombinant protein, in order to obtain a high purity of the recombinant protein for use in the diagnostic test.

In the present study a more efficient purification step has been developed using IMAC (compared to the manufacturer's protocol, QIAGEN, Germany) by varying the imidazole, salt concentration and volume of wash buffer. The quality of the recovered target antigen has also been assessed for its purity level, as well as its sensitivity and specificity towards the detection of both bancroftian and brugian filariasis.

Materials and Methods

Strain and plasmid—The host strain used was *E. coli* TOP10F', and the expression vector was pPROEXHT™HTa (Life Technologies, USA). The recombinant bacteria, *Bm*SXP/pPROEXHTa/TOP10F', was previously constructed in the laboratory[4] by cloning the open reading frame (ORF) of the SXP1 gene (462 bp, GenBank accession no. *M* 98813) from *B. malayi* cDNA library. After subcloning into the expression vector, the length of the nucleotides from start codon of the vector to the stop codon of the gene is 585 bp, which was translated to the *Bm*SXP recombinant antigen. Inclusive of the 6xHis-tagged region, this sums up to a size of the recombinant antigen of approximately 21.8 kDa[4].

Sample preparation

Protein expression—Protein expression was performed in a 5 L round bottom stirred fermenter (B. Braun, Germany) of 160 mm vessel diameter containing 1,800 mL modified Terrific broth (TB). The fermenter was fitted with *p*H and dissolved oxygen sensors (Mettler Toledo, USA). Temperature was controlled at 37°C via a water-filled double jacketed system. Agitation was provided by a centrally mounted six-bladed Rushton turbines positioned 40 mm above the base of the vessel. Aeration occurred through a perforated pipe sparger ring. Dissolved oxygen (DO) was controlled at 30% of air saturation via pure oxygen injection into the sparged air at 2 L/min. The *p*H was controlled at 7.0 using 2 *M* H_2SO_4 and 25% ammonia solution. Antifoam A (Sigma, USA) was added automatically to control the foaming. The inoculum used consisted of 200 mL modified TB medium[10] in 1 L Erlenmeyer flask which has been incubated at 37°C until optical density (OD) attained 5.0 at 600 nm wavelength. The feeding rate was controlled by the automated MFCS/Win software (B. Braun, Germany) based on the exponential feeding strategy formula whereby μ_{set} is set at a constant of 0.20 h^{-1}. IPTG (1m*M*) induction was carried out on the culture when it attained OD_{600} 25. The parameters stated above have been optimized to yield the best protein expression level for *Bm*SXP/pPROEXHTa/TOP10F' (data not shown).

Lysate preparation—Cells were harvested at 5 h post-induction and suspended using lysis buffer (*p*H 8.0) containing 50 m*M* NaH_2PO_4, 300 m*M* NaCl, 10 m*M* imidazole and a cocktail of protease inhibitor (serine, cysteine, calpains, and metalloproteases) (Roche, Diagnostics, Germany). Lysozyme (Amresco, USA) at 0.5 mg/mL was also added to the suspension and incubated on ice for 30 min. The suspended cell was then disrupted in a high-pressure homogeniser (Thermo Spectronic, USA) at 1,700 psi, then centrifuged at 12,000 *g* (Kubota, Japan) for 30 min at 4°C. The supernatant was then treated with DNase1 (Amresco, Ohio) to a final concentration of 0.5 mg/mL. Following incubation for 15 min at 4°C, centrifugation was carried out at 13,000 *g* (Kubota, Japan) for 30 min at 4°C. The supernatant containing the soluble proteins was filtered through a 0.2 µm filter membrane (Millipore, USA).

Immobilized metal affinity chromatography (IMAC)—IMAC technique was performed with HisTrap column (GE Health Care, United Kingdom) connected to a FPLC protein purification system (AKTA™*prime*, GE Health Care, United Kingdom). The HisTrap column consisted of iminodiacetic acid group as a chelating ligand, coupled to the agarose beads (Nickel-Sepharose Fast Flow) that was used as the chosen matrix. The binding capacity was reported by the manufacturer to be 40 mg of histidine-tagged protein for every mL of medium. Four different

conditions were studied, the first was the optimization of imidazole concentration in the lysis buffer (5, 10, 15 and 20 m*M*). Next is the optimization of imidazole concentration in the washing buffers (10, 20, 30, 40, 45 and 50 m*M*), followed by the optimization of wash buffer volume [5 and 10 column volumes (CV)]. Subsequently, the effects of two different salt concentrations in the wash buffer solutions were compared i.e. 300 and 500 m*M*. The sample lysates was loaded into the IMAC column at a flow rate of 1 mL/min. Fractions containing protein at concentration >200 µg/mL, as determined by Bio-Rad Protein Assay Reagent (BioRad, USA), were pooled.

Protein analysis—The selected protein fractions (OD_{595} >0.4) were pooled together and the final protein concentration of the eluted *Bm*SXP recombinant antigen concentration was estimated by Bio-Rad Protein Assay Reagent (BioRad, USA)[11], with various concentrations of bovine serum albumin (Sigma, USA) as the protein standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of protein samples were carried out as described by Laemmli[12] followed by Coomassie blue staining (BioRad, USA). Low molecular weight marker proteins were used as standards (BioRad Precision Plus Protein Standards Unstained, BioRad, USA). For a quantitative comparison of band intensities, AlphaEase® FC Software (Cell Biosciences, USA) with "Spot Denso" feature was used.

Western blot—The immunoreactivity of the *Bm*SXP recombinant antigen obtained from the optimized protein purification conditions was demonstrated by Western blot analysis probed with human sera. The sera panel came from patients infected with both bancroftian and brugian filariasis (positive samples) (n = 16 of each kind). In addition, sera from other parasitic infections (n = 16) and healthy individuals (negative samples) (n = 16) were also tested. All serum samples used in this study were those stored in our sera bank and coded to maintain anonymity. They were previously obtained with consent according to the requirements of Universiti Sains Malaysia Human Research Ethics Committee, and permission to use these stored samples were obtained from the same committee. Parameters for the Western blot analysis include 10 µg protein amount loaded per lane, serum samples at 1:200 dilution and monoclonal anti-human antibody IgG4-HRP at 1:2000 dilution.

Enzyme linked immunosorbent assay (ELISA)—ELISA assay was performed to determine the sensitivity and specificity of the recombinant protein for the detection of bancroftian and brugian filariasis. The recovered *Bm*SXP recombinant antigen was diluted to 20 µg per well with 0.02 *M* bicarabonate buffer at a final volume of 100 µL per well. The solution was then coated onto a 96-well ELISA plate (BD, USA). The sealed 96-well ELISA plate was then placed into a humid chamber and stored overnight at 4°C. The unbound sample solution was discarded and the surface of the wells was blocked for 2 h with 3% BSA at 200 µL per well followed by PBS-T wash for three times. Then serum samples were added at 100 µL per well (1:50 dilution with PBS), these comprised the same sera panel as those used in the Western blot. Results using LF sera were used for determination of sensitivity of the recombinant protein, and the results of the non-LF sera were used for determination of the protein specificity.

The 96-well ELISA plate was then incubated for 2 h at 37°C, followed by a washing step. Next, 100 µL per well (1:4500 dilution with PBS) of monoclonal anti-human antibody IgG4-HRP (Zymed, USA) was added to each of the well and was incubated for 1 h at 37°C. Following a final wash, the colour development was accomplished using ABTS substrate (Roche Diagnositics, Germany) for 30 min at 37°C. The plate was then read at 410 nm test filter and 490 nm reference filter using an ELISA plate reader (Tecan Systems, USA).

All sera were tested in duplicate and results were expressed as OD values. An OD of ≥0.3 was employed as the cut-off value for positive sample, this was previously derived from the mean OD plus three standard deviations after testing 50 serum samples of normal individuals from a filariasis endemic area in Malaysia[4]. The results were then analyzed using the *t*-test to determine whether there was significant difference between the ODs of the positive and negative samples.

Analytical methods—Biomass was monitored by measuring optical density at 600nm using a spectrophotometer (Thermo Spectronic, USA). The dry cell weight (DCW) was estimated from a calibration curve that correlated experimentally measured dry weight to spectrophotometric measurement of optical density.

Results

Immobilized metal affinity chromatography (IMAC)—Protein purification performed under constant settings of 10 column volume (CV) wash buffer containing 45m*M* imidazole and 300 m*M* salt concentration with 5, 10, 15, 20 m*M* imidazole concentration in lysis buffer revealed that 10 m*M* imidazole concentration in lysis buffer yielded the highest recovery rate of *Bm*SXP recombinant antigen at 9.36 mg/g Dry Cell Weight (DCW) (Table 1). This gave almost 11% recovery improvement over the next best parameter (15 m*M* imidazole concentration in lysis buffer which recovered 8.40 mg/g DCW *Bm*SXP recombinant antigen). Table 1 also demonstrated that there is an effective range between 15 m*M* imidazole concentrations in lysis buffer (with 10 m*M* being its best) before the recovery rate dropped drastically at 20 m*M*.

There was a significant difference in the recovery of *Bm*SXP recombinant antigen concentrations when five different imidazole concentrations in the wash buffer were used namely 20, 30, 40, 45 and 50 m*M*. Other parameters such as salt concentration (300 m*M*) and column volume (10 CV) were maintained as constants. As can be observed (Table 1), the highest recovery of *Bm*SXP recombinant antigen was obtained when gradient washing was performed with 30 m*M* imidazole wash buffer, followed by 20 and 45 m*M*. The recoveries of *Bm*SXP recombinant antigen were 11.82, 11.16 and 8.12 mg/g DCW respectively.

As for the column volume and salt concentration in the wash buffer optimization, 10 CV and 300 m*M* salt concentration recorded higher recovery rate at 11.82 mg/g DCW compared to 5 CV and 300 m*M* parameter which gave 9.23 mg/g DCW. Washing was kept constants at 30 m*M* imidazole in wash buffer. Recovery of target antigen was recorded at a similar range of 10.05 to 10.31 mg/g DCW for 10 CV with 500 m*M* salt concentration wash buffer and 5 CV with 500 m*M* salt concentration wash buffer. The best parameter was observed with 10 CV and 300 m*M* salt concentration in wash buffer as it recovered an additional 15% of *Bm*SXP recombinant antigen.

The chromatogram output of purification with 30 m*M* imidazole and 10 CV is shown in Fig. 1. A wide peak can be observed between breakpoint 2 and 3 which represents the elution of contaminants proteins during washing step with Buffer A. The binding of the His-tag recombinant antigen in the lysate was optimum as there was no peak observed between breakpoint 3 to 4. This implied that there was no removal of bound proteins during the washing step. The main chromatography peak was observed during the elution step from breakpoint 4 to 5 which was found to be at approximately 900 mAu.

SDS-PAGE—Analysis of the protein fractions using imidazole concentrations from 20-50 m*M* in wash buffer was performed using SDS-PAGE. As shown in Fig. 2, compared to 50 m*M* concentration of imidazole, the 30 and 20 m*M* concentrations exhibited higher amounts of protein. The AlphaEase® FC Software (Cell Biosciences, USA) estimated the integrated density value of the band in lane 2 (20 m*M* imidazole concentration wash buffer) at 1,637,948 density value, while the integrated density value of the band in lane 3 (30 m*M* imidazole concentration wash buffer) was at 1,732,483 density value.

Western blot—The results of Western blot clearly showed specific reactivity of *Bm*SXP recombinant antigen since it only reacted with sera from positive samples. The ~21.8 kDa *Bm*SXP protein band was clearly seen in all strips probed with primary antibody of patients' serum diagnosed with bancroftian and brugian filariasis (Figs 3 and 4). Slight detection of non-specific or contaminating protein bands could be seen in some strips, however, specificity was still high as there was no reaction when probed with sera from other helminthic infections and healthy individuals (Figs 5 and 6).

Table 1—Recovery of *Bm*SXP recombinant antigen obtained when different imidazole concentrations were used in lysis buffer and wash buffer

Imidazole concentration in lysis buffer (m*M*)	*Bm*SXP recombinant antigen concentration (mg/g DCW)	Imidazole concentration in wash buffer (m*M*)	*Bm*SXP recombinant antigen concentration (mg/ gDCW)
5	7.88 ± 0.46	20	11.16 ± 0.64
10	9.36 ± 0.86	30	11.82 ± 0.40
15	8.40 ± 0.28	40	8.09 ± 0.25
20	6.64 ± 0.43	45	8.12 ± 0.54
		50 (Manufacturer's Protocol)	7.68 ± 0.36

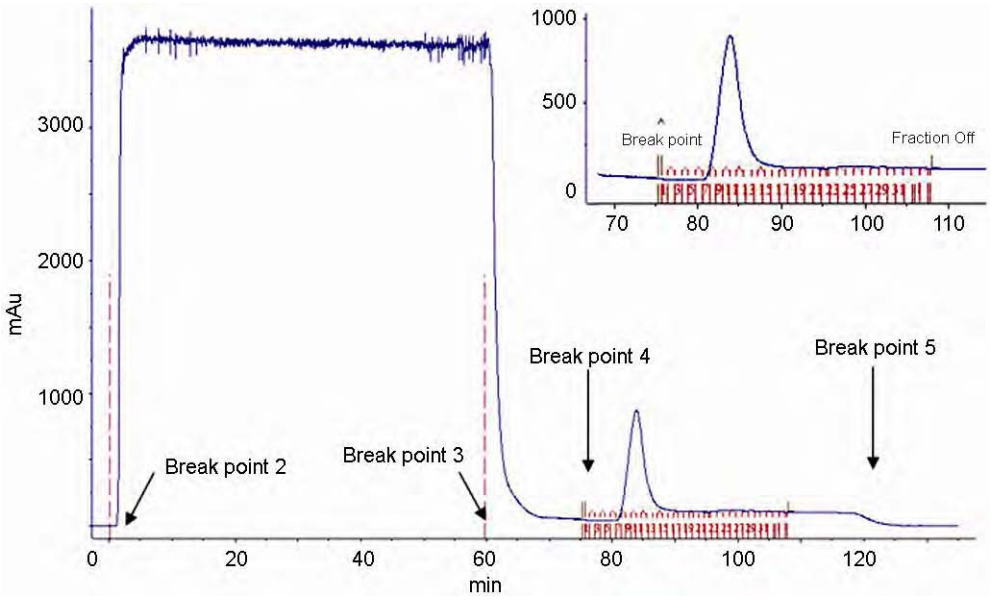

Fig. 1—Chromatogram output of the purification using wash buffer containing 30 m*M* imidazole, 300 m*M* salt concentration and 10 CV

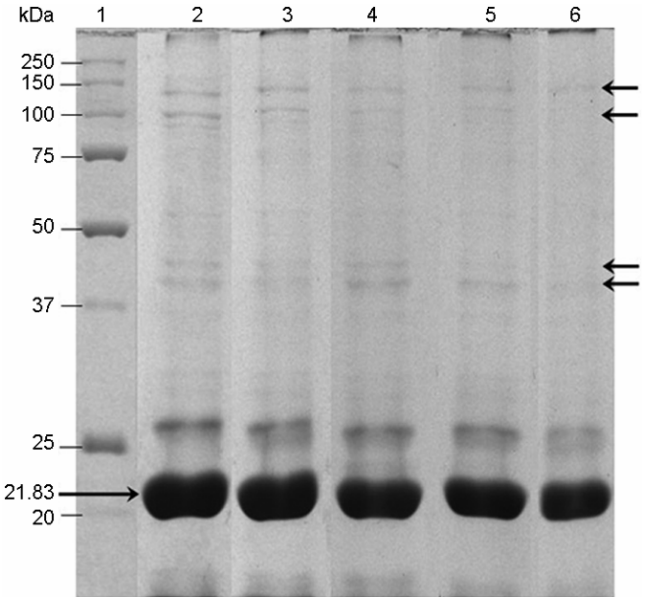

Fig. 2—SDS-PAGE (10%) analysis of the pooled fractions (fractions 5-30) using affinity chromatography with wash buffers containing 20, 30, 40, 45 and 50 m*M* imidazole, 10 CV and 300 m*M* salt concentration. Gel was stained with Coomassie blue [Lane 1: molecular weight protein marker; Lane 2–6: 20, 30, 40, 45 and 50 m*M* imidazole concentration washing buffer respectively]

ELISA—The immunoreactivity of the recovered *BmSXP* recombinant antigen obtained from the optimized protein purification conditions (30 m*M* imidazole, 300 m*M* salt and 10 CV of wash buffer) was evaluated via ELISA method. The results showed 93.75 and 100% sensitivity and specificity respectively in the detection of brugian and bancroftian LF (Table 2). The *t-test* analysis *P*-value of 0.001, thus there is a significant difference between the ODs of the positive and negative serum samples.

Discussion

The efficiency of IMAC purification technique is dependent on many factors, such as the natural properties of the target protein, culture conditions and purification conditions[13]. Since there is a high potential for the binding of background contaminating cellular proteins (which also contained histidine amino acid as its peptide basic building blocks) to the active binding sites of the nickel resin, the usage of low concentrations of imidazole in the lysis and wash buffers is recommended to improve purity[14]. This is because the imidazole ring resembles part of the structure of histidine, which would act as a direct competitor of histidine where it would bind to the Ni^{2+} immobilized on the matrix (resin) and disrupt the binding of dispersed histidine residues in non-tagged background proteins. At low imidazole concentrations, non-specific, low affinity binding of background proteins is prevented, while 6xHis-tagged antigens still bind strongly to the resin. This is consistent with the results of the present study which showed that too low a concentration of imidazole in the lysis buffer (5 m*M*) led to low recovery of *Bm*SXP recombinant antigen because the presence

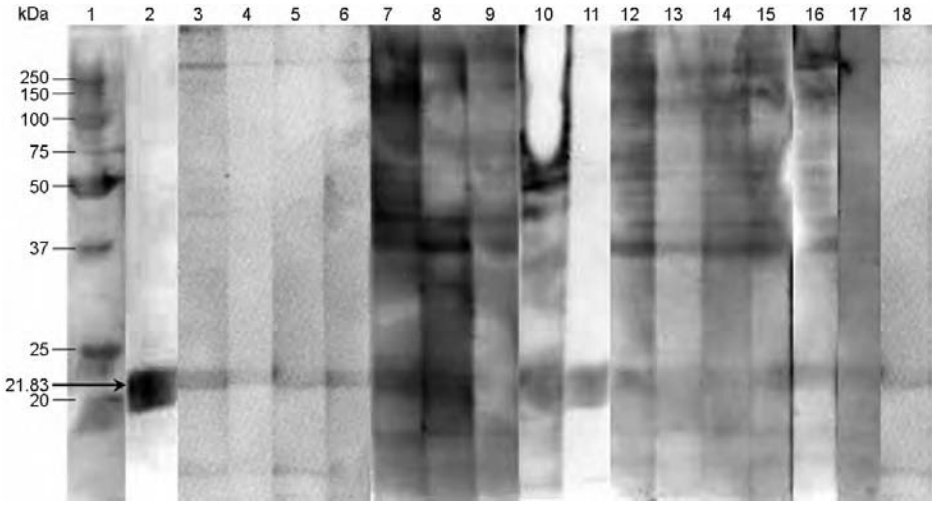

Fig. 3—Western blot analysis of *Bm*SXP recombinant antigen using patients' serum samples diagnosed with *W. bancrofti* infection [Nitrocellulose membrane strip in Lane 2 was probed with anti-histidine HRP antibody at dilution of 1:1500. Lanes 3-18 were probed with patient serum sample diagnosed with *W. bancrofti* infection (bancroftian filariasis) at the dilution of 1:200. Lane 1: Molecular weight marker]

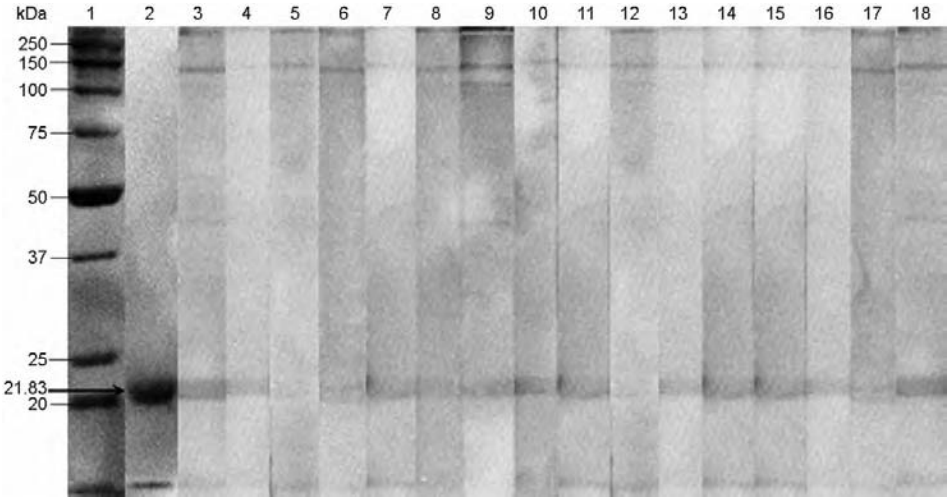

Fig. 4—Western blot analysis of *Bm*SXP recombinant antigen using patients' serum samples diagnosed with *B. malayi* infection [Nitrocellulose membrane strip in Lane 2 was probed with anti-histidine HRP antibody at dilution of 1:1500. Lanes 3-18 were probed with patient serum sample diagnosed with *B. malayi* infection (brugian filariasis) at the dilution of 1:200. Lane 1: molecular weight marker]

of other histidine-containing cellular proteins (contaminating proteins) have created a strong competition by taking up most of the active binding sites of the nickel resin. Similarly, high concentration of imidazole in the lysis buffer (15 m*M* onwards) also led to a low recovery of the antigen of interest due to the competition imposed by the high concentration of imidazole.

Excessive washing volume and high imidazole concentration in washing buffer could possibly elute not only the unbound proteins (contaminants), but also the loosely bound target antigen, leading to low recovery of *Bm*SXP recombinant antigen obtained at the end of the purification process[15]. Too low a concentration (30 m*M*) led to lower purity, but higher recovery rate (11.82 mg/g DCW); too high a concentration (50 m*M*) led to slightly greater purity, but lower recovery rate (7.68 mg/g DCW). This finding was consistent with Gupta and Chatterjee[16] whose study revealed that the purity might be enhanced by increasing imidazole concentration in the washing buffer. Thus, the optimization of washing buffer was an important factor in affecting the recovery rate of *BmSXP* recombinant antigen while maintaining the purity level at an acceptable range suitable for diagnostic purposes.

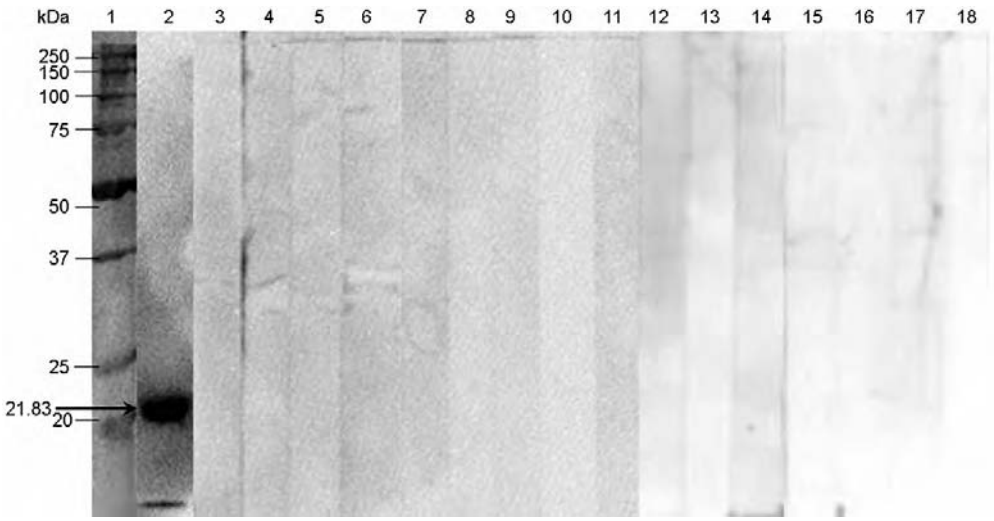

Fig. 5—Western blot analysis of *Bm*SXP recombinant antigen using patients' serum samples diagnosed with other parasitic infections [Nitrocellulose membrane strip in Lane 2 was probed with anti-histidine HRP antibody at dilution of 1:1500. Lanes 3-18 were probed with patients' serum samples diagnosed with other helminthic infections (*Entamoeba histolytica, Ascaris lumbricoides, Trichuris trichiura*, hookworm, *Strongyloides stercoralis*, *Toxoplasma gondii*) at the dilution of 1:200. Lane 1: Molecular weight marker]

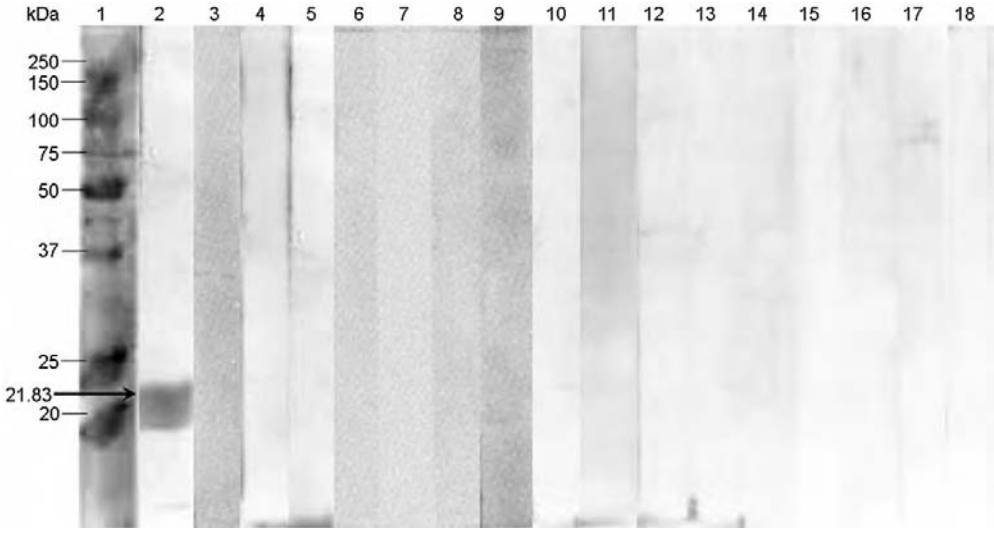

Fig. 6—Western blot analysis of *Bm*SXP recombinant antigen using healthy individuals' serum samples [Nitrocellulose membrane strip in Lane 2 was probed with anti-histidine HRP antibody at dilution of 1:1500. Lanes 3-18 were probed with healthy individuals' serum samples at the dilution of 1:200. Lane 1: Molecular weight marker]

Table 2—Result for the *t*-test analysis of positive and negative serum samples

Serum	Infection	Results	Mean	Standard Deviation	*P* value
Positive	*W. bancrofti*	15/16	1.611	1.131	
	B. malayi	16/16			
Negative	Other helminthic diseases	0/16	0.045	0.085	0.001
	Healthy	0/16			

Gupta *et al.*[17] have also stated that the common factors influencing IMAC performance were not only limited to the sorbent matrix, the chelating agent, method of immobilization and metal ion, but also heavily depend on the solution conditions (buffer solution, salt concentration, and *p*H). In the present study, the best parameter was clearly the protocol with 10 column volume (CV) and 300 m*M* salt concentration in wash buffer since there was

an additional recovery of 15% of the *Bm*SXP recombinant antigen. The combination of 300 m*M* salt concentration and the amount of imidazole concentration in 10 CV of wash buffer resulted in an equilibrium that further strengthened the binding between the target antigen with the immobilized nickel resin[18,19]. This could possibly explain why even though higher volume of wash buffer ran through the immobilized Ni-Sepharose resin matrix, the target antigens still remained tightly bounded to the nickel resin and were only eluted out when eluting buffer was applied.

The concentration of the protein is in direct proportion with the SDS-PAGE band intensity and width. The SDS-PAGE band of *Bm*SXP recombinant antigen revealed that recovery rate was the best, when purification was performed with wash buffer containing 20 and 30 m*M* imidazole concentrations. In this case, the concentration of imidazole in the wash buffer regulated the amount of non-specific binding, thus increasing the purity of the target antigen as the concentration of imidazole increases. Since the recovery rate of both 20 m*M* and 30 m*M* imidazole concentration wash buffer were comparable i.e. 11.16 and 11.82 mg/g DCW respectively, this explained why both lane 2 and lane 3 had similar band intensities and width of the target antigen in the SDS-PAGE. However the software analysis showed that sample from 30 m*M* imidazole wash buffer was 5.77% denser than that of 20 m*M* imidazole wash buffer. Since the objective of the present study was to mass produce the *Bm*SXP recombinant antigen for diagnostic test kits application, the best recovery with sufficient purity was attained with 30 m*M* imidazole concentration and 10 CV of wash buffer.

In Western blot, anti-His_6-peroxidase was used as a positive control to prove the presence of the *Bm*SXP recombinant antigen. Slight detection of non-specific or contaminating protein bands could be seen in some strips that were probed with primary antibody of patients' serum diagnosed with bancroftian and brugian filariasis, as well as in some negative samples of patients' serum diagnosed with other helmenthic diseases and healthy individuals' serum, but this do not affect the specificity of the recombinant antigen. This was proven when further analysis of the *Bm*SXP recombinant antigen by ELISA showed no cross reaction with other helminthic infections and healthy individuals, while demonstrating high sensitivity of the antigen (97%). This is consistent with previous studies on WB rapid (which also uses *Bm*SXP recombinant antigen) and panLF Rapid which showed sensitivities of 97.6% (n = 165) and 96.5% (n = 276) respectively; and the average specificities of 99.6% (n = 441)[4]. It is also comparable to sensitivities of *Wb*SXP, a recombinant antigen similar to *Bm*SXP, which were reported to able to detect 91% (n = 33)[20], 91.4% (n = 140)[21], and 100% (n = 72)[22] of sera from bancroftian filariasis patients.

Conclusion

In conclusion, high recovery of *Bm*SXP recombinant antigen was successfully obtained using IMAC. The results showed that under optimized downstream purification process conditions, the recovery of the expressed *Bm*SXP recombinant antigen was raised by 54% compared to the manufacturer's protocol without affecting the purity, sensitivity and specificity as assessed by Western blot and ELISA.

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