CHAPTER IV

RESULTS

Developing a *Wolbachia* isolation method for filarial nematodes

1. *Wolbachia* purified from *Dirofilaria immitis*

In an attempt to study *Wolbachia* genome architecture, an isolation method for *Wolbachia*, including *Wolbachia* of the filarial nematode *Brugia malayi* and *Dirofilaria immitis* has been developed (Sun *et al.*, 2001). Adult filarial worms were homogenized using physiological saline supplemented with 0.001% Nonidet P-40 (NP-40) non-ionic detergent, and *Wolbachia* cells were isolated by differential centrifugation. Inclusion of this very low concentration of detergent in the homogenization buffer is found to decrease the worm tissues co-purified with *Wolbachia*, and does not cause any noticeable DNA degradation (Sun *et al.*, 2001). We hypothesized that inclusion of higher concentration of NP-40 to the homogenization buffer (0.85% NaCl) would help increase yield of the *Wolbachia* bacterial extraction, as well as decrease host parasite contamination, and provide suitable materials for protein analysis. The optimal NP-40 concentration in 0.85% NaCl would help the physical treatment to disrupt parasite cell membranes, and to release *Wolbachia* cells from host materials efficiently.

Due to the limitation of *B. malayi* parasite specimen, we used adults of filarial dog heartworm *D. immitis* for the set-up experiments of *Wolbachia* isolation by varying concentrations of NP-40 (ranging from 0.005% to 0.04%) supplemented to the homogenization buffer. Each *Wolbachia* isolate was checked for intact cells under microscopic examination. *Wolbachia* in an isolate were also characterized by immunofluorescence with specific anti-recombinant *Wolbachia* surface protein

(rWSP) antibodies. To determine which homogenization buffer presented the optimal amount of *Wolbachia* cells, we analyzed the results of Western blot of *Wolbachia* extracts by specific anti-rWSP antibodies, which were achieved from the use of different concentrations of NP-40. Protein profiles of *Wolbachia* isolation from each homogenization were also analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). In some experiments, we studied protein profiles by Western blot analysis with anti-*Onchocerca volvulus* antibodies. The antibodies were raised against extracts of *O. volvulus*, a filarial nematode containing *Wolbachia*. Therefore, there would be antibodies to *O. volvulus* proteins and antibodies to *O. volvulus Wolbachia* proteins as well. Antibodies to *O. volvulus* proteins should cross-react to conserved *D. immitis* proteins, and to *D. immitis Wolbachia* proteins. The immunostaining with the *Onchocerca* antibodies could be used to check both protein profiles of *D. immitis*, and *Wolbachia*.

1.1 Intact *Dirofilaria immitis Wolbachia* cells under microscopy

Under microscopy, a lot of shinny bacteria cells with cocco-bacilli and pleomorphic shapes were identified, most arranged in single and double cells in the wet-mount preparation of *Wolbachia* isolate (**Figure 8**).

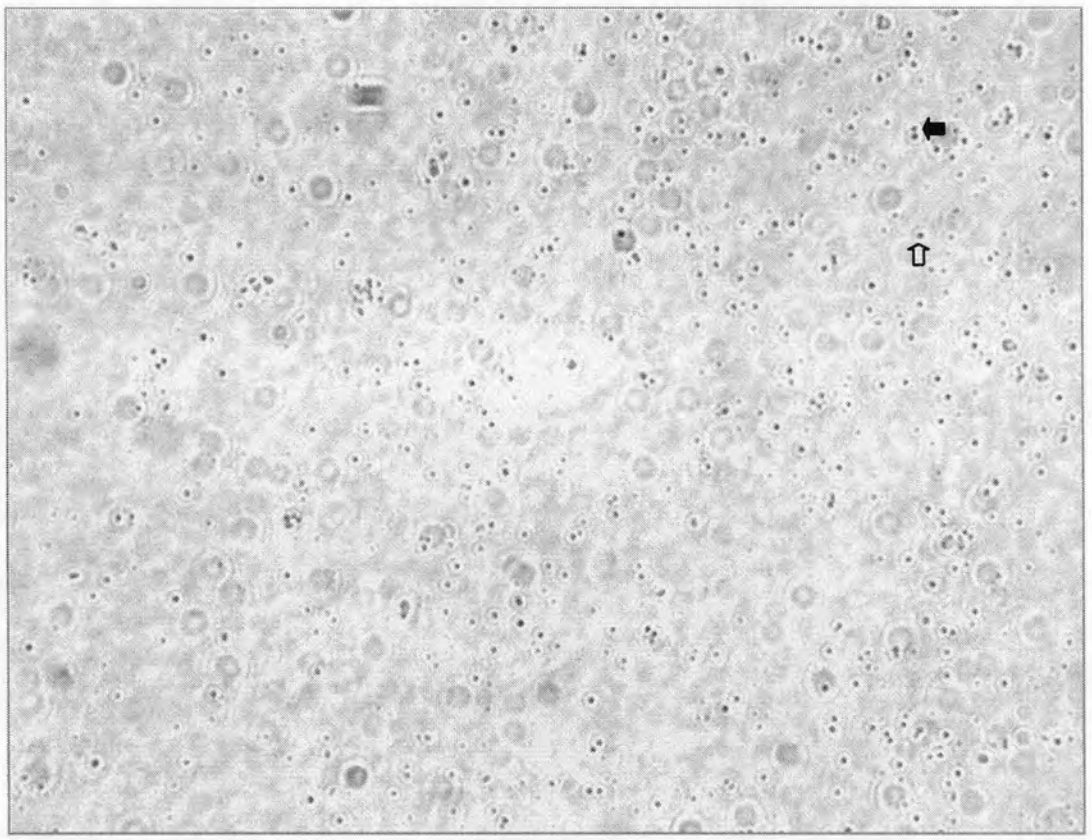

Figure 8 Wet-mount preparation of the enriched *Wolbachia* cells from *Dirofilaria immitis*. *Wolbachia* appear as single (white-arrow head) or double cells (black-arrow head).

Immunofluorescence by specific anti-rWSP antibodies confirmed that those cocco-bacilli and pleomorphic bacteria cells were *Wolbachia* (**Figure 9**). The positive immunofluorescence staining was specific for anti-rWSP antibodies. The immunofluorescence of the *Wolbachia* cells was negative with the staining of FITC-rabbit anti-chicken IgG antibodies only (data not shown).

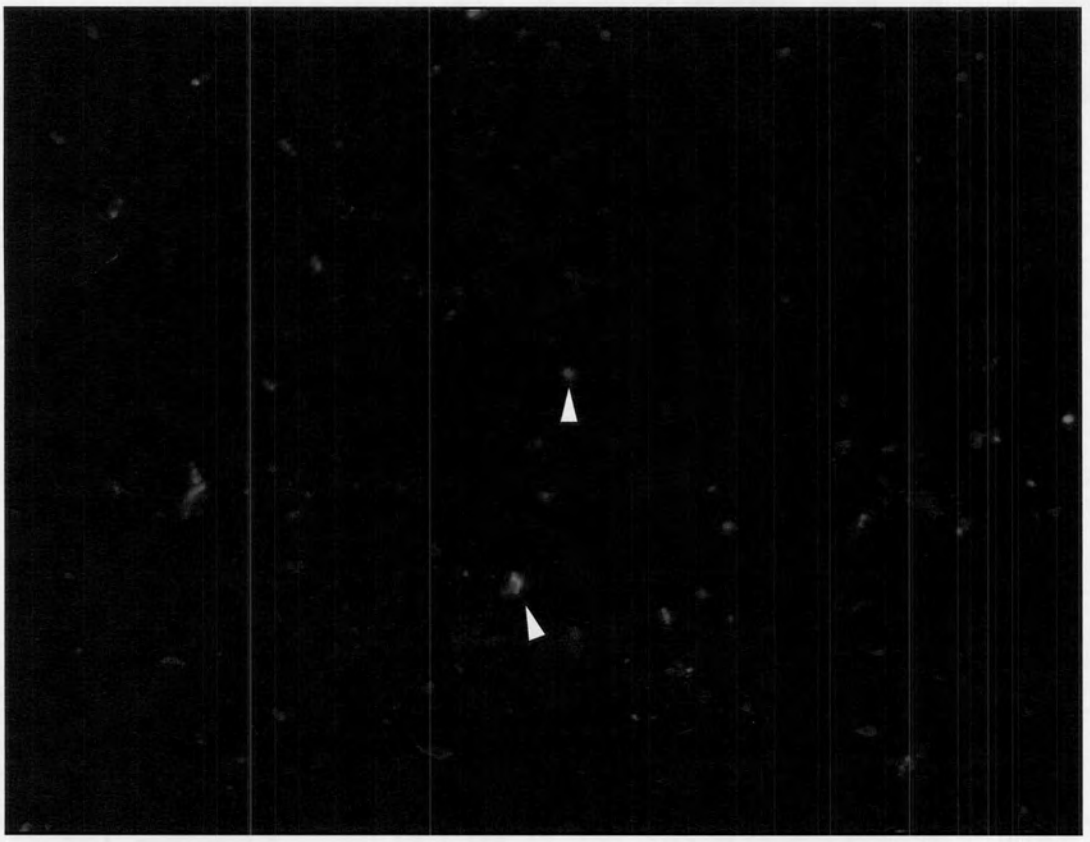

Figure 9 Immunofluorescence of *Wolbachia* purified from *Dirofilaria immitis* by using anti-rWSP antibodies. *Wolbachia* precipitates were prepared with 1:100 anti-rWSP antibodies, and with 1:20 FITC-rabbit anti-chicken IgG antibodies. *Wolbachia* cells with positive staining are indicated (arrow).

1.2 *Wolbachia* purified from *Dirofilaria immitis* by using 0.85% NaCl and 0.005%, 0.01%, and 0.02% Nonidet P-40 as homogenization buffers

To determine which homogenization buffer presented the optimal amount of *Wolbachia* cells, we analyzed the results of Western blot of *Wolbachia* extracts by using specific anti-rWSP antibodies. The diagram of *Wolbachia* isolation from *D. immitis* representing the experimental outline is shown in **Figure 10**.

Total parasite extracts, *Wolbachia*-depleted supernatants, and isolated *Wolbachia* cells from each homogenization (by using 0.85% NaCl and 0.005%, 0.01%, or 0.02% NP-40) were collected. Protein profiles were then analyzed by SDS-PAGE, and by immunoblotting with anti-rWSP antibodies. The WSP concentration was determined in *Wolbachia* extracts isolated by using 0.85% NaCl and NP-40 at the different concentrations as homogenization buffers. The supernatants obtained after *Wolbachia* sedimentation (*Wolbachia*-depleted supernatants) should contain no WSP protein, when analyzed by Western blot analysis, to ensure efficiency of the centrifugation force in sedimentation of *Wolbachia* cells, or checked none of broken *Wolbachia* cells. The total parasite extracts (without enriching of *Wolbachia*) were determined for WSP concentration compared to the *Wolbachia* extracts (with enriching of *Wolbachia*) to validate the effectiveness of the isolation procedures.

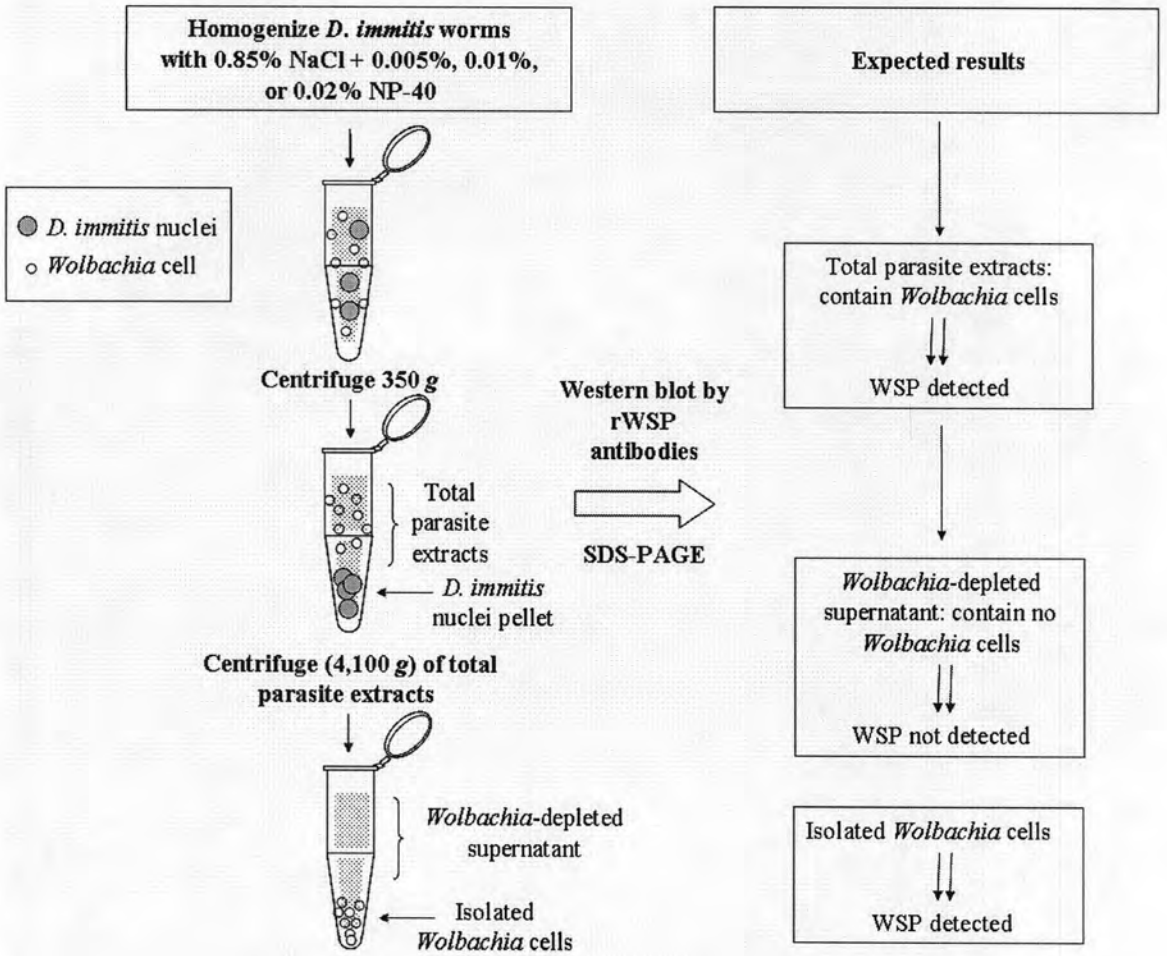

Figure 10 Diagram of *Wolbachia* isolation procedure from *Dirofilaria immitis*. Total parasite extracts, *Wolbachia*-depleted supernatants, and isolated *Wolbachia* cells from each homogenization, by using 0.85% NaCl and 0.005%, 0.01%, or 0.02% NP-40 as a homogenization buffer, were collected, and analyzed by SDS-PAGE and immunoblotting with anti-rWSP antibodies.

Protein profiles of *Wolbachia* isolation, when extracted with 0.85% NaCl and 0.005%, 0.01%, and 0.02% NP-40 as homogenization buffers, and analyzed by Coomassie-blue staining of SDS-PAGE gels, showed higher concentration of NP-40 supplemented to the homogenization buffer provided more protein concentration in the *Wolbachia* enriched extracts (**Figure 11A**; Lane 3, 6 and 9, respectively). Western blot analysis with specific anti-rWSP antibodies indicated that extraction with 0.85% NaCl and 0.02% NP-40 provided the highest WSP concentration (Lane 9), while the WSP protein could not be detected in the *Wolbachia*-depleted supernatant (Lane 8) (**Figure 11B**), implying that there was no *Wolbachia* cell, nor soluble WSP in the supernatants.

Higher NP-40 concentration supplemented to 0.85% NaCl would provide more isolated *Wolbachia* cells. From the experiments, the use of 0.02% NP-40 provided the highest total protein concentration, and WSP concentration in the *Wolbachia* enriched extracts. However, higher *Wolbachia* cells archived could come with higher co-purified parasite proteins.

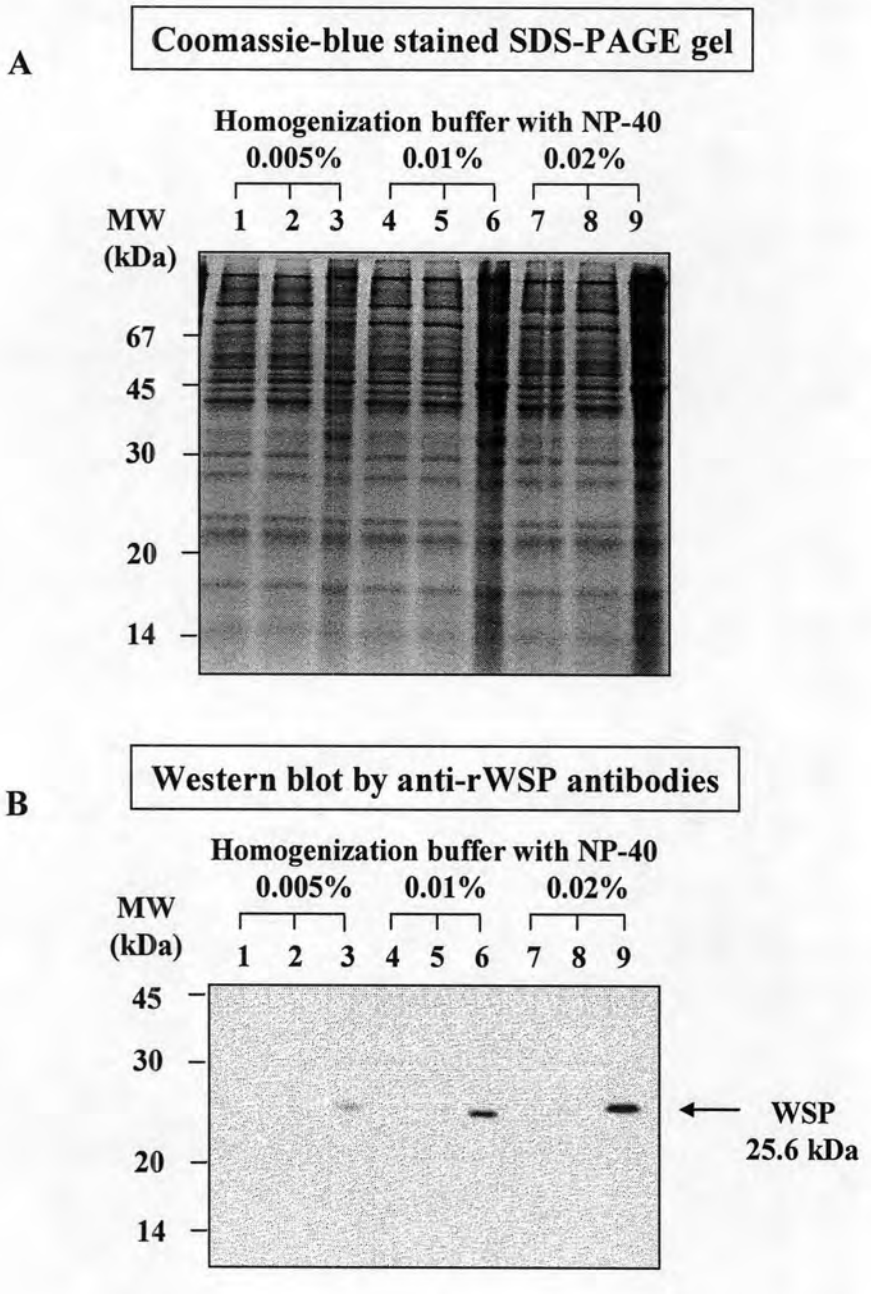

Figure 11 SDS-PAGE and Western blot analysis of *Wolbachia* purified from *Dirofilaria immitis* by using anti-rWSP antibodies; Coomassie-blue stained SDS-PAGE gel (A) and Western blot by anti-rWSP antibodies (B). Lane 1-3, 4-6, and 7-9, *Wolbachia* extraction by using 0.85% NaCl and 0.005%, 0.01%, and 0.02% NP-40 as homogenization buffers, respectively; Lane 1, 4, and 7, total parasite extracts; Lane 2, 5, and 8, *Wolbachia*-depleted supernatants; Lane 3, 6, and 9, *Wolbachia* extracts.

1.3 *Wolbachia* purified from *Dirofilaria immitis* by using 0.85% NaCl and 0.02%, and 0.04% Nonidet P-40 as homogenization buffers

To further adjust the best condition for *Wolbachia* extraction, we increased NP-40 concentration supplemented to 0.85% NaCl, and after homogenization, we incubated the samples on ice before the first centrifugation. Furthermore, we suspended isolated *Wolbachia* cells with a homogenization buffer, and repeated the last centrifugation for collection of *Wolbachia* cells. The usefulness of 0.04% NP-40 were tested by the *Wolbachia* isolation from *D. immitis* using 0.85% NaCl and 0.04% NP-40 compared to 0.02% NP-40 (**Figure 12**). The *Wolbachia*-washed buffers were also analyzed for protein concentration, and for WSP protein, to validate the washing step included.

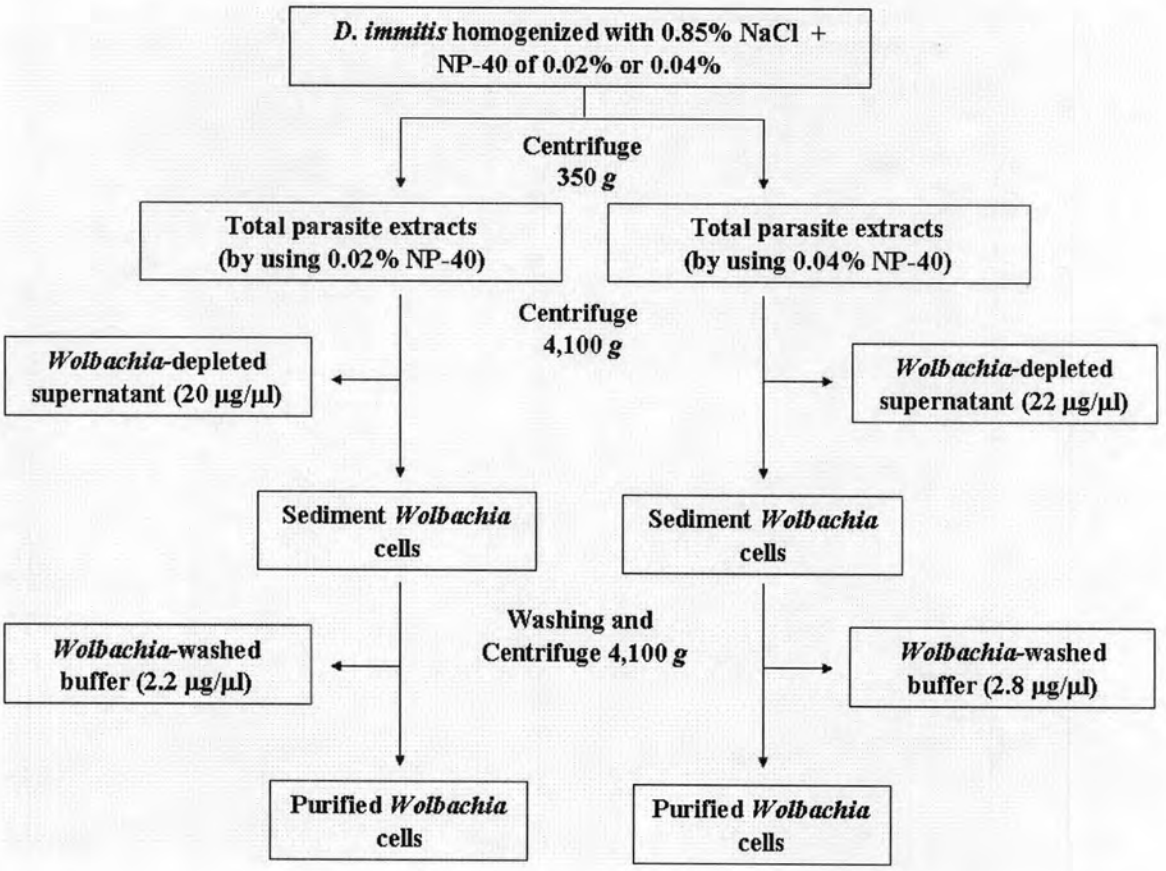

Figure 12 Diagram of *Wolbachia* isolation procedure from *Dirofilaria immitis*. After homogenized worms by using 0.85% NaCl and NP-40 (0.02% and 0.04%), each fraction was collected, and analyzed by immunoblotting with anti-rWSP antibodies, and with anti-*Onchocerca volvulus* antibodies. Protein concentrations were indicated in parentheses.

Protein profiles of the *Wolbachia* extraction by using 0.85% and NP-40 of 0.02%, and 0.04% were analyzed by Western blot with anti-rWSP antibodies (**Figure 13A**), and with anti-*O. volvulus* antibodies (**Figure 13B**). As introduced above, the antibodies to *O. volvulus* proteins should have cross-reaction with some *D. immitis* parasite proteins, and with proteins of *Wolbachia* in *D. immitis*. The immunostaining with the antibodies could be used to examine total protein profiles of the isolated *Wolbachia*. In addition, when comparing reactive patterns to the antibodies, we should be able to obtain unique reactive patterns of each fraction, suggesting for *D. immitis* proteins and *Wolbachia* specific proteins.

Inclusion of 0.04% NP-40 to the homogenization buffer yielded the highest WSP concentration in protein extracted from the isolated *Wolbachia* cells (**Figure 13A**; Lane 5), while the WSP protein could not be detected in the *Wolbachia*-depleted supernatant (**Figure 13A**; Lane 6).

Based on the detection of the anti-*Onchocerca* antibodies, overall reactive profiles of the *Wolbachia* extracts by using 0.85% NaCl and 0.04% NP-40 were not different from that using 0.02% NP-40, and intensity of proteins did not represent difference in total protein concentrations (**Figure 13B**; Lane 5 and 2, respectively). From all reactive profiles, it was more likely that the anti-*Onchocerca* antibodies were more reactive to parasite proteins, and less reactive to the WSP protein. The explanation would be more parasite antigens than *Wolbachia* antigens. Together, purity of the isolated *Wolbachia* may not different from that using 0.02%, and 0.04% NP-40.

For washing step included in the isolation protocol, it was found that the *Wolbachia*-washed buffers contained proteins that could react with the *Onchocerca*

antibodies (**Figure 13B**; Lane 1 and 4). The washing step likely helped to clean co-purified parasite proteins from the isolated *Wolbachia*.

The use of 0.04% NP-40 was more likely to provide optimal *Wolbachia* cells of starting parasite materials. An effort to test NP-40 at higher concentrations has been limited with unavailability of *D. immitis* adult worms in the laboratory. The optimized condition with 0.04% NP-40 supplemented into the homogenization buffer was further test in isolation of *Wolbachia* from *Brugia malayi* adult worms.

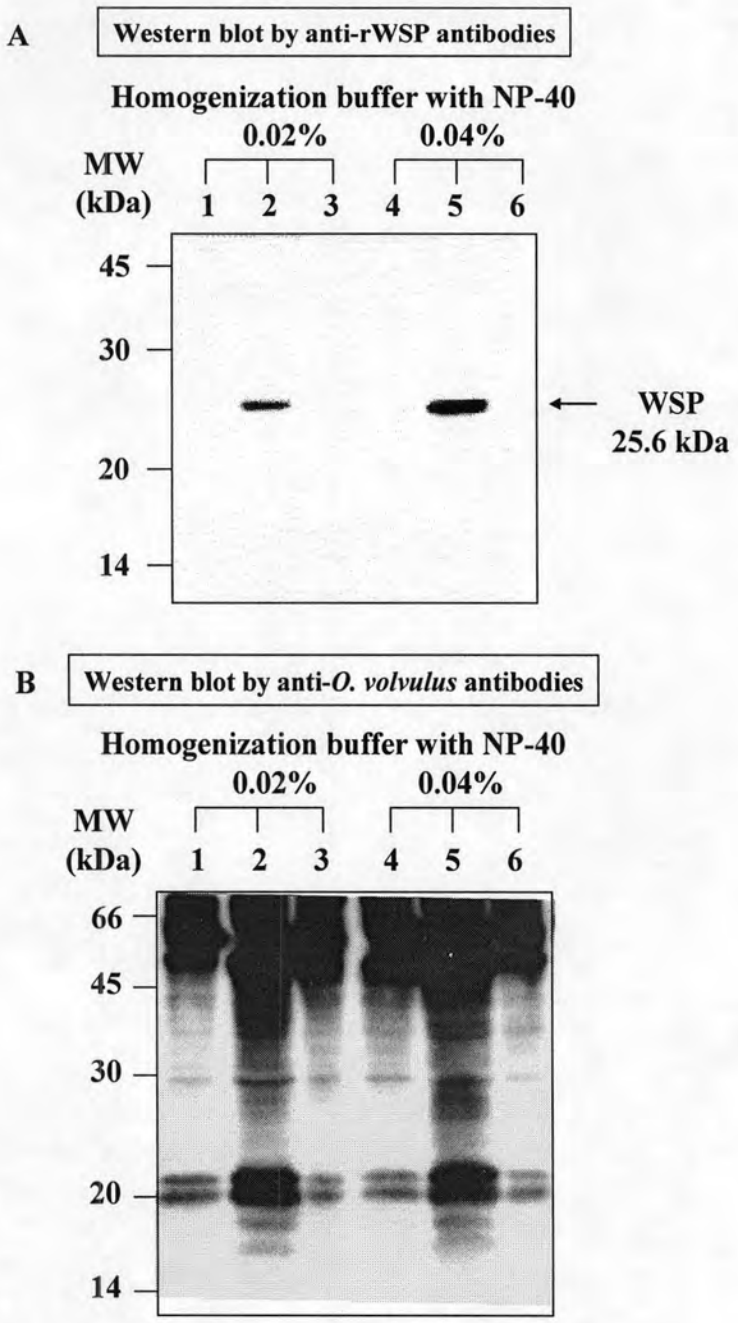

Figure 13 Western blot analysis of *Wolbachia* purified from *Dirofilaria immitis*, by using anti-rWSP antibodies and anti-*Onchocerca volvulus* antibodies. After homogenized worms by using 0.85% NaCl and 0.02% (Lane 1-3), and 0.04% (Lane 4-6) NP-40, each fraction was collected, and analyzed by immunoblotting with anti-rWSP antibodies (A) and anti-*Onchocerca volvulus* antibodies (B); Lane 1 and 4, *Wolbachia*-washed buffers; Lane 2 and 5, *Wolbachia* extracts; Lane 3 and 6, *Wolbachia*-depleted supernatants.

2. *Wolbachia* purified from *Brugia malayi*

2.1 *Wolbachia* purified from *Brugia malayi* by using 0.85% NaCl and 0.04% NP-40 as a homogenization buffer

To apply the developed protocol to *B. malayi Wolbachia* extraction, 0.85% NaCl and 0.04% NP-40 was used as a homogenization buffer. Protein profiles were detected by Coomassie-blue staining of the SDS-PAGE gel (**Figure 14A**), and antigenic pattern was analyzed by using Western blot by anti-rWSP antibodies (**Figure 14B**). The total protein profiles detected by Coomassie-blue staining of the SDS-PAGE gel showed that total protein amount of the *Wolbachia* extracts were lower than that of the total parasite extracts (**Figure 14A**; Lane 1), and the *Wolbachia*-depleted supernatant (Lane 2). Nevertheless, the *Wolbachia* enriched fraction showed the highest WSP amount (**Figure 14B**). Thus, the isolation method was useful to purify *Wolbachia* from *B. malayi* adult worms.

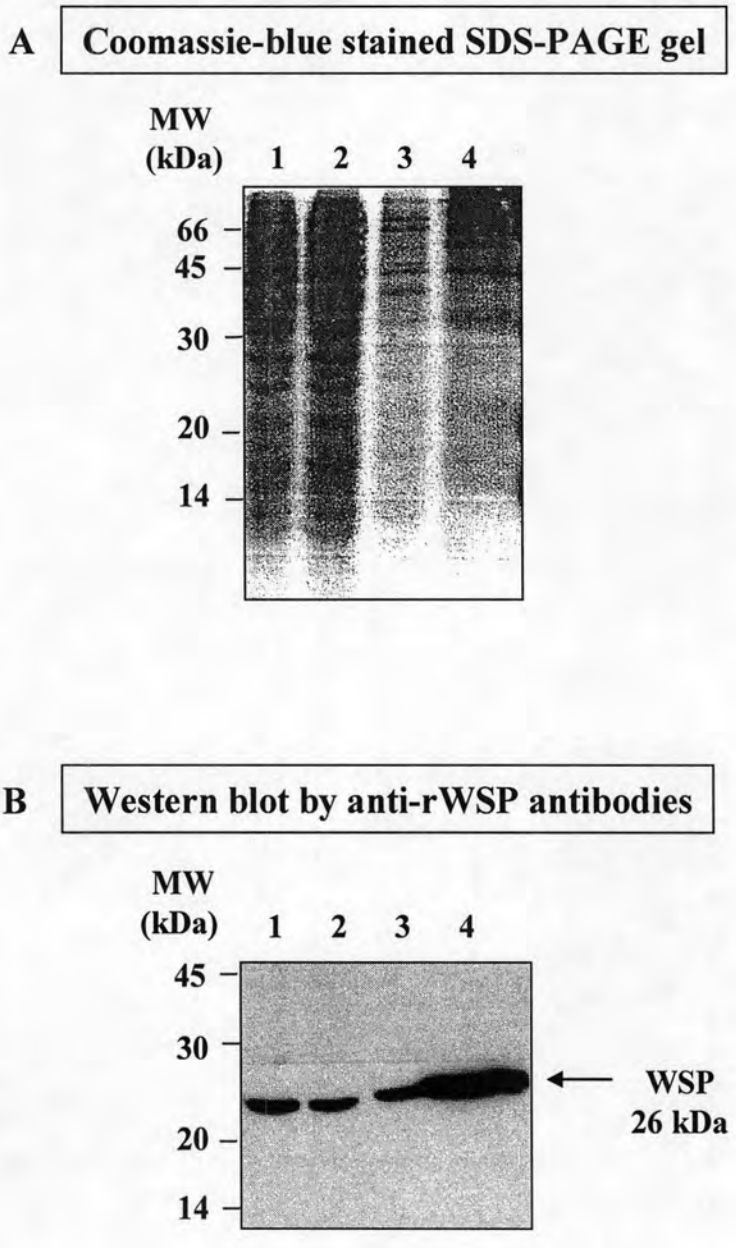

Figure 14 SDS-PAGE and Western blot analysis of *Wolbachia* purified from *Brugia malayi* by using anti-rWSP antibodies. After homogenized worms by using 0.85% NaCl and 0.04% NP-40, each fraction was collected, and analyzed by Coomassie-blue stained SDS-PAGE gel (A) and Western blot by anti-rWSP antibodies (B); Lane 1, total parasite extracts; Lane 2, *Wolbachia*-depleted supernatant; Lane 3, *Wolbachia*-washed buffer; Lane 4, *Wolbachia* extracts.

However, the results revealed that the WSP protein was found both in the *Wolbachia*-depleted supernatant (**Figure 14B**; Lane 2), and the *Wolbachia*-washed buffer (Lane 3). The explanation would be that there were remaining *Wolbachia* cells, due to the centrifugation force insufficient to entirely sediment *Wolbachia*, since a centrifugation force is influenced by various factors, including viscosity of a solution, temperature, and pH (Rickwood, 1984). The centrifuge force used in the original protocol could not be applied in the *B. malayi Wolbachia* extraction. Alternatively, there was WSP protein from the bacterial cells that were ruptured during processes of worm collection and/or extraction processes using the homogenization buffer. To prove that there were *Wolbachia* cells in the supernatant after the step of the bacteria collection, the higher centrifugation force was used in the next experiment, and WSP protein was analyzed in *Wolbachia* extracts after and before the centrifugation.

2.2 Effect of the centrifugation force at 6000 *g* in *Wolbachia* isolation from *Brugia malayi*

To optimize the *Wolbachia* isolation protocol with higher centrifugation force, the centrifugation was adjusted to 6,000 *g* at the final step instead of 4,100 *g* (**Figure 14**). The *Wolbachia*-depleted supernatant, and the *Wolbachia*-washed buffer were re-centrifuged at 6,000 *g*. The samples at before and after the centrifugation, including the pellet obtained were analyzed by Western blot with anti-rWSP antibodies (**Figure 15**). The WSP protein was identified in all samples. Interestingly, after the centrifugation, the pellet samples were positive for WSP from both *Wolbachia*-depleted supernatant (Lane 3), and *Wolbachia*-washed buffer (Lane 6). Since the intact *Wolbachia* cells were observed under a microscopy from the pellet preparations, the centrifugation force is likely helpful in sediment more *Wolbachia* cells.

However, the analysis by anti-rWSP antibodies did not illustrate difference in WSP protein concentration of both supernatant samples collected before (Lane 1 and 4) and after (Lane 2 and 5) the centrifugation. Such difference in WSP concentration protein of the supernatants before and after collection of *Wolbachia* cells would exist, but be small to be demonstrated any difference by the Western blot analysis.

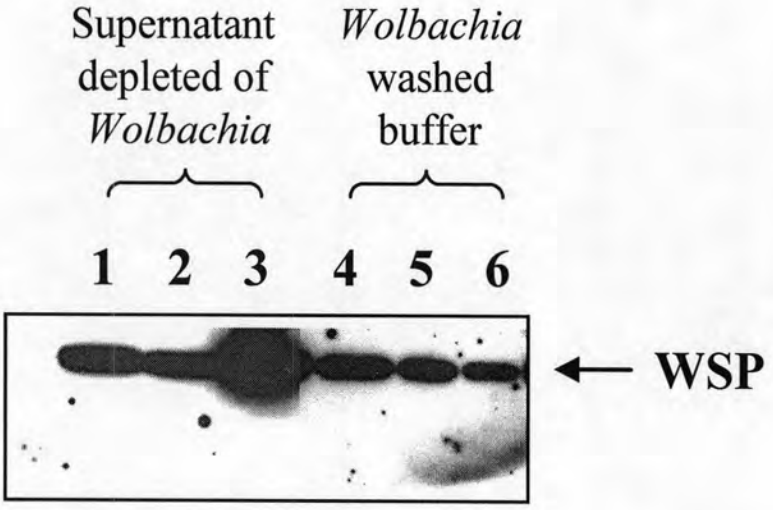

Figure 15 Effect of the centrifugation force at 6000 *g* in *Wolbachia* isolation from *Brugia malayi*, by analysis of Western blot with anti-rWSP antibodies; Lane 1-3 and 4-6, the centrifugation of *Wolbachia*-depleted supernatant and *Wolbachia*-washed buffer, respectively; Lane 1 and 4, samples before the centrifugation; Lane 2 and 5, samples after the centrifugation; Lane 3 and 6, the sediments.

2.3 Effect of the centrifugation force at 16,000 *g* in *Wolbachia* isolation from *Brugia malayi*

Since the WSP protein was still detected after 6,000 *g* centrifugation, we determined effect of the centrifugation force at 16,000 *g*. The *Wolbachia*-depleted supernatant, and the *Wolbachia*-washed buffer were re-centrifuged, and all fractions were collected to analyze by Western blot with anti-rWSP antibodies (**Figure 16**). The results showed that we still obtained the pellet sample positive for WSP from the centrifugation of the *Wolbachia*-depleted supernatant (Lane 3). In addition, WSP protein concentration was lower after the centrifugation of the *Wolbachia*-washed buffer (Lane 4 and 5). However, the antibodies did not detect difference in WSP protein concentration in the *Wolbachia*-depleted supernatant before (Lane 1), and after (Lane 2) the centrifugation.

The presence of WSP protein in both supernatant samples obtained from the centrifugation force at 16,000 *g* may explain soluble WSP molecules. We hypothesized that using frozen worms could break *Wolbachia* cells, and result in WSP detected in the supernatants. Thus, live worms would be more suitable for *Wolbachia* isolation. To test the hypothesis, *Wolbachia* were purified from live *B. malayi* adult worms instead of the frozen worms.

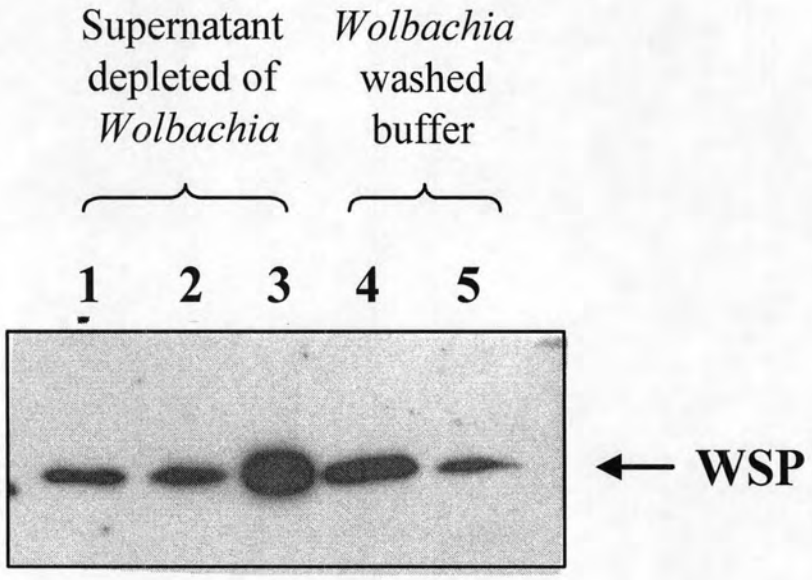

Figure 16 Effect of the centrifugation force at 16,000 *g* in *Wolbachia* isolation from *Brugia malayi*, by analysis of Western blot with anti-rWSP antibodies; Lane 1-3 and 4-5, the centrifugation of *Wolbachia*-depleted supernatant, and of *Wolbachia*-washed buffer, respectively; Lane 1 and 4, samples before the centrifugation; Lane 2 and 5, samples after the centrifugation; Lane 3, the sediments.

2.4 *Wolbachia* purified from *Brugia malayi* adult alive worms

In this experiment, *B. malayi* adult alive worms, instead of frozen worms, were used as the source of *Wolbachia*. The WSP profile of the *Wolbachia* extraction was analyzed by Western blot with anti-rWSP antibodies (**Figure 17**). The antibodies detected the WSP protein, not only in the *Wolbachia* enriched preparation (Lane 3), but also in the *Wolbachia*-depleted supernatant (Lane 1). However, the WSP protein was not found in the *Wolbachia*-washed buffer (Lane 2). When WSP profiles of the *Wolbachia* isolation obtained from frozen and alive worms were compared, we concluded that freezing and thawing processes could explain WSP protein found in the *Wolbachia*-washed buffer. However, the WSP protein found in the *Wolbachia*-depleted supernatant cannot be clarified from these experiments.

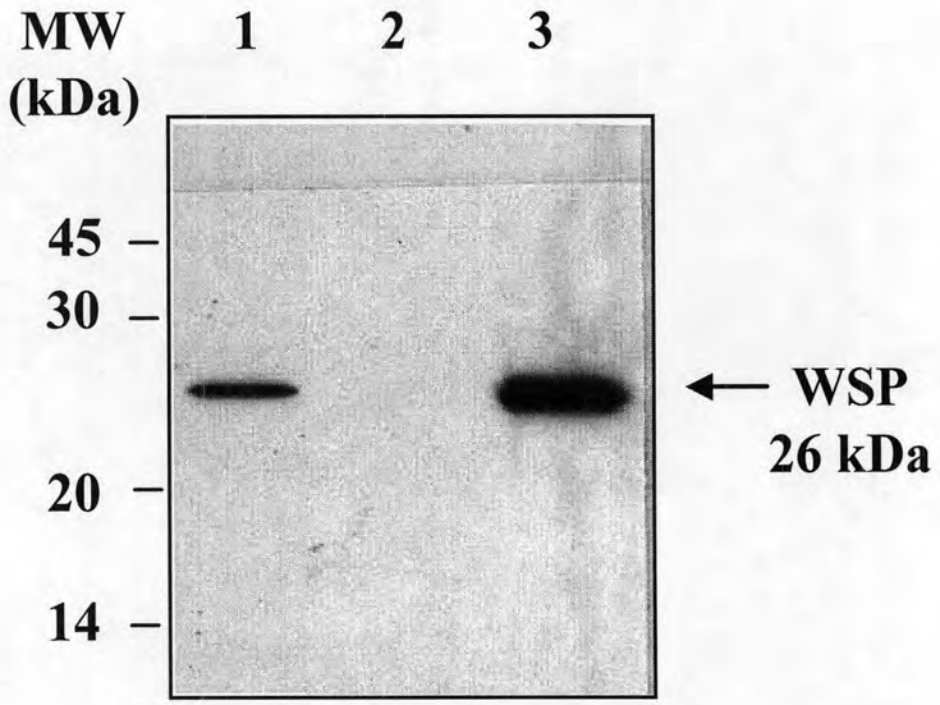

Figure 17 Western blot analysis of *Wolbachia* purified from *Brugia malayi* adult alive worms, by using anti-rWSP antibodies. The *Wolbachia* extraction was performed by using 0.85% NaCl and 0.04% NP-40 as a homogenization buffer; Lane 1, *Wolbachia*-depleted supernatant; Lane 2, *Wolbachia*-washed buffer; Lane 3, *Wolbachia* extracts.

We have optimized the *Wolbachia* isolation protocol from the filarial nematodes, *D. immitis* and *B. malayi* by using 0.85% NaCl and 0.04% NP-40 as a homogenization buffer. For *Wolbachia* isolation from *B. malayi*, it was likely that the higher centrifugation force at 16,000 *g* helped collect more purified *Wolbachia* cells. Moreover, *B. malayi* adult alive worms are more suitable source for *Wolbachia* isolation than frozen worms.

Characterization of proteins of filarial nematode *Wolbachia*

1. Proteins of *Wolbachia* of *Dirofilaria immitis*

1.1 2D-gel electrophoresis and silver staining

In order to characterize proteins of *Wolbachia* of *B. malayi* with limitation of the parasite materials, a pilot study of *D. immitis Wolbachia* proteins analyzed by 2D-gel electrophoresis (2-DE), and matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS) was carried out. Proteins in the preparation of *Wolbachia* purified from *D. immitis* were separated by 2-DE, IPG range 3-10. Silver staining was used to visualize the protein spots (**Figure 18**).

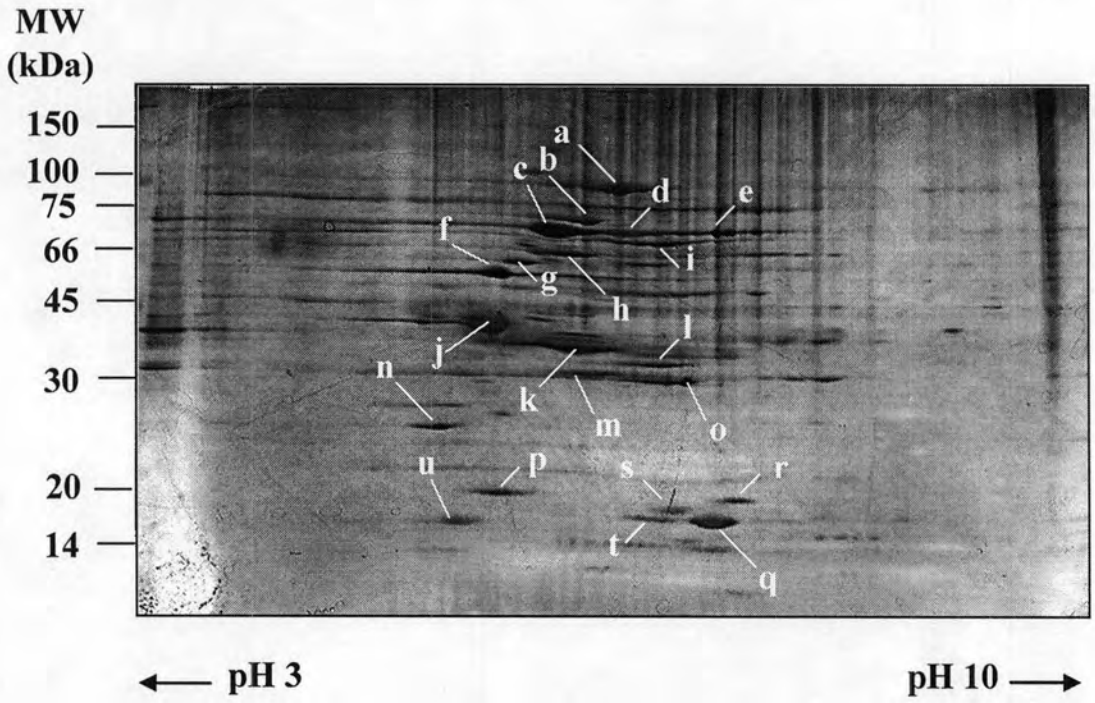

Figure 18 2-DE gel of *Wolbachia* extracts from *Dirofilaria immitis*. The 2-DE gel, IPG range 3-10, was visualized by silver staining; spots a-r, major stained spots; spot n predicted as WSP.

The major spots on the silver stained-2D-gel were identified as spots a-r, and their observed MW (18-94 kDa) and pI (5.1-7.5) were listed in **Table 5**.

Table 5 Major protein spots identified on the silver stained 2-DE gel of *Wolbachia* of *Dirofilaria immitis* with their observed MW and pI.

Spot	Observed MW (kDa)	Observed pI
a	94	6.5
b	75	6.2
c	72	6.0
d	72	6.6
e	72	7.2
f	56	5.7
g	63	5.8
h	66	6.1
i	71	6.8
j	40	5.6
k	35	6.2
l	34	6.8
m	32	6.2
n (predicted WSP)	26	5.1
o	30	6.9
p	20	5.7
q	18	7.2
r	19	7.5

The 26 kDa, pI 5.1 (spot n), spot was corresponding to the calculated MW (25.6 kDa) and pI (4.8) of WSP of *D. immitis Wolbachia*. For setting-up the MALDI-TOF-MS analysis, protein spots with their observed MW and pI corresponding to theoretical MW and pI of proteins available in the databases of the *D. immitis*, and the *Wolbachia* were selected. This could help practice MALDI-TOF-MS analysis, and peptide mass searching with a positive identification. The spots a, f, and h would represent the parasite proteins available in the databases. In addition, based on the protein analysis of *Wolbachia* purified from *D. immitis* by Western blot with the anti-*Onchocerca* antibodies, the reactive bands having MW of 18-20 and 22-30 kDa were probably belonging to *Wolbachia* of *D. immitis*. Therefore, the major spots a, f, h, n, and p-r were chosen for MALDI-TOF-MS analysis. We thus also excised spots n, and p-u for the protein identification.

1.2 Protein identification by MALDI-TOF-MS and peptide mass fingerprint searching

The excised spots were digested with enzyme trypsin in-gel. Tryptic peptides of each spot were subsequently extracted, and measured their masses by MALDI-TOF-MS. Peptide mass fingerprints were used to search against NCBI protein databases using MS-Fit program. Interpretation for protein identification includes concordance between observed MW/pI of each analyzed protein, and theoretical MW/pI of a matched protein difference within 20%, and concordance between analyzed species, and matched species in the protein databases. In addition, we considered the protein identification based on the criteria, including $\geq$ 4 matched peptides, and 20% sequence coverage by matched peptides.

The results of protein identification by MALDI-TOF-MS, and peptide mass fingerprint searching are shown in **Table 6**. The spots a, f, h, and n were successfully identified by the MALDI-TOF-MS analysis; spot n were identified as a surface protein of *Wolbachia* (WSP) of *D. immitis*. In addition to the bacterial-derived spot, we detected proteins of *D. immitis* identified as paramyosin (spot a), beta-tubulin (spot f), and chaperonine HSP60 (spot h). Spots p, q, r, s, t, and u were not able to be identified.

Table 6 Identification of *Dirofilaria immitis* and *Wolbachia* proteins by MALDI-TOF-MS and peptide mass fingerprint searching.

Spot	Observed MW (kDa)/pI	Species	Proteins	Calculated MW (kDa)/pI	Note
a	94/6.5	*D.immitis*	paramyosin	98.0/5.3	Eukaryote
f	56/5.7	*D.immitis*	Beta-tubulin	50.1/4.8	Eukaryote
h	66/6.1	*O.volvulus*	Chaperonine HSP60	64.3/5.7	Eukaryote
n	26/5.1	*Wolbachia* of *D.immitis*	Surface protein	25.6/4.8	Bacteria
p	20/5.7	*Arabidopsis thaliana*	~disease resistant protein	91.5/6.6	Eukaryote
q	18/7.2	*Moraxella catarrhalis*	Outer membrane protein	82.6/9.0	Bacteria
r	19/7.5	*Sulfolobus solfataricus*	Glutamate synthase (NADPH)	81.7/6.4	Archaea
s	20/7.0	*Mus Musculus*	Translation elongation factor 2	95.3/6.4	Eukaryote
t	18/6.9	*Porphyra purpurea*	ORF621	73.8/9.7	Eukaryote
u	18/5.3	*Myxococcus xanthus*	Hypothetical proteins	88.9/5.78	Bacteria

For protein identification by the MALDI-TOF-MS analysis of *Wolbachia* of *D. immitis* as preliminary data, most of the other spots analyzed were not yield the protein identification. In addition, the mass spectra of such spots showed low quality. This would be from the fact that samples from silver-stained protein gel are most problematic to obtain the high sensitivity, and quality of mass spectra (Gharahdaghi *et al.*, 1999). The silver staining has more sensitivity to detect protein spots compared to Coomassie-blue staining. However, the compatible staining method, Coomassie-blue G250 staining was used for the next MALDI-TOF-MS analysis. Moreover, we created the Mascot program*, a program for peptide mass fingerprint searching, containing the local database of *Wolbachia* of *B. malayi* to facilitate the unambiguous protein identification.

To report as the reference peptide mass fingerprint, the peptide mass fingerprint and peptide masses of the WSP of *D. immitis Wolbachia* were shown in **Figure 19** and **Table 7**, respectively.

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* Mascot program is a software packages to facilitate protein database searches. Protein data obtained from MALDI-TOF-MS or tandem MS analysis can be searched to identify interesting proteins. To facilitate rapid PMF searching, Mascot can be downloaded locally, and created with a group of protein databases of interest.

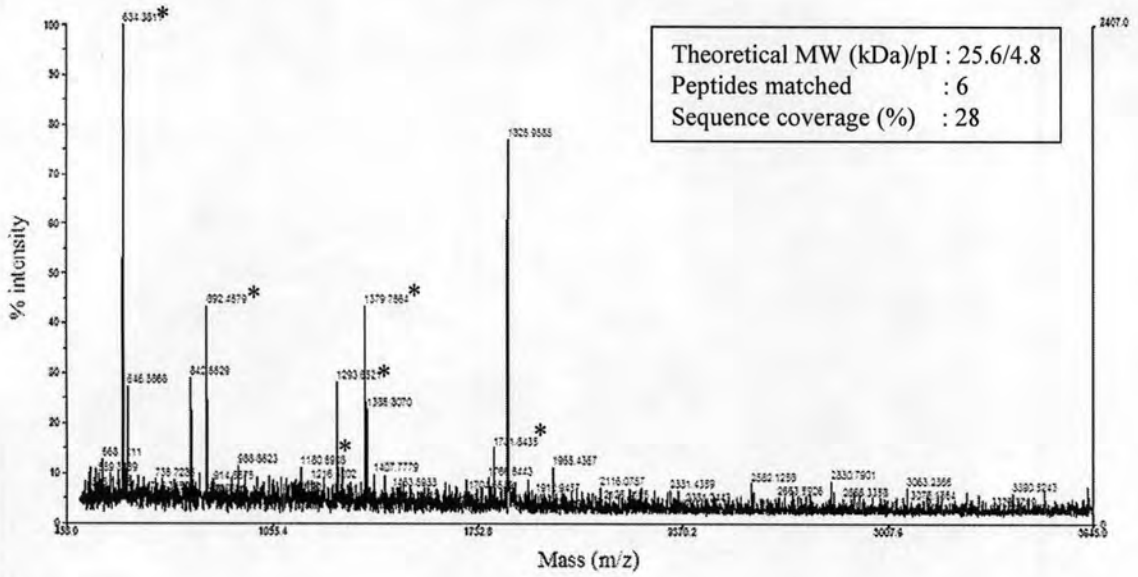


Figure 19 MALDI-TOF-MS spectra of *Wolbachia* surface protein (WSP) of *Dirofilaria immitis Wolbachia*. The spot n was excised, and analyzed by MALDI-TOF-MS; matched peptide masses are indicated (asterisk).

Table 7 Peptide mass data of *Wolbachia* surface protein (WSP) of *Dirofilaria immitis Wolbachia*. The matched peptides from the peptide mass fingerprint searching of mass lists obtained from the MALDI-TOF-MS analysis of spots n were shown.

Residue numbers	Measured mass	Calculated mass	Database sequence
1	634.3811	634.3677	LFAGAR
2	892.4579	892.4205	YFGSYGAK
3	1292.6930	1292.6738	AGISYDVTPEIK
4	1293.6521	1293.5938	ASFMAGGSAFGYK
5	1379.7564	1379.7171	VDIEGLYSQLSR
6	1780.8807	1780.8546	VTGDKEYGFGFAYQAK

1.3 2D-blot analysis by anti-rWSP antibodies

To characterize WSP separated by 2D-PAGE with anti-rWSP antibodies, the 2D blot of *Wolbachia* extracts from *D. immitis* was immunostained with the antibodies. Anti-rWSP antibodies detected one major protein spot (b) on the blot of *Wolbachia* extracts (**Figure 20**).

The identification result by anti-rWSP antibodies was concordant with the MALDI-TOF-MS analysis; the observed pI (5.0) and MW (25.6 kDa) of spot b were corresponding with the observed pI (5.1) and MW (26 kDa) of WSP revealed on the silver-stained 2D-gel. In addition to the major reactive spot, the antibodies recognized the spot 25.6 kDa, pI 4.4 (a), suggesting the isoforms of WSP as a result of secondary changes, such as post-translational modifications.

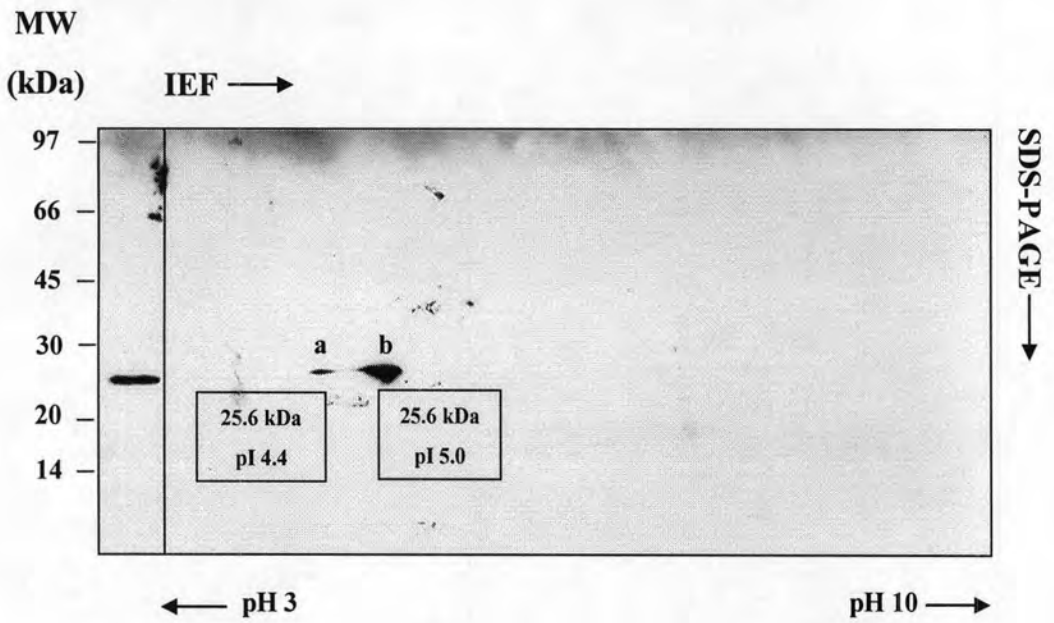


Figure 20 2D-blot analysis of *Wolbachia* extracts from *Dirofilaria immitis* by using anti-rWSP antibodies. The antibodies were identified 2 WSP spots with MW of 25.6 kDa, but different in pI values; pI 4.4 (a); pI 5.0 (b).

1.4 Immunoblot analysis by anti-*Wolbachia* antibodies

From the preliminary protein identification data, the *Wolbachia* extracts of *D. immitis* were consisted of not only proteins derived from *Wolbachia*, but also from the filarial nematode. It was possible to excise all protein spots on a 2D-gel, and analyze by MALDI-TOF-MS. However, all protein spots belonging to the parasite would not be identified, since the genome project of *D. immitis* was incomplete, and it seemed not reasonable to do the strategy without studying on protein complexity of *Wolbachia* protein materials.

In order to detect *Wolbachia* proteins, we performed immunoblots with anti-*Wolbachia* antibodies prepared against *Wolbachia* of *Drosophila* spp. The antibodies should be less reactive with parasite proteins, but react strongly with *Wolbachia* proteins. The blots with varied antibody dilutions have been tried. The 26-kDa band of *Wolbachia* extracts prepared from *B. malayi* (Lane 2), and the 25.6-kDa band of *Wolbachia* purified from *D. immitis* (Lane 3) were reacted strongly by the anti-*Wolbachia* antibodies, a dilution of 1: 2,000 (**Figure 21**).

As expected, the antibodies did not recognize any parasite proteins from total extracts of *Acanthocheilonema viteae* (Lane 1), a filarial nematode with free of *Wolbachia*. Nevertheless, only one major band on both *Wolbachia* preparations was detected.

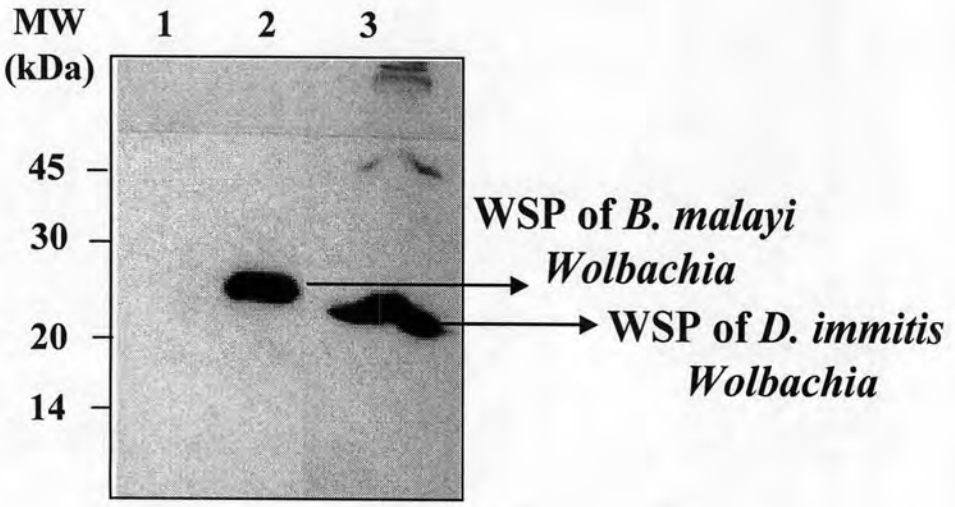


Figure 21 Optimization of Western blot analysis of *Dirofilaria immitis* and *Brugia malayi Wolbachia*, by using anti-*Wolbachia* antibodies. The antibody dilution of 1: 2000 was used; Lane 1, crude *Acanthocheilonema viteae* extracts; Lane 2, *Wolbachia* purified from *Brugia malayi*; Lane 3, *Wolbachia* purified from *Dirofilaria immitis*.

Because the antibodies did not recognize other *Wolbachia* proteins, we probed with higher concentration. The higher antibody concentration, a dilution of 1: 150, was found to detect more protein bands (**Figure 22**) as compared to the dilution of 1: 2,000. Therefore, Western blot by anti-*Wolbachia* antibodies was optimized with antibody dilution of 1: 150.

However, the antibodies showed cross reactivity with proteins of *A. viteae* (**Figure 22**; Lane 1). Any recognized band of *Wolbachia* extracts might be either bacterial proteins or parasite proteins. We should use *D. immitis* strain that is free of *Wolbachia* to control co-purified parasite proteins in a *Wolbachia* extract preparation, but there is no such host strain that can be applied ideally as a control. Alternatively, to study proteins specific for *Wolbachia* of *D. immitis*, we analyzed the reactive profiles of the *Wolbachia* enriched extracts (Lane 2) as compared to the reactive bands of *A. viteae* extracts.

Although the reaction of proteins with MW higher than 25.6 kDa in the *Wolbachia* extracts could not provide a result for comparison, this strategy to detect *Wolbachia* proteins is more likely reliable. The immunorecognition profile of *D. immitis* extracts (**Figure 22**; Lane 3) showed that the protein bands of 97-78 kDa, 62 kDa, and 32 kDa were also found in the preparation of *A. viteae* extracts. These protein bands are possibly cross-reactive parasite proteins, and suggested that parasite proteins of *A. viteae* and *D. immitis* that can be cross-reactive with anti-*Wolbachia* antibodies having conserved MW. Therefore, unique reactive bands of a sample containing *Wolbachia* as well as parasite proteins to the antibodies that are not comparable with reactive bands of *A. vitreae* extracts would be identified as *Wolbachia*-derived proteins.

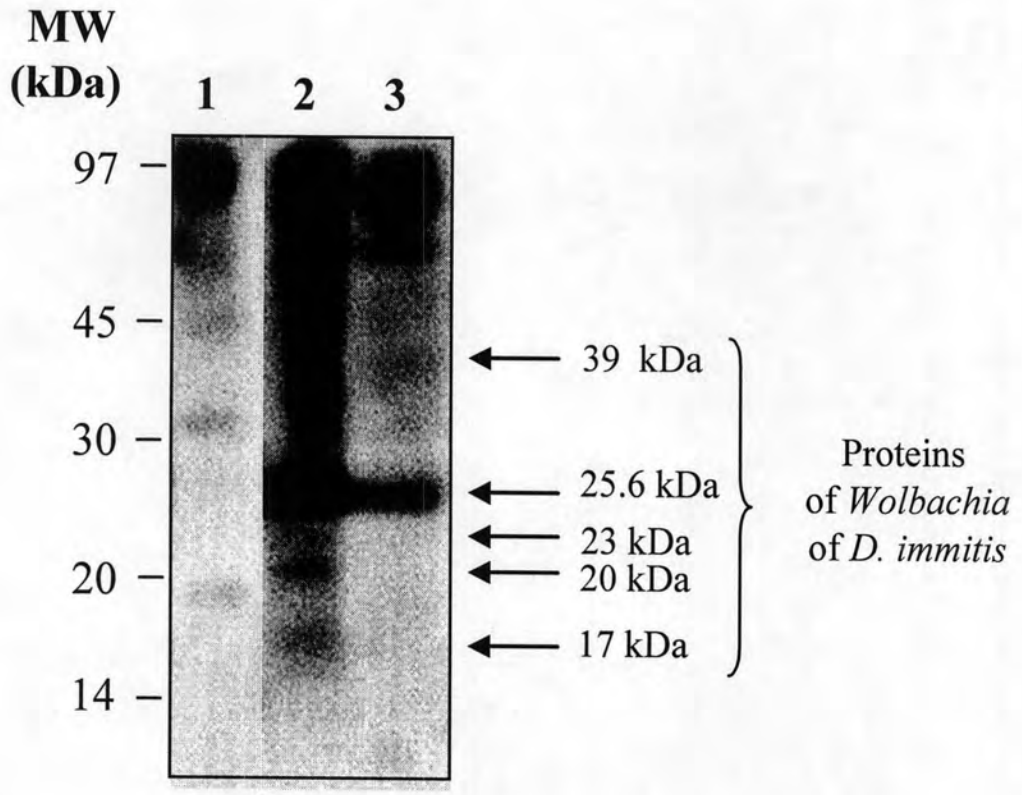


Figure 22 Optimization of Western blot analysis of *Dirofilaria immitis Wolbachia*, by using anti-*Wolbachia* antibodies. The antibody dilution of 1: 150 was used; Lane 1, crude *Acanthocheilonema viteae* extracts; Lane 2, *Wolbachia* purified from *D. immitis*; Lane 3, *D. immitis* extracts. Reactive protein bands of *Wolbachia* of *D. immitis* are indicated.

Reactivity to proteins ranged of 97-78 kDa, proteins of 62, 45, 32 and 19 kDa was detected in the crude *A. viteae* extracts. In *Wolbachia* extracts prepared from *D. immitis*, anti-*Wolbachia* antibodies reacted with 17-kDa, 19-kDa, 20-kDa, 23-kDa, 25.6-kDa, and higher molecular weight proteins. Since the protein bands with MW of 17, 20, 23, and 25.6 kDa were not found in the reactive pattern of *A. viteae* extracts, the reactive bands would be derived from *Wolbachia* of *D. immitis*. The additionally unique 39-kDa antigen was detected in the *D. immitis* extracts, and would belong to *Wolbachia*. The most highly reactive antigen (25.6 kDa) detected was corresponding with calculated MW of WSP of *D. immitis Wolbachia*. The 25.6-kDa band could be the WSP.

2. Proteins of *Wolbachia* of *Brugia malayi*

2.1 Immunoblot analysis by anti-*Wolbachia* antibodies

As we have mentioned regarding the characterization of proteins of *D. immitis Wolbachia*, we should use *B. malayi* strain that is free of *Wolbachia* to control co-purified parasite proteins in a *Wolbachia* extract preparation. However, there is no such host strain that can be applied ideally as a control. In addition, although *Wolbachia*-depleted filarial nematodes by antibiotic treatment seem to be an alternative choice, the published protocol is not able to eliminate all intact *Wolbachia* (Turner *et al.*, 2006). With the previous finding suggested that proteins of *A. viteae* and *D. immitis* are conserved in their MW and in recognition of anti-*Wolbachia* antibodies, protein extracts of *A. viteae* could be used as an alternative control in study of *B. malayi Wolbachia* proteins.

Initially, to detect proteins specific for *Wolbachia* of *B. malayi*, the strategy was implemented to analyze in crude extracts of *B. malayi*. Results of Western blot analysis by anti-*Wolbachia* antibodies in *A. viteae* and *B. malayi* extracts were evaluated (**Figure 23**). The immunorecognition bands of *A. viteae* (Lane 1) were all comparable in their observed MW with the reactive bands that were a subset in the reactive profile of *B. malayi* extracts (Lane 2). This implied that proteins of *A. viteae* and *B. malayi* are conserved in recognition of anti-*Wolbachia* antibodies, and in their MW. The unique antigens found in the *B. malayi* extracts were 83-kDa, 48-kDa, 36-kDa, 26-kDa, 20-kDa and 17-kDa antigens, and could be of *Wolbachia*. The highly reactive antigen (26 kDa) detected was corresponding with calculated MW of WSP of *B. malayi Wolbachia*. The 26-kDa band could be the WSP.

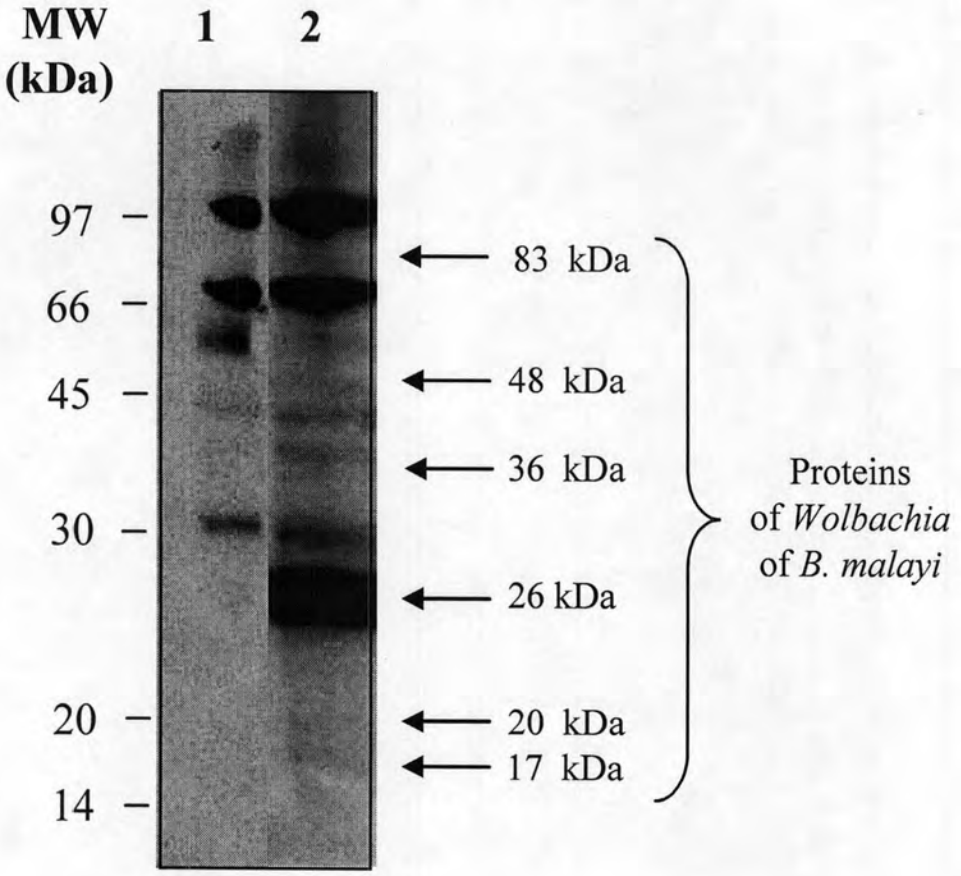


Figure 23 Western blot analysis of *Wolbachia* of *Brugia malayi*, by using anti-*Wolbachia* antibodies. Lane 1, crude *Acanthocheilonema viteae* extracts; Lane 2, crude *B. malayi* extracts. Reactive protein bands of *Wolbachia* of *B. malayi* are indicated.

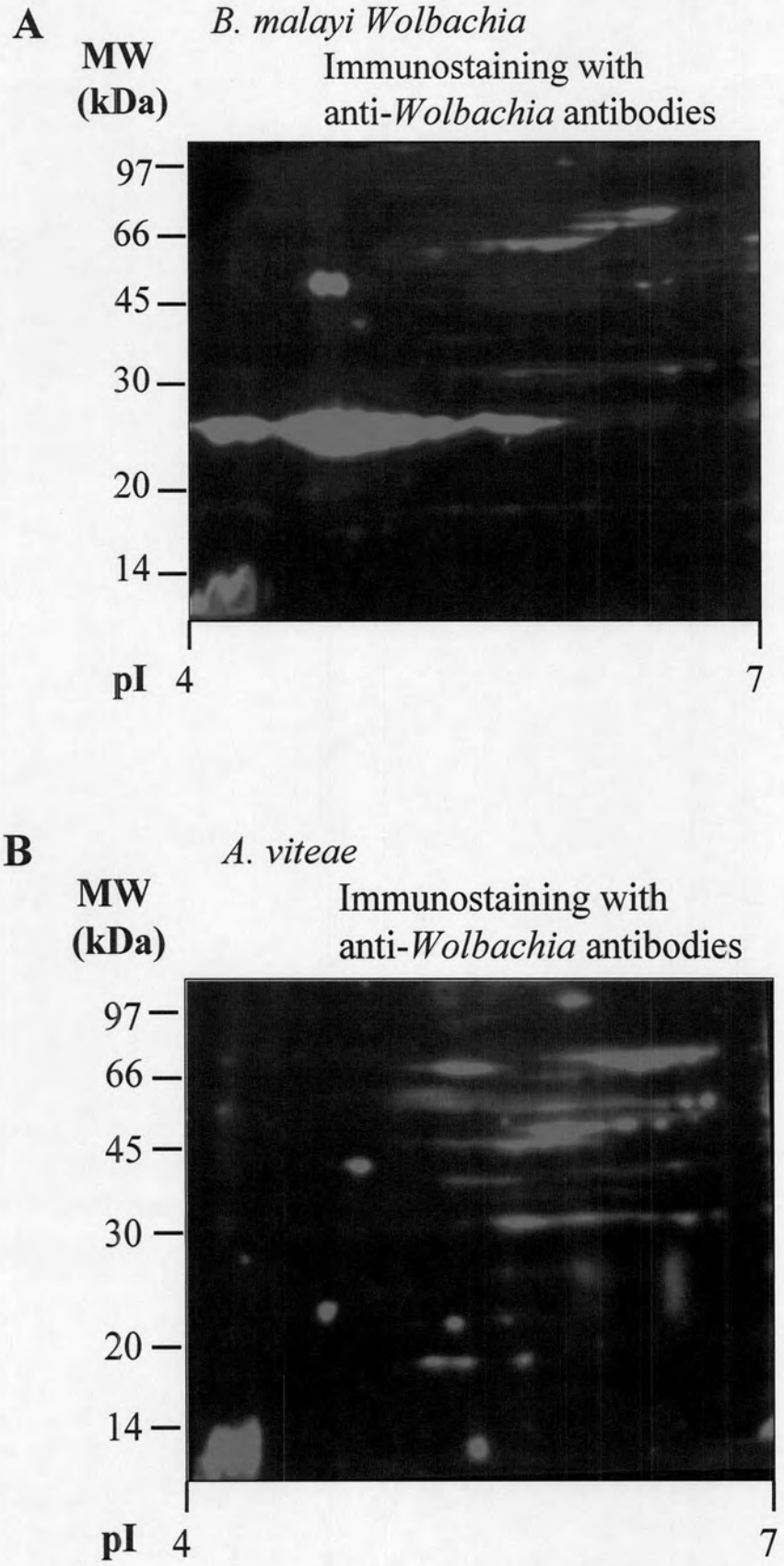
In addition, when compared with the results of antigens specific for *Wolbachia* of *D. immitis* (**Figure 22**), common reactive antigens to both *Wolbachia* of *B. malayi* and *D. immitis* would be identified with MW of 26/25.6, 20, and 17 kDa.

2.2 Western blot analysis of *Brugia malayi Wolbachia* and 2D-PAGE of *Wolbachia* specific proteins on IPG range 4-7

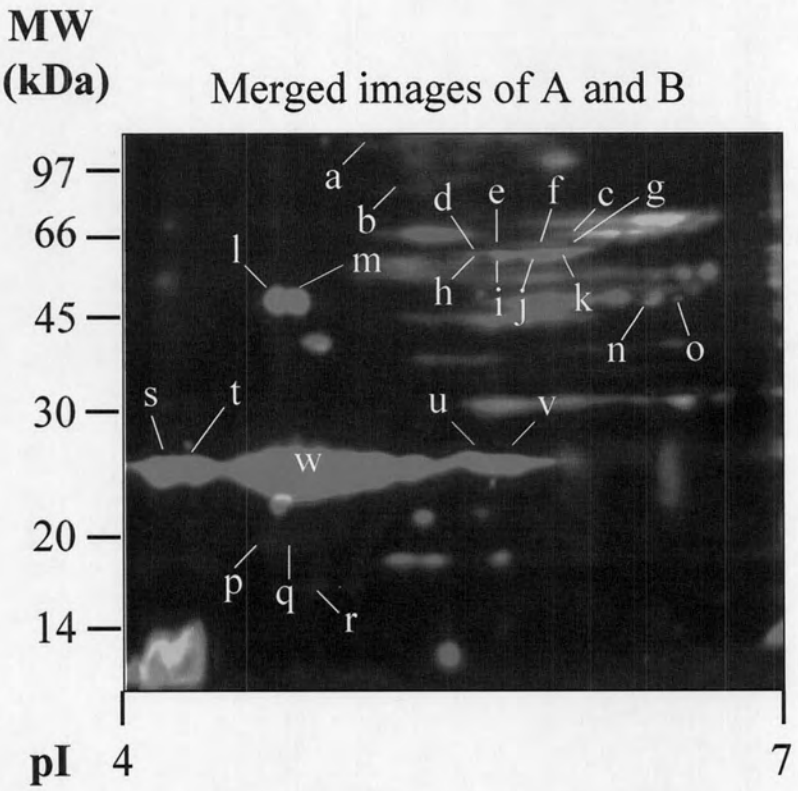
The approach could be applied to study proteins of *Wolbachia* of *B. malayi* separated by 2D-PAGE (Porksakorn *et al.*, 2003). Protein extracts were separated by 2-DE, and proteome profiles of *B. malayi Wolbachia* were visualized either by Western blot analysis by anti-*Wolbachia* antibodies, or Coomassie-blue staining. The resolution of separated proteins was achieved by using IPG range 4-7 (**Figure 24**).

On IPG range 4-7, the 2D-immunoblot results of *Wolbachia* of *B. malayi* (**Figure 24A**) were compared with the immunoreactive profiles of *A. viteae* (**Figure 24B**). The comparable reactive patterns of *A. viteae* were as a subset of the reactive profiles of *B. malayi Wolbachia*. By merging of images A and B, a number of recognized spots on *B. malayi Wolbachia* blots were, therefore, subtracted with the reactive spots of *A. viteae*, and led to the identification of antigens specific for *Wolbachia* as indicated (**Figure 24C**). We could detect totally 23 reactive spots, including the most highly reactive spots (spots s, t, w, u and v) located at pI range 4-6. An additional 13 strong (spots c-o), and 5 weak spots (spots a, b, p, q and r) were detected at the pI range 4.7-6.3.

By comparing the Coomassie blue-stained 2-DE gels with the respective immunoblots, 16 protein spots expressed by *Wolbachia* of *B. malayi* were mapped (**Figure 24D**). The spots were distributed over the pI range of 4.7-6.3, and the MW range of 20-62 kDa.


A
B. malayi Wolbachia
MW
(kDa)
Immunostaining with
anti-Wolbachia antibodies
97
66
45
30
20
14
pI 4
7
B
A. viteae
MW
(kDa)
Immunostaining with
anti-Wolbachia antibodies
97
66
45
30
20
14
pI 4
7

C


MW
(kDa)
Merged images of A and B
97
66
45
30
20
14
a
b
d
e
f
c
g
l
m
h
i
j
k
n
o
s
t
u
v
w
p
q
r
pI 4
7

D

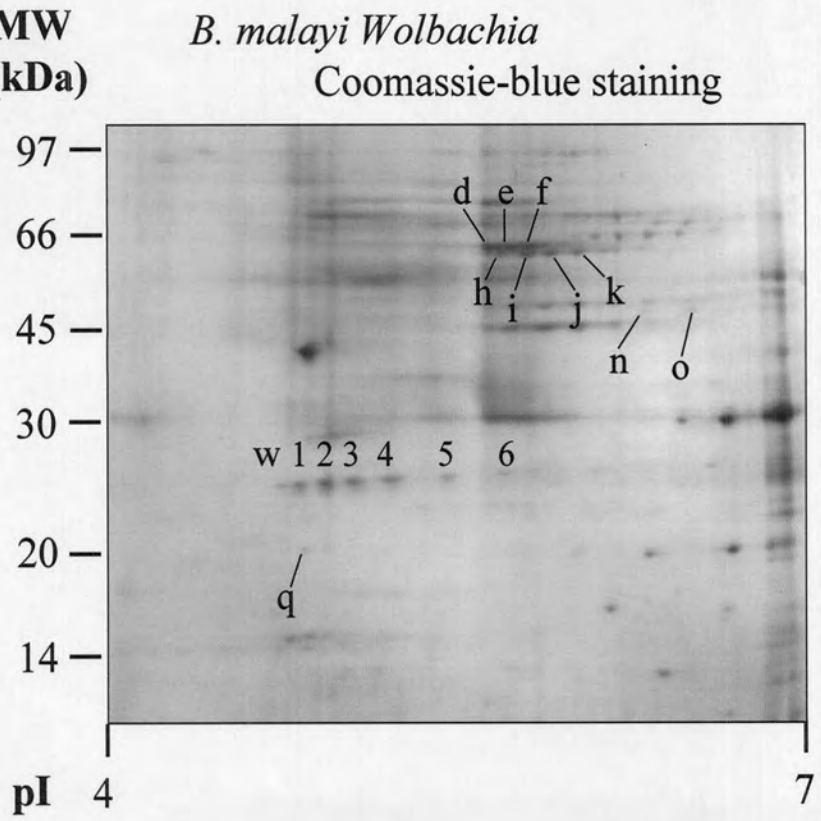
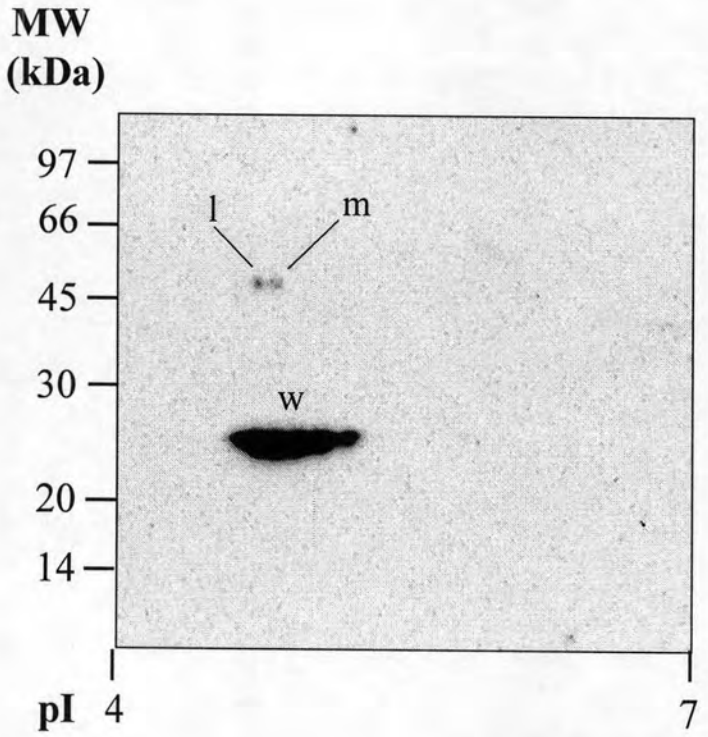

MW
(kDa)
B. malayi Wolbachia
Coomassie-blue staining
97
66
45
30
20
14
d
e
f
h
i
j
k
n
o
w 1 2 3 4 5 6
q
pI 4
7

Figure 24 Western blot analysis and 2D-PAGE of *Wolbachia* of *Brugia malayi* (IPG range 4-7). The *Wolbachia* enriched extracts of *B. malayi* (A and D), and control extracts of *Acanthocheilonema viteae* (B) were separated by 2-DE. The 2D gels were visualized by immunostaining with anti-*Wolbachia* antibodies (A and B) or Coomassie-blue staining (D). Mapping of antigens specific for *Wolbachia* of *B. malayi* was performed by merging the images of the *B. malayi Wolbachia*, and *A. viteae* blots (C). The *Wolbachia* antigenic spots are indicated, and identified on the corresponding Coomassie-blue stained gel.

2.3 Identification of the major antigenic spots l, m, and w as WSP by anti-rWSP antibodies

The major antigenic w spots with MW of 26 kDa, and pI range over 4.5-5.5 on the 2D-blots of *Wolbachia* of *B. malayi* immunostained with anti-*Wolbachia* antibodies were identified as WSP by specific reactivity to anti-rWSP antibodies (**Figure 25A**). In addition to the 26-kDa WSP spots, the reactive 51-kDa spots with pI of 4.7 (spot l) and 4.8 (spot m) were identified by anti-rWSP antibodies as WSP. The w1-6 spots on the Coomassie-blue stained gels of *Wolbachia* of *B. malayi* were identified as WSP by another immunoblotting with anti-rWSP antibodies (**Figure 25B**).

A


MW
(kDa)
97
66
45
30
20
14
l
m
w
pI 4
7

B

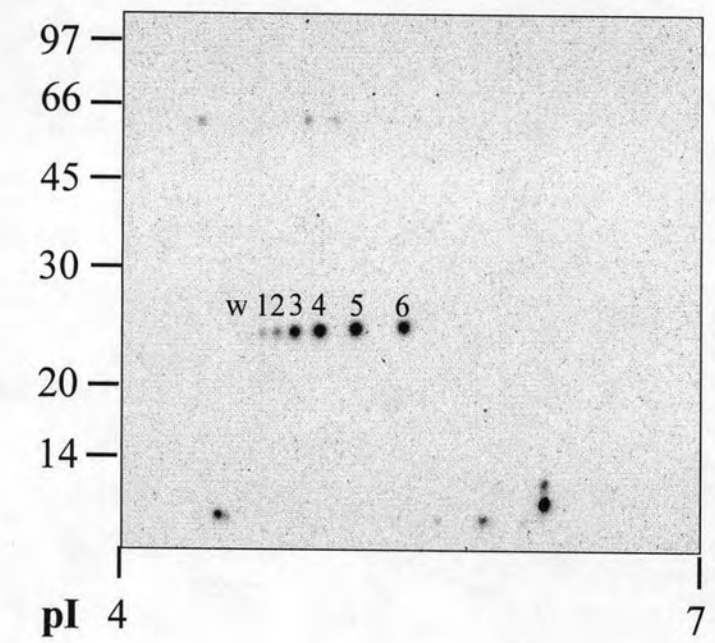

MW
(kDa)
97
66
45
30
20
14
w 1 2 3 4 5 6
pI 4
7

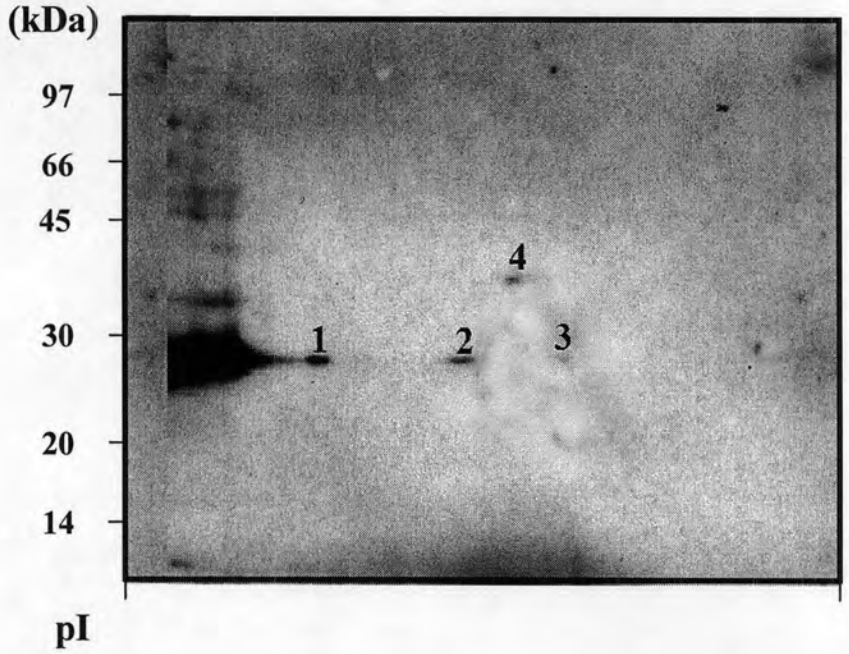
Figure 25 Identification of high antigenic spots as WSP from 2D-blots of *Brugia malayi Wolbachia*, by using anti-rWSP antibodies. The identical 2-DE gels of *Wolbachia* enriched extracts of *B. malayi* were immunostained by anti-rWSP antibodies. The major l, m, and w antigenic spots of *Wolbachia* of *B. malayi* were identified as WSP by specific reactivity with the antibodies (A). Another immunoblotting with anti-rWSP antibodies (B) specified spots w 1-6 on the Coomassie-blue stained gels as WSP.

2.4 Western blot analysis of *Brugia malayi Wolbachia* and 2D-PAGE of *Wolbachia* specific proteins on IPG range 6-11

On the IPG range 6-11, the Western blot analysis showed 4 spots specific for *Wolbachia* of *B. malayi* on the blot of *Wolbachia* enriched extracts (**Figure 26A**). None of proteins with pI range 6-11 of the *A. viteae* extracts was reacted with the antibodies. Based on the 2D-blot analysis, only one specific spot (spot 1) could be matched within the Coomassie-blue stained gels (**Figure 26B**).

A

B. Malayi Wolbachia
Immunostaining with
anti-*Wolbachia* antibodies



B

B. Malayi Wolbachia
Coomassie-blue staining

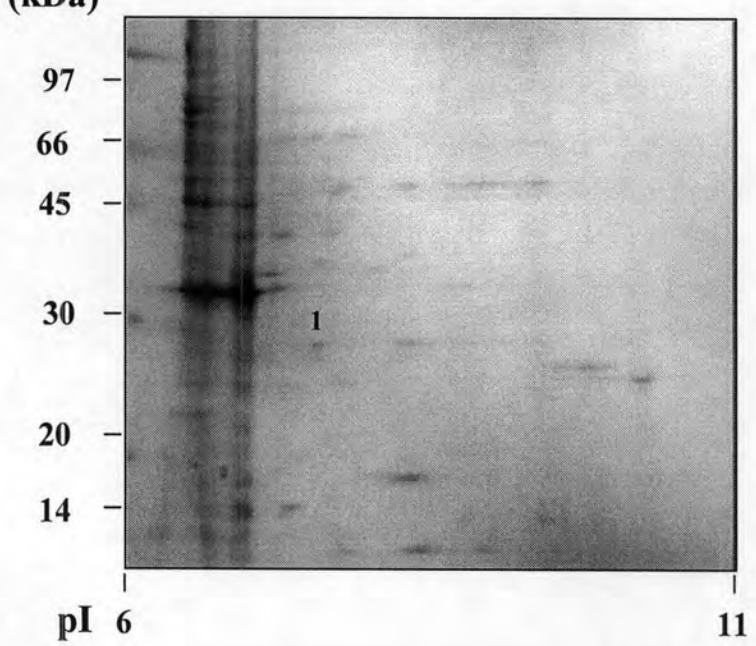


Figure 26 Western blot analysis and 2D-PAGE of *Wolbachia* of *Brugia malayi* (IPG range 6-11). The *Wolbachia* enriched extracts of *B. malayi* were separated by 2-DE; A, Western blot by anti-*Wolbachia* antibodies; B, Coomassie blue staining.

2.5 Protein identification of *Wolbachia* of *Brugia malayi* by MALDI-TOF-MS and peptide mass fingerprint searching

As we have mapped 16 *Wolbachia*-derived spots, including 6 WSP spots, on the Coomassie blue-stained 2D gels with IPG range 4-7, and one spot on the IPG range 6-11. To identify proteins, the spots were excised, digested in-gel with trypsin, and extracted tryptic peptides. After the analysis of the prepared samples by MALDI-TOF-MS, peptide mass fingerprints of protein spots were obtained and used to search against the Mascot program linked to the local *B. malayi Wolbachia* protein databases. However, the databases did not contain fully deduced protein sequences of the *Wolbachia* genome because sequencing project of the *Wolbachia* genome was ongoing at that time. Evidences for interpretation are concordance between observed MW/pI of the analyzed protein and matched protein in the databases with a significant score (Mascot score ≥ 40).

The results of peptide mass fingerprint searching against the *Wolbachia* protein databases are shown in **Table 8**. Spots w2 and w3 were successfully identified as WSP of *B. malayi Wolbachia* by the peptide mass fingerprint searching. Therefore, 2 isoforms out of 6 WSP isoforms identified by anti-rWSP antibodies were confirmed by MALDI-TOF-MS analysis.

Table 8 Protein identification of spots specific for *Wolbachia* of *Brugia malayi* by MALDI-TOF-MS and peptide mass fingerprint searching.

Spots	Observed MW (kDa)/pI	Identified proteins	Calculated MW (kDa)/pI	Matched peptides (n)	Coverage (%)	Scores
w1	26/4.7	ribosomal protein L29 (rpmC)	8.0/10.1	2	20	24
w2	26/4.8	Surface protein	26.1/4.9	4	25	40*
w3	26/4.9	Surface protein	26.1/4.9	5	22	48*
w4	26/5.1	Surface protein	26.1/4.9	3	17	32
w5	26/5.3	Surface protein	26.1/4.9	9	31	31
w6	26/5.5	NADH dehydrogenase I chain D	44.6/5.5	11	23	29
d	62/5.4	Transposase, IS5,	7.3/9.6	4	54	18
e	62/5.5	family OrfB	12.4/10.2	4	16	10
f	62/5.6	Ribosomal protein S16	48.9/7.1	19	37	26
h	58/5.5	Adenylosuccinate lyase	12.7/10.4	10	61	15
i	58/5.6	Ribosomal protein L22	12.6/10.2	9	48	14
j	58/5.7	Ribosomal protein L14	11.2/10.2	13	71	19
k	58/5.8	Ribosomal protein L23	7.9/8.0	6	55	26
n	48/6.1	Ribosomal protein L31 Transcriptional regulator, putative	10.8/10.2	2	17	23
o	48/6.3	Hypothetical protein	15.9/9.4	4	27	16
q	20/4.7	Hypothetical protein	13.1/4.8	1	36	17
l	27/7.3	Heme exporter protein CcmB	24.1/6.3	2	34	15

*a significant score

We did not found any significant matches in the database for the remaining spots. Spots d, e, f, and 1 yielded medium mass spectra while spots h, i, j, k, n, o, q, w1, w4-6 yielded weak mass spectra. Therefore, the main reason could be either there were no such proteins in the *Wolbachia* databases, or we did not achieve suitable mass spectra to yield unambiguous identification.

The experimental peptide mass fingerprints of spot w2 and w3 were matched significantly with theoretical peptide mass fingerprints of WSP in the *Wolbachia* databases. Detailed results of the protein identification by MALDI-TOF-MS analysis of spots w2 and w3 are shown in **Figure 27** and **Table 9**.

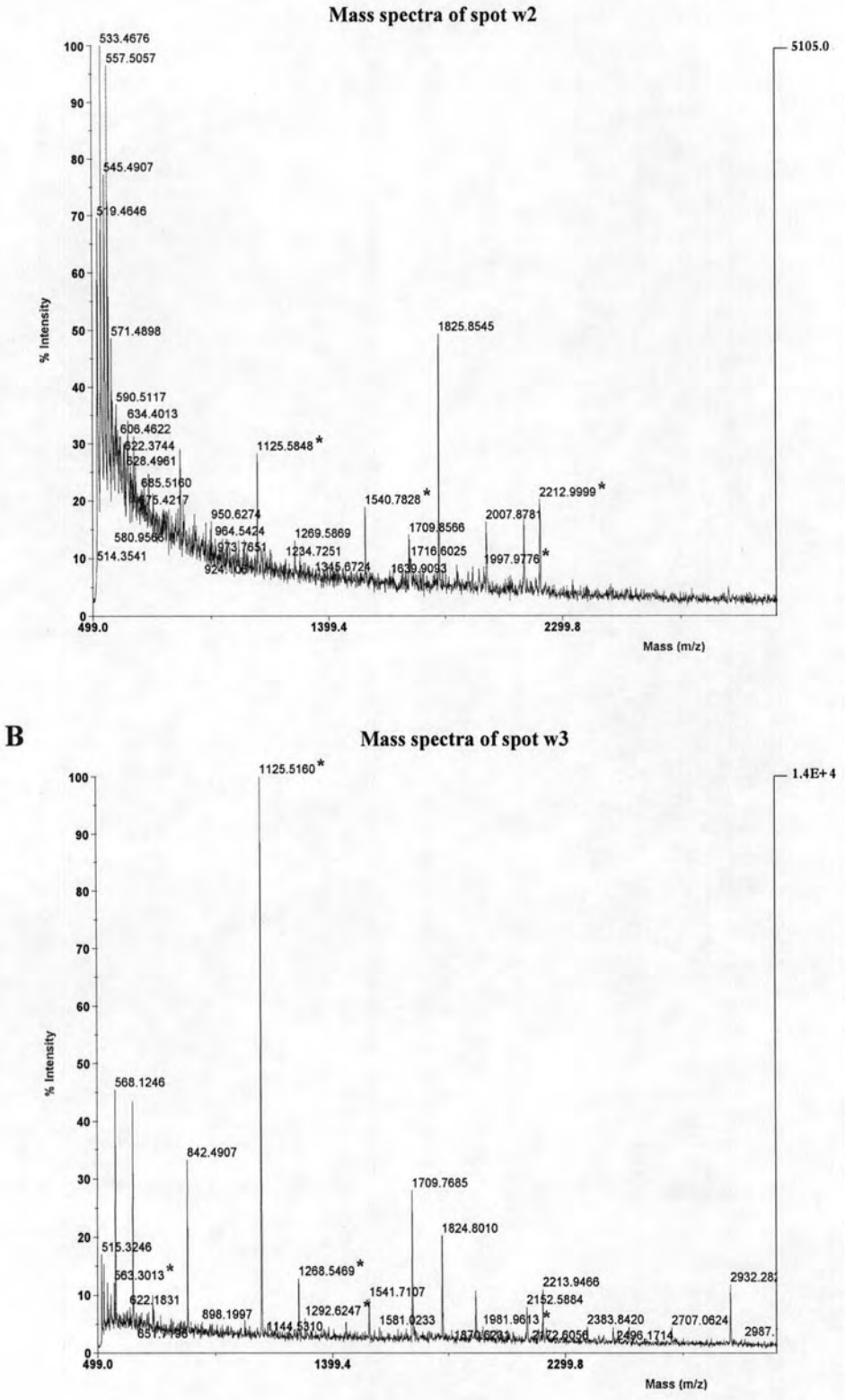


Figure 27 MALDI-TOF spectra of *Wolbachia* surface protein (WSP) isoforms of *Brugia malayi Wolbachia*. The measured masses matched obtained from w2 (A) or w3 (B) were asterisked.

Table 9 Peptide mass data of *Wolbachia* surface protein (WSP) isoforms of *Brugia malayi Wolbachia*. The measure masses matched obtained from spot w2 (A) or w3 (B) were shown.

A

Residue numbers	Measured mass	Calculated mass	Database sequence
1	1125.58	1124.54	HGFGFAYQAK
2	1540.78	1539.76	LQYNSEFSPLNTK
3	1997.98	1996.98	MDDIRVDIEGLYSQLSK
4	2213.00	2211.99	DTNDLYKPSFMAGGSAFGYR

B

Residue numbers	Measured mass	Calculated mass	Database sequence
1	563.30	562.31	STLSR
2	1125.52	1124.54	HGFGFAYQAK
3	1292.62	1291.67	AGISYDVTPEIK
4	1268.55	1267.55	YFGSYGANFDK
5	1981.96	1980.98	MDDIRVDIEGLYSQLSK

2.6 Preliminary data of phosphorylation on WSP

Interestingly, WSP exhibited difference in pI values. Since WSP is encoded by the single copy gene, post-translational modifications may associate with the observed pI heterogeneity of WSP (Braig *et al.*, 1998). Protein phosphorylation is one of the most abundant post-translational modifications, and a key biological process regulating a great number of essential biochemical reactions in living organisms, including prokaryotes (Stock *et al.*, 2000; Kennelly 2002; D'Ambrosio *et al.*, 2006). Identification of WSP phosphorylation would purpose a novel biological function of WSP. To identify phosphorylated WSP, the identical 2-DE gels of *Wolbachia* enriched extracts of *B. malayi* were probed by anti-phosphorylated protein antibodies (**Figure 28**). Interestingly, WSP spots 1-6 were detected by the antibodies.

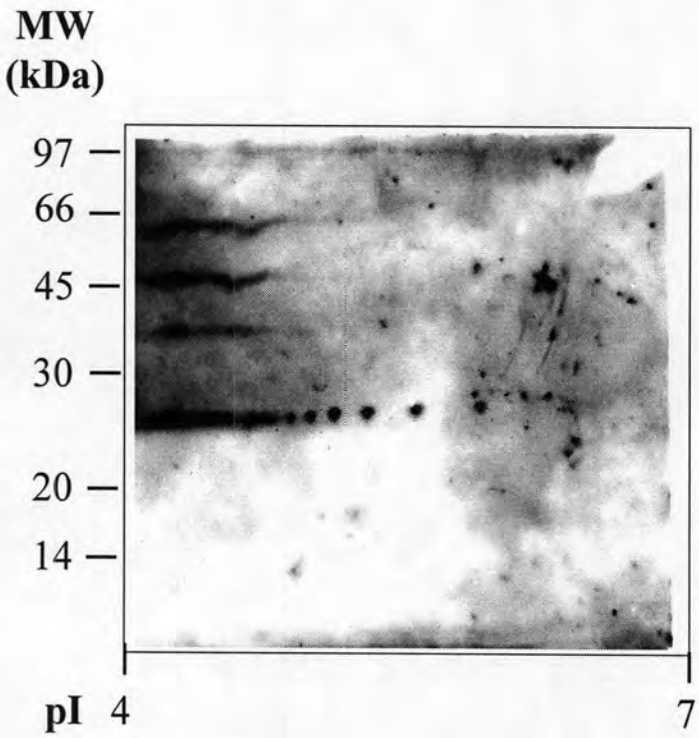


Figure 28 Western blot analysis of *Wolbachia* of *Brugia malayi* by anti-phosphorylated protein antibodies.

To confirm the existing phosphorylation, we observed pI shifts of WSP containing samples treated by phosphatases as compared with untreated WSP samples. If WSP is phosphorylated, dephosphorylated WSP should shift in their pI. A protocol for protein dephosphorylation has been tried, and subsequently we could set-up a suitable condition. Due to limitation of *Wolbachia* enriched extract samples, we used crude *B. malayi* extracts as a WSP containing sample. Both untreated and treated *B. malayi* extracts were separated by 2D-PAGE and immunostained by anti-rWSP antibodies (**Figure 29**). In untreated *B. malayi* extracts, 2 WSP spots were detected as of the treated samples. However, separating resolution was not proper for the 2-DE gels. It seems not clear that there were pI shifts of WSP (arrow).

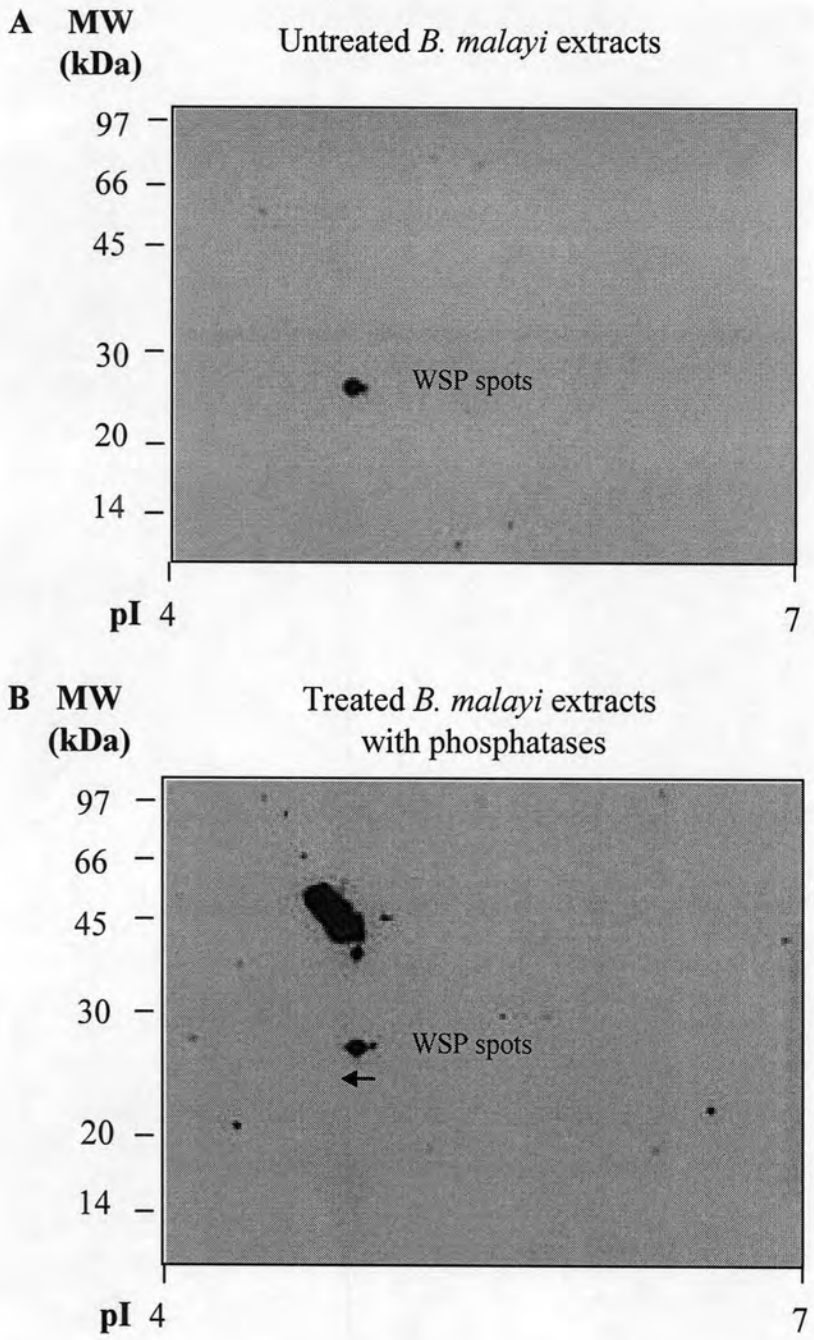


Figure 29 Effect of calf intestinal alkaline phosphatase treatment on pI shift of WSP from *Brugia malayi Wolbachia*. *Brugia malayi* extracts were not treated (A), or treated (B) with calf intestinal alkaline phosphatase, and proteins were then analyzed by Western blot with anti-rWSP antibodies.

WSP may be phosphorylated by the evidence from anti-phosphorylated protein antibodies. However, further confirmation by, such as phosphoprotein specific stains, MALDI-TOF-MS and phosphatase treatment, and tandem MS, are required.

Characterization of proinflammatory activity of WSP to murine macrophages

1. Characteristics of WSP of *Brugia malayi Wolbachia*

The WSP was selected as a candidate *Wolbachia* protein that could be an inflammatory molecule for the murine macrophage RAW 264.7 cells. WSP of *Wolbachia* from *B. malayi* shares conserved regions with that of *Wolbachia* from other filarial parasites, and with outer membrane proteins of the closely related bacteria. Furthermore, the WSP appeared to be a major protein antigen in our protein analysis. The *wsp* gene was cloned, and induced to express as a recombinant protein in *Escherichia coli* cells, and purified rWSP protein was used to stimulate the murine macrophages.

1.1 Deduced amino acid sequence of *wsp* gene

The deduced amino acid sequence of *wsp* gene of *Wolbachia* of *B. malayi* was retrieved from the databases (GenBank, EMBL, SWISS-PROT, PDB, and dbEST) distributed by NCBI. The accession number of *wsp* gene and WSP sequence are AJ252061 and CAB95872, respectively. The predicted mass of WSP protein was 26.02 kDa with the pI of 4.89 (**Table 10**).

Table 10 Predicted characteristics of WSP of *Brugia malayi Wolbachia*.

Analysis	WSP
Length	240 aa
Molecular weight	26016.85 Da
Isoelectric point (pI)	4.89

Whole protein composition analysis

Amino acid (s)	% residue composition
A Ala	10.0
C Cys	0.0
D Asp	7.1
E Glu	4.6
F Phe	5.4
G Gly	8.8
H His	0.8
I Ile	6.7
K Lys	6.7
L Leu	6.2
M Met	1.7
N Asn	4.2
P Pro	4.2
Q Gln	2.5
R Arg	2.1
S Ser	9.2
T Thr	5.8
V Val	7.1
W Trp	0
Y Tyr	7.1

1.2 Predicted antigenic peptides of WSP

The hydrophilicity, surface probability, and antigenicity algorithms were used to predict WSP antigenic determinants. With considering sequences that were upward on all three graphs (**Figure 30B**), two antigenic regions in the peptide were identified (**Figure 30A**). One sequence was at the N-terminus (amino acids 59-72, 14 aa); the other was at the C-terminus (amino acids 201-218, 18 aa).

In addition, BLAST sequence homology was performed for both antigenic peptides to determine their specificity for WSP protein. As expected, the homology was found in other filarial nematode *Wolbachia*. The one at the N-terminus showed significant homology to the surface protein of *Wolbachia* in *B. pahangi* (Identities = 100%), *W. bancrofti* (Identities = 100%), and *Litomosoides sigmodontis* (Identities = 100%). The other peptide (C-terminus) also showed significant homology to the surface protein of *Wolbachia* in *B. pahangi* (Identities = 100%) and *W. bancrofti* (Identities = 100%).

A

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1   ATG CAT TAT AAA AAG TTT TTT TCA GCA ACC GCT TTA GTA ATG TTG   45
1    M   H   Y   K   K   F   F   S   A   T   A   L   V   M   L    15
46  CTA AGT TTA TCA AAC TCT GCT TTT TCA GAT CCT GTT GGT CCA ATA   90
16   L   S   L   S   N   S   A   F   S   D   P   V   G   P   I    30
91  GCT GAT GAG GAA ACT AGT TAC TAC ATT CGC TTG CAG TAC AAT AGT  135
31   A   D   E   E   T   S   Y   Y   I   R   L   Q   Y   N   S    45
136 GAG TTT TCA CCT TTG AAT ACA AAG GTT GAT GGT ATC ACA GGA GCT  180
46   E   F   S   P   L   N   T   K   V   D   G   I   T   G   A    60
181 CAA AAG GAT AGT AAA GAC ACT AAT GAC CTT TAT AAG CCT TCT TTC  225
61   Q   K   D   S   K   D   T   N   D   L   Y   K   P   S   F    75
226 ATG GCT GGT GGT AGT GCA TTT GGT TAT AGA ATG GAT GAT ATC AGA  270
76   M   A   G   G   S   A   F   G   Y   R   M   D   D   I   R    90
271 GTG GAC ATT GAA GGA CTT TAT TCA CAA TTA AGT AAA AGT ACT CTT  315
91   V   D   I   E   G   L   Y   S   Q   L   S   K   S   T   L   105
316 TCA CGA GCT CCT ACT CCA GAT ATT GTA GAT AAT TTA ACA GCA ATT  360
106  S   R   A   P   T   P   D   I   V   D   N   L   T   A   I   120
361 TCA GGA CTA GTT AAT GTG TAT TAT GAT GTA GTA ATT GAA GAT ATA  405
121  S   G   L   V   N   V   Y   Y   D   V   V   I   E   D   I   135
406 CCT ATT ACT CCA TAT GTT GGT GTT GGT CTT GGT GTA GCA TAT ATC  450
136  P   I   T   P   Y   V   G   V   G   L   G   V   A   Y   I   150
451 AGC AAC CCT GCA AAG GCA CAA GTT ATT GCT GAT CAA AAA CAT GGG  495
151  S   N   P   A   K   A   Q   V   I   A   D   Q   K   H   G   165
496 TTT GGT TTT GCT TAC CAG GCG AAA GCT GGT ATT AGC TAT GAT GTA  540
166  F   G   F   A   Y   Q   A   K   A   G   I   S   Y   D   V   180
541 ACC CCA GAA ATT AAA CTC TTT GCT GGA GCT CGC TAC TTT GGT TCT  585
181  T   P   E   I   K   L   F   A   G   A   R   Y   F   G   S   195
586 TAT GGC GCT AAC TTT GAT AAA AGT GAA GAA GTA AAT AAA GGA ACT  630
196  Y   G   A   N   F   D   K   S   E   E   V   N   K   G   T   210
631 AGT GAG GAT AAA GAA ACA AAA GTT ACT GCG GGT GCA TAT AAA GTT  675
211  S   E   D   K   E   T   K   V   T   A   G   A   Y   K   V   225
676 CTT TAT AGC ACT ATT GGT GCA GAA GCT GGA ATA GCG TTT AAT TTC  720
226  L   Y   S   T   I   G   A   E   A   G   I   A   F   N   F   240
721 TAA                                                        723
241  *
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B

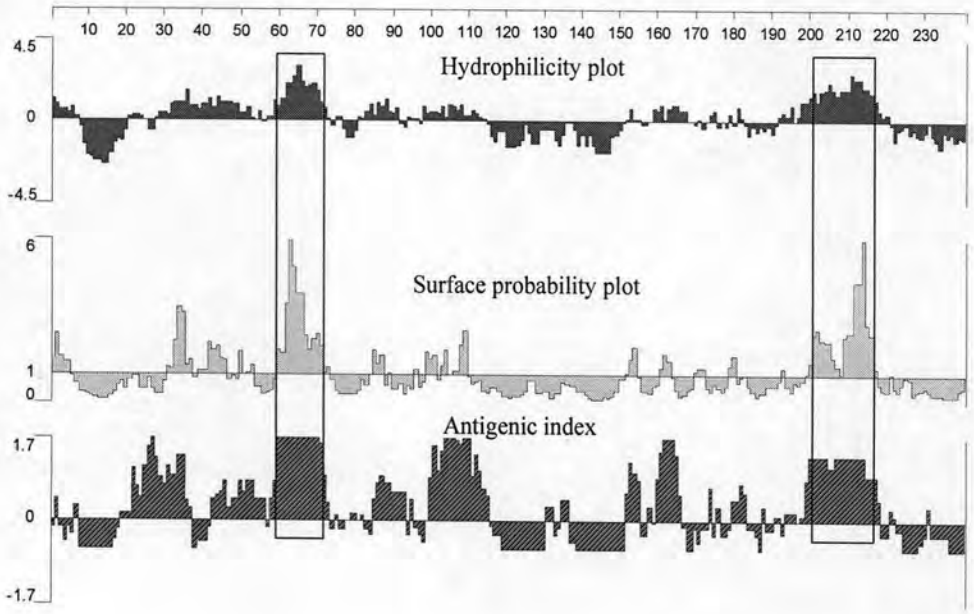


Figure 30 Predicted antigenic peptides of thr WSP of *Brugia malayi Wolbachia*. Deduced amino acid sequence of *wsp* gene of *Brugia malayi Wolbachia* and two predicted antigenic regions (underline) (A), and hydrophilicity, surface probability, and antigenicity plots of WSP (B).

2. Production of rWSP fusion protein

2.1 Calculated molecular mass of rWSP under expression in pET100/D-TOPO vector

The calculated mass of rWSP was 27.60 kDa with the pI of 5.00 (**Table 11**).

Table 11 Predicted characteristics of the rWSP fusion protein derived from *Brugia malayi Wolbachia*.

Analysis	WSP
Length	253 aa
Molecular weight	27598.31 Da
Isoelectric point (pI)	5.00

2.2 Cloning of *wsp* gene of *Wolbachia* from *Brugia malayi*

The 658-bp DNA fragment containing *wsp* gene minus its N-terminal signal sequence was amplified by PCR from crude *B. malayi* DNA extracts (**Figure 31**). The PCR products were directionally cloned into the pET100/D-TOPO expression vector.

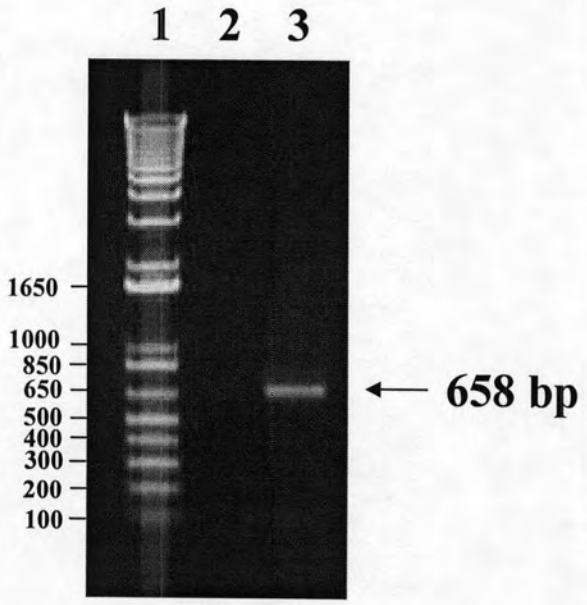


Figure 31 PCR amplification of *wsp* gene derived from *Brugia malayi Wolbachia*; Lane 1, DNA ladder. Lane 2, negative control; Lane 3, crude *B. malayi* DNA extracts as template. PCR products were run onto 1% agarose gel.

Positive transformants growing on Luria-Bertani plates containing amplicilin were analyzed by PCR (**Figure 32**). Transformants containing inserts (clones 8, 11, and 12) were identified. The plasmids containing inserts with correct orientation were confirmed by sequencing analysis technique.

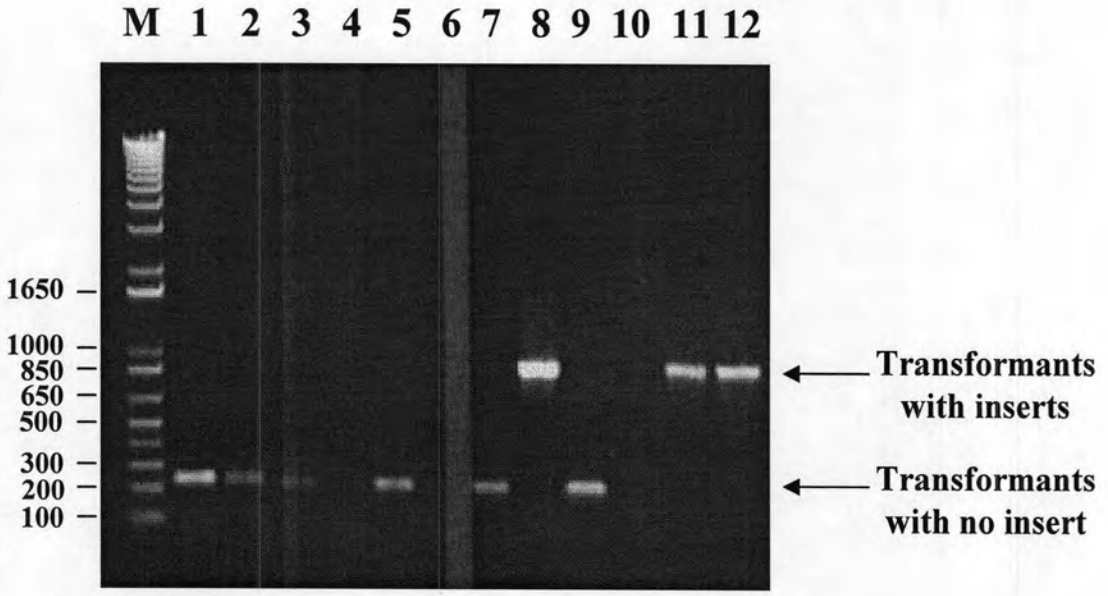


Figure 32 Analysis of the rWSP pET 100/D-TOPO expression vector by PCR; Lane M, DNA ladder; Lane 1-12, plasmid templates extracted from colonies going on Luria-Bertani plates containing amplicilin. PCR products were run onto 1% agarose gel.

2.3 Expression of rWSP fusion protein

Expression of rWSP fusion protein was performed by induction with 1 mM IPTG (**Figure 33**). After induction for 1h or 4h, *E. coli* BL21 Star (DE3) cells were harvested to analyze the expression profile by SDS-PAGE. At 1-h induction, rWSP was abundantly expressed as much as at the 4-h induction. To determine whether rWSP was expressed as a soluble, insoluble, or secreted form to culturing medium, we analyzed a protein profile of medium, prepared pellet, and supernatant as described in Materials and Methods by SDS-PAGE. rWSP fusion protein was expressed as a protein aggregate abundant in the precipitates. An approximated wet weight of the pellet was 6 mg/ml of cell culture with an expression yield of rWSP 30-40%.

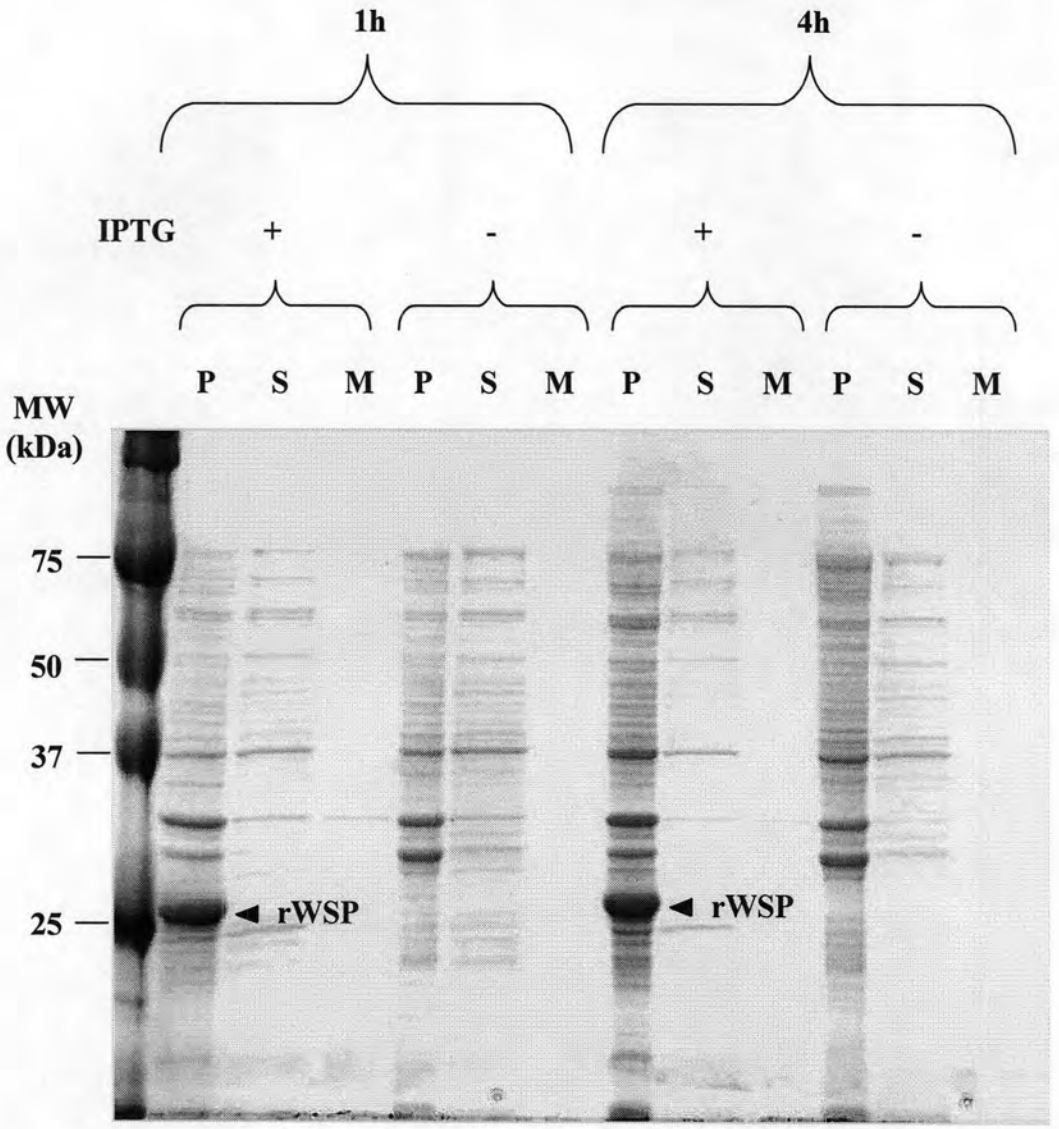


Figure 33 SDS-PAGE profile of rWSP derived from *Brugia malayi Wolbachia* expressed in *Escherichia coli* BL21 (DE3) cells. The rWSP expression was induced with 1 mM IPTG for 1 h, or 4 h; P, precipitated pellet; S, supernatant; and M, culturing medium were collected to be analyzed by SDS-PAGE.

2.4 Purification of rWSP fusion protein

The initial purification step for the recombinant protein was to extract the highly aggregated protein, inclusion bodies, from *E. coli* cell lysates by treatment of B-PER bacteria protein extraction reagent and lysozyme (**Figure 34**). The *E. coli* contaminating components were removed from the isolated inclusion bodies (label) by washing with B-PER bacteria protein extraction reagent (Lane 2).

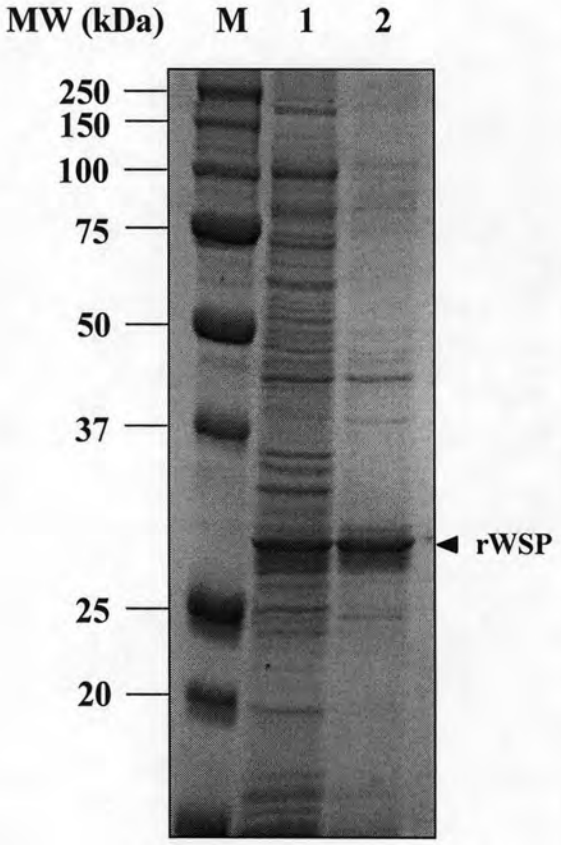


Figure 34 SDS-PAGE of purification of inclusion bodies enriched with rWSP of *Brugia malayi Wolbachia*. The inclusion bodies were purified by using B-PER bacterial protein extraction reagent: Lane M, protein standards; Lane 1, inclusion bodies from cell lysates; Lane 2, washed inclusion bodies. Samples were analyzed by SDS-PAGE, and stained with Coomassie-blue G250.

The rWSP fusion protein was further purified by an affinity chromatography with Ni-NTA resin (**Figure 35**). The purification was archived by optimization of imidazole concentrations supplemented in denaturing buffers. Low amount of imidazole in the binding buffer helped to block non-specific binding as can be found in *E. coli* contaminants with the flow-through fraction (Lane F). The washing buffer with higher concentration of imidazole was capable to remove more *E. coli* proteins (Lane W1 and W2). The purified rWSP was obtained by treatment of column with the elution buffer, and the fractions were combined to further refold.

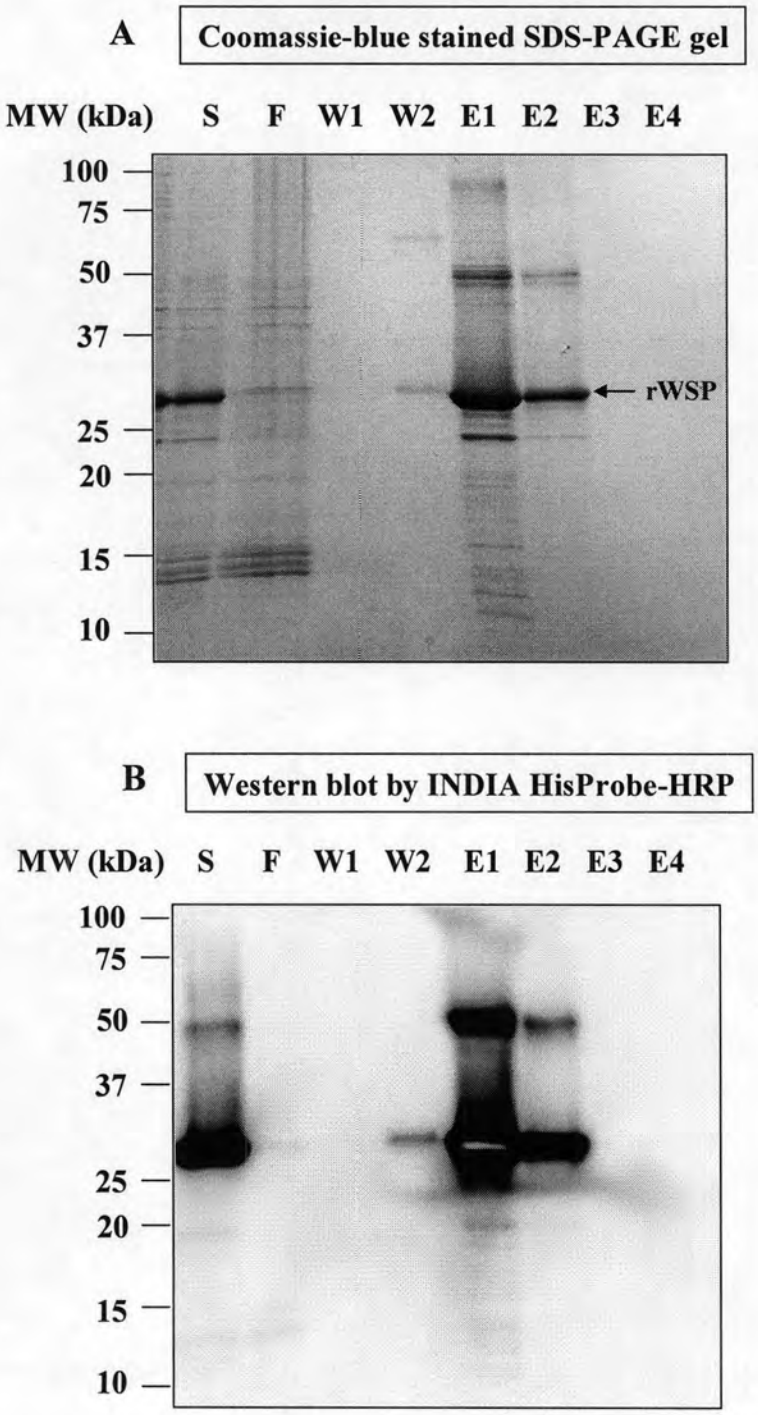


Figure 35 SDS-PAGE and Western blot analysis by INDIA HisProbe-HRP of purification of the rWSP derived from *Brugia malayi Wolbachia* by affinity chromatography with Ni-NTA resin: A, Coomassie blue stained gel of the protein profile; B, Western blot by INDIA HisProbe-HRP; S, loading sample; F, flow-through; W1, 5 mM imidazole wash; W2, 20 mM imidazole wash; E, elutes.

2.5 Purified and refolded rWSP fusion protein

Refolding of the purified rWSP fusion protein was performed by dialysis. Aggregation was protected by passing elutes through a 0.2 or 0.45 μm-nylon membrane, and starting with very low concentration of the purified rWSP. The purified and refolded rWSP was concentrated, and analyzed by SDS-PAGE and immunoblotting (**Figure 36**). The SDS-PAGE gel revealed two protein bands (**Figure 36A**). Both bands were detected by antibodies to 6x-histidine tag (**Figure 36B**) and rWSP (**Figure 36C**), suggesting that the preparation contains mature rWSP (the major band), and truncated rWSP (the minor one). The rWSP fusion protein was purified and concentrated with yield approximated 200 μg/mg of the loading sample.

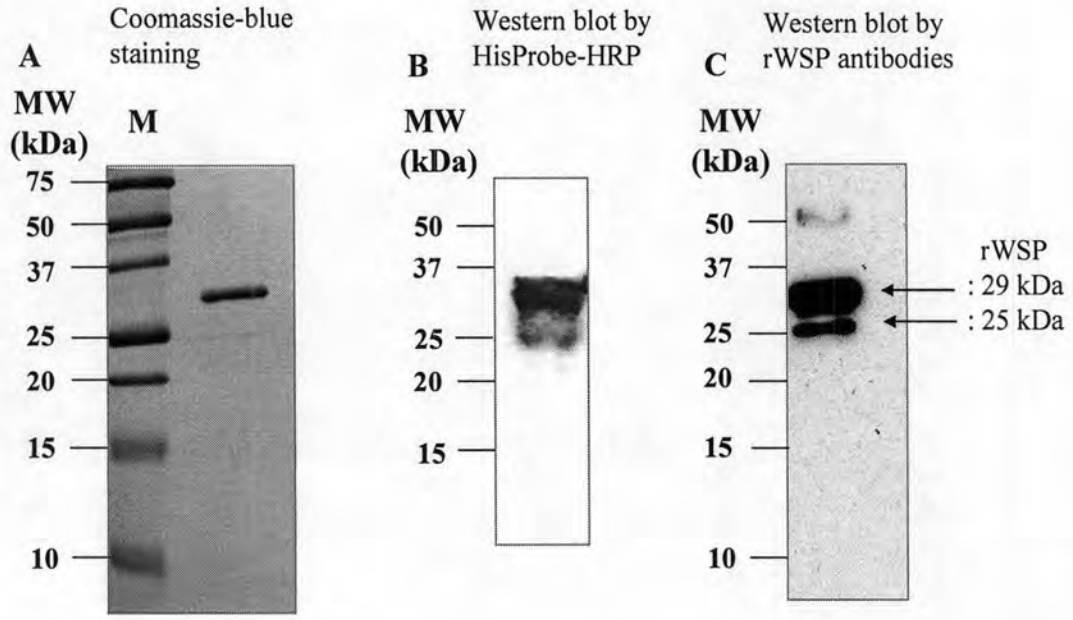


Figure 36 SDS-PAGE and Western blot analysis of the refolded rWSP of *Brugia malayi Wolbachia*: A, Coomassie-blue stained gel; B, Western blot by INDIA HisProbe-HRP; C, Western blot by anti-rWSP antibodies.

Purity of the rWSP preparation was also determined on 2D-gels with IPG range 4-7, and revealed by Coomassie-blue staining (**Figure 37**). There were only 4 major spots (pI range over 5.0-5.4) distributed on MW of 29 kDa, and the 4 protein spots were all identified as WSP by MALDI-TOF-MS (**Table 12**).

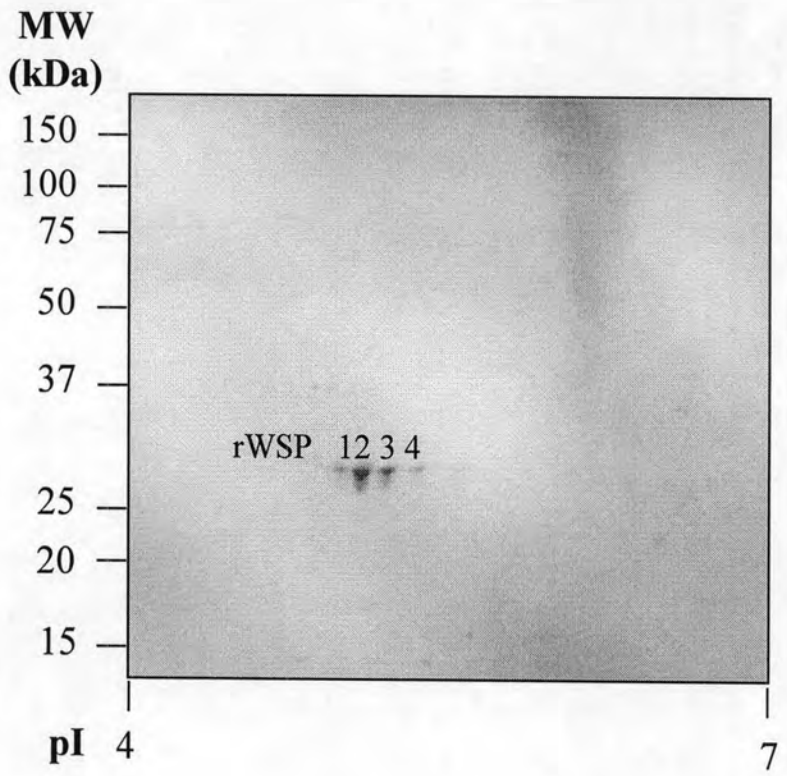


Figure 37 2-DE analysis of rWSP of *Brugia malayi Wolbachia*. The 2-DE gel was stained by Coomassie blue staining; spots rWSP 1-4 were identified as surface protein of *Wolbachia* from *B. malayi*.

Table 12 Identification of four rWSP spots of *Brugia malayi Wolbachia* by MALDI-TOF-MS and peptide mass fingerprint searching.

rWSP No.	Observed MW (kDa)/pI	Calculated MW (kDa)/pI	Matched peptides (n)	% Coverage	Significant score
1	29/5.0	27.5/5.09	8	35	67
2	28.6/5.1	27.5/5.09	12	44	139
3	28.6/5.3	27.5/5.09	12	46	134
4	29/5.4	27.5/5.09	8	39	66

MALDI-TOF-MS analysis was performed to analyze intact mass of rWSP and purity of the rWSP preparation (**Figure 38**). The intact mass of rWSP was 27.6 kDa which is corresponding to the theoretical MW of rWSP. Based on the mass analysis range from 15-70 kDa, the purity of rWSP was > 90%. The additional peak of 55.3 kDa would be a dimer of rWSP.

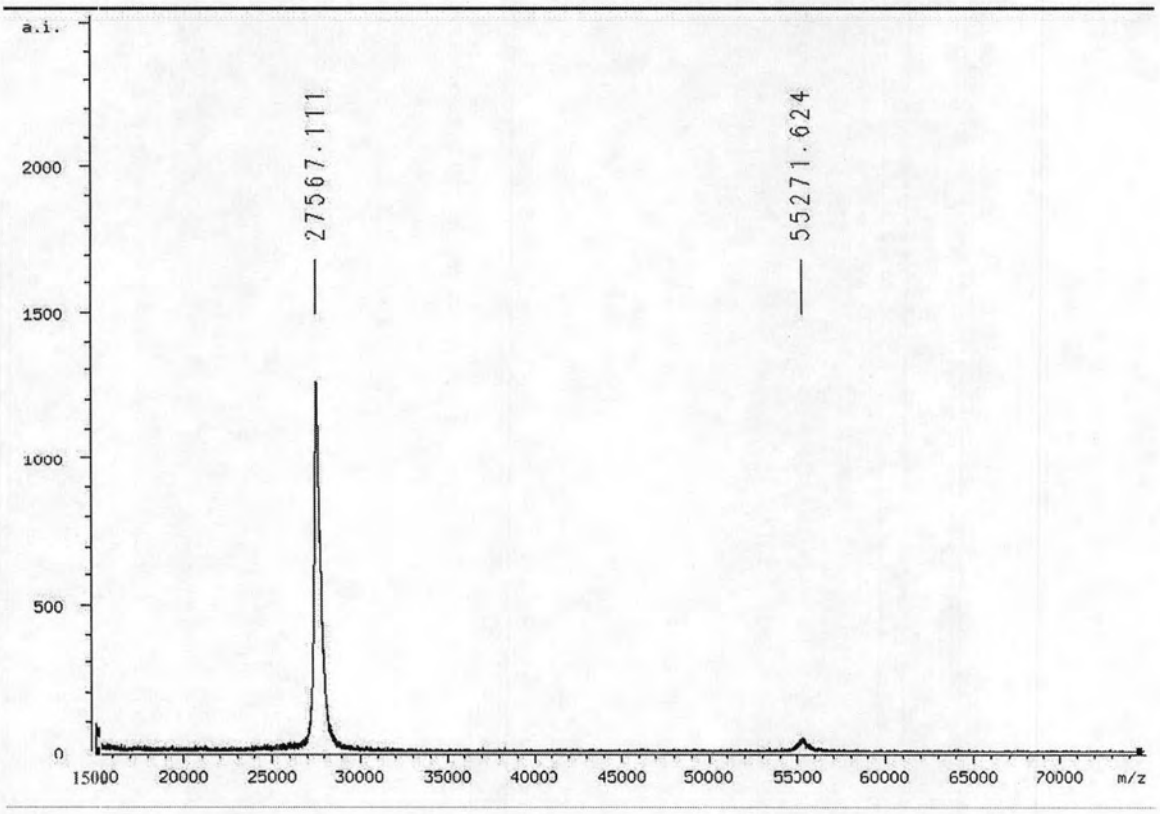


Figure 38 MALDI-TOF-MS analysis of intact purified rWSP of *Brugia malayi Wolbachia*.

2.6 Proinflammatory activity of rWSP of *Brugia malayi Wolbachia* to RAW 264.7 murine macrophage cell line

Dose response of IL-1β, IL-6, and TNF-α mRNA induction against the rWSP

The expression of IL-1β, IL-6, and TNF-α mRNA in the RAW 264.7 cells in response to the rWSP was dose-dependent (**Figure 39**) (Porksakorn *et al.*, 2004). The IL-1β transcription was upregulated (>5 folds) with as little as 1 μg/ml rWSP, in contrast to the activation of the macrophage cell line to expressed IL-6 and TNF-α mRNA that required higher rWSP concentration (3 μg/ml rWSP). When 9.0 μg/ml rWSP was supplemented to the cell cultures, IL-1β, IL-6 and TNFα mRNA expression increased from 1.9 to 295.0, 0.5 to 81.3 and 1.8 to 9.2 folds, respectively.

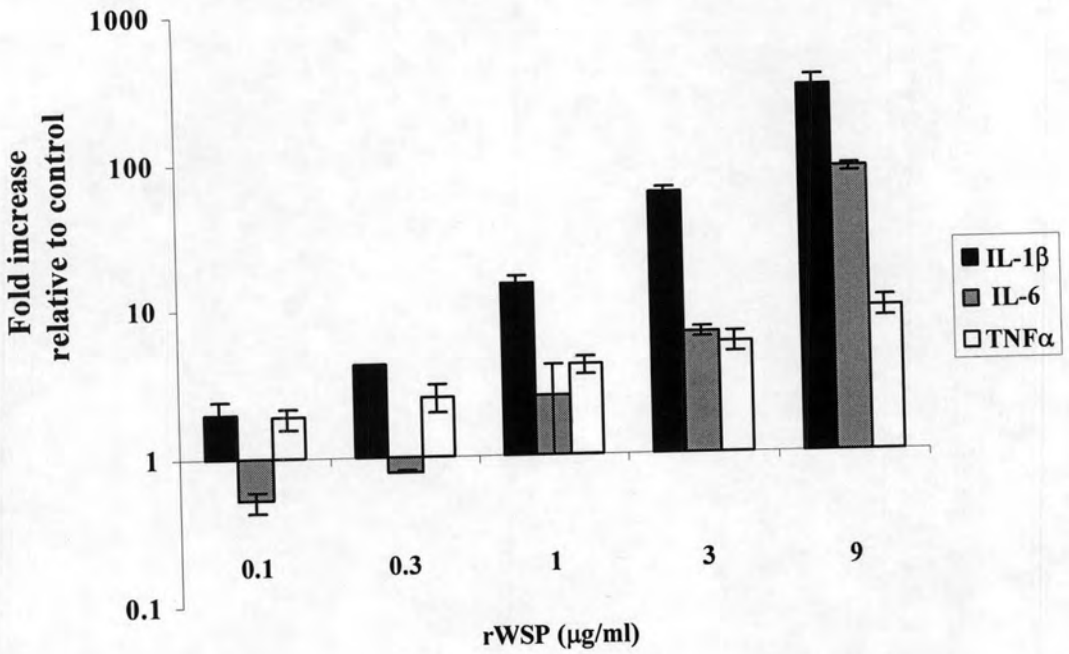
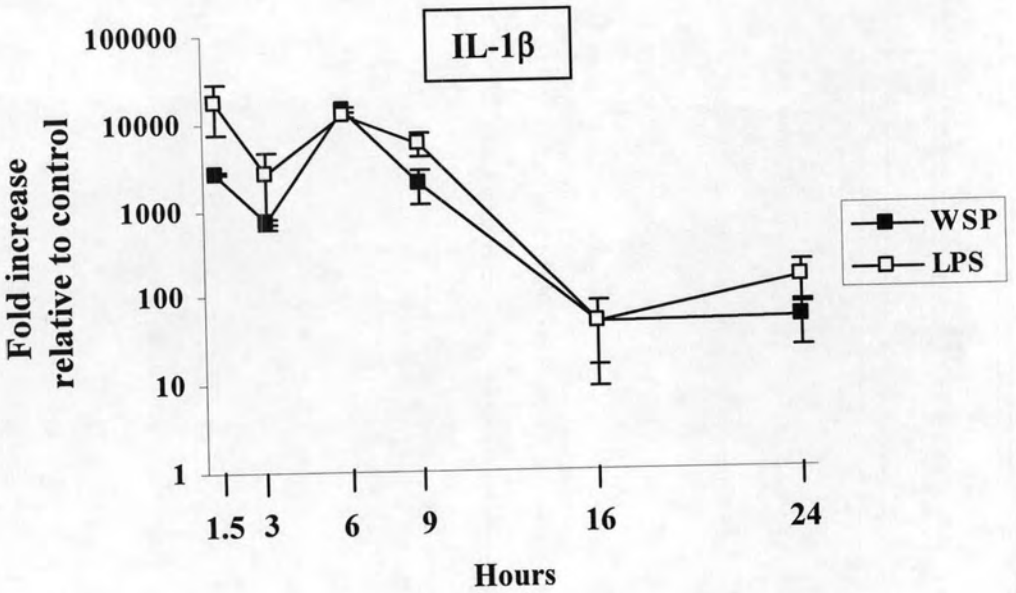
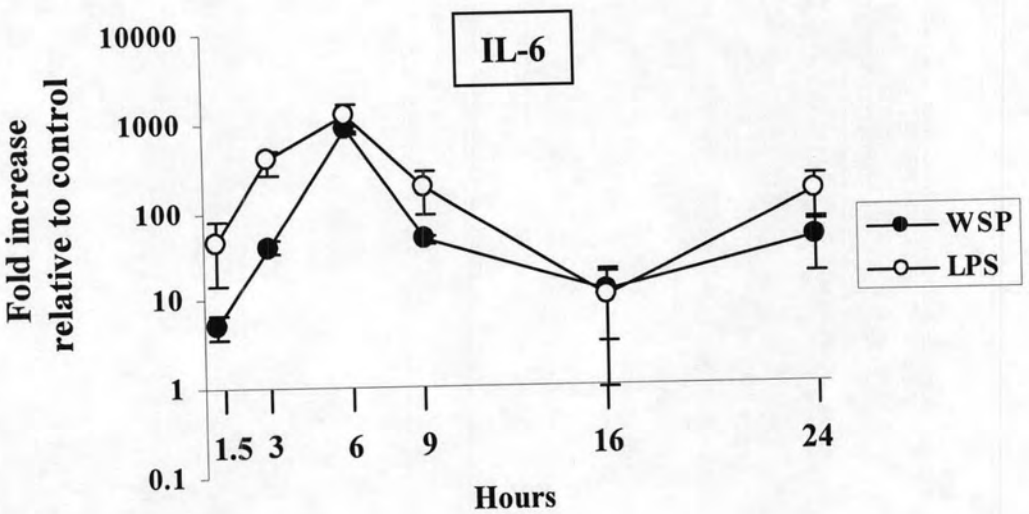


Figure 39 Dose responses of IL-1β, IL-6, and TNFα mRNA production in the RAW 264.7 macrophage cell line to rWSP of *Brugia malayi Wolbachia*. The murine macrophage RAW 264.7 cells were incubated with various concentrations of rWSP for 3 h. The data represent the mean of fold increases of cytokine transcripts relative to negative control ± SD.

Early peak of TNF-α mRNA accumulation followed by IL-1β and IL-6 mRNA peaks in response to the rWSP

To analyze the kinetics of proinflammatory responses in the RAW 264.7 cells to the rWSP, the transcription was determined at various time-points upon stimulation of 9 μg/ml rWSP. An analysis of the kinetics revealed that, as early as 1.5 h-incubation of RAW 264.7 cells with rWSP, mRNA expression of IL-1β (2684.8 fold increase relative to control), IL-6 (6.7 folds), and TNFα (19.3 folds) was highly induced (**Figure 40**). Expression of TNF-α mRNA peaked at 3 h after stimulation, whereas IL-1β and IL-6 peaked at 6 h post-exposure. Cytokine transcription decreased between 6 h and 24 h post-exposure, but remained elevated as compared to controls. The levels of IL-1β, IL-6, and TNF-α transcription at their peaks were comparable to that induced by *E. coli* LPS (0.1 μg/ml).


IL-1β
Fold increase relative to control
100000
10000
1000
100
10
1
1.5
3
6
9
16
24
Hours
WSP
LPS


IL-6
Fold increase relative to control
10000
1000
100
10
1
0.1
1.5
3
6
9
16
24
Hours
WSP
LPS

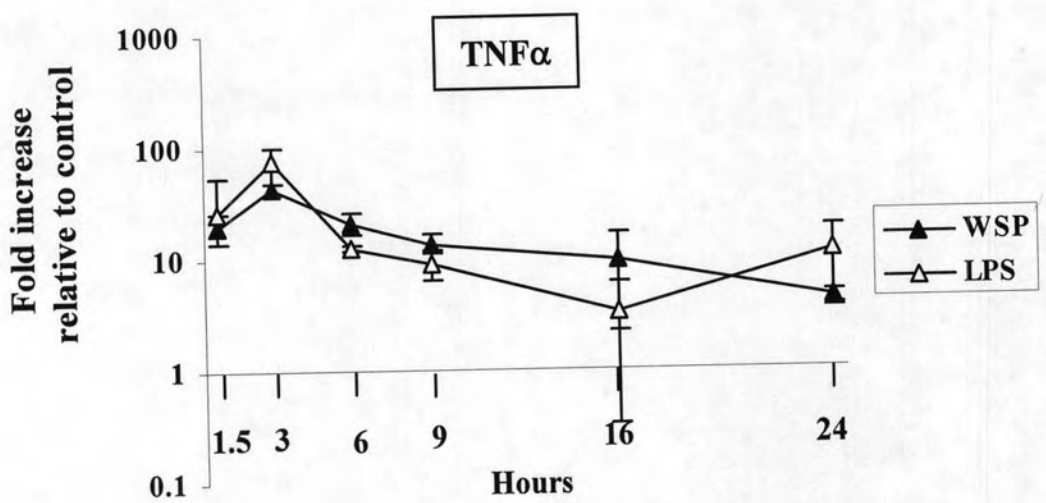

TNFα
Fold increase relative to control
1000
100
10
1
0.1
1.5
3
6
9
16
24
Hours
WSP
LPS

Figure 40 Time-course analysis of IL-1β, IL-6, and TNFα mRNA induction in the RAW 264.7 macrophage cell line induced with rWSP of *Brugia malayi Wolbachia*. The murine macrophage RAW 264.7 cells incubated with 9.0 μg/ml rWSP (black symbols), or 0.1 μg/ml *E. coli* LPS (white symbols) were performed at various time-points. The data represent the mean fold increases of cytokine transcripts relative to negative control ± SD.

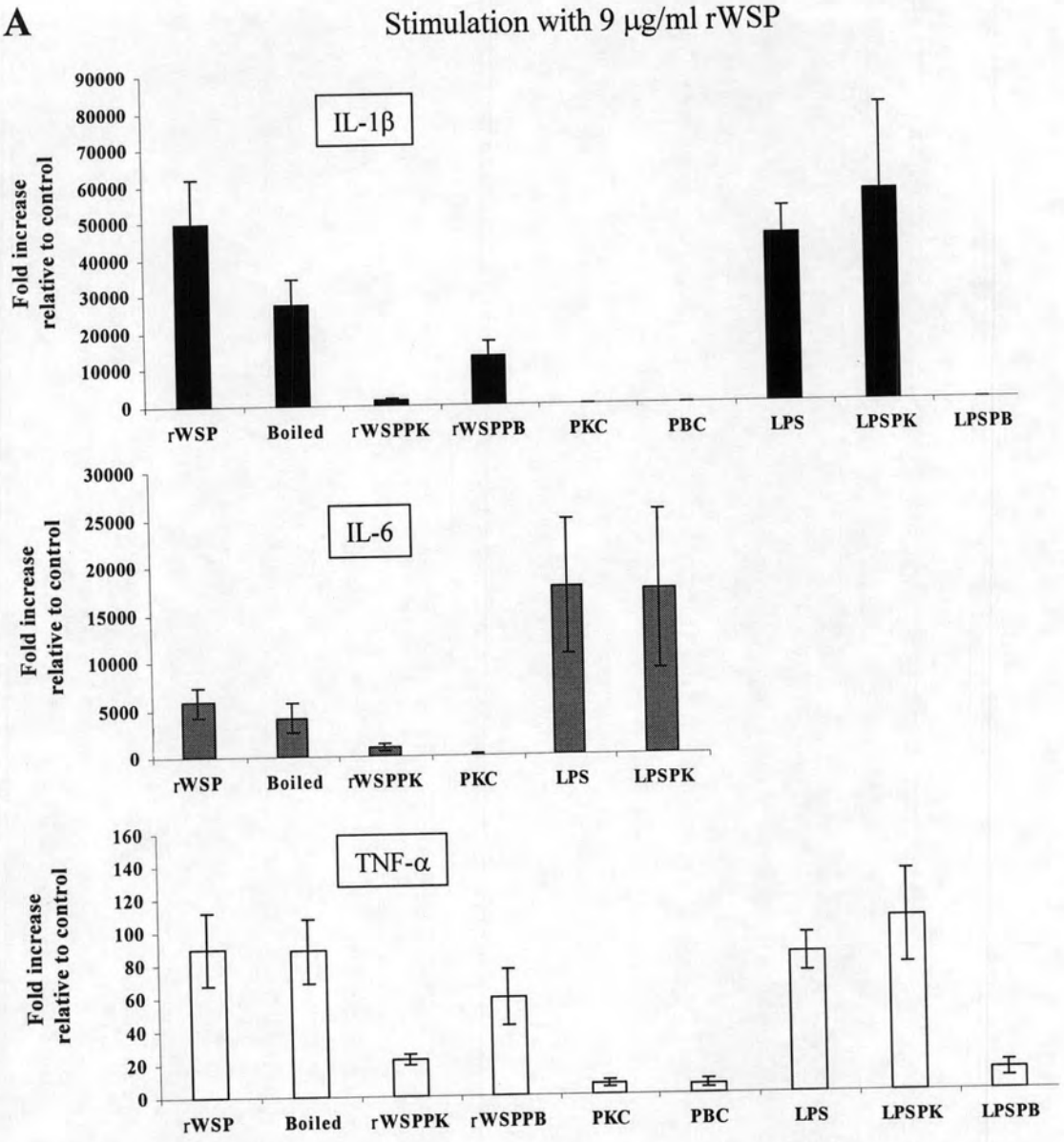
Upregulation of IL-1β, IL-6, and TNF-α mRNA is directly induced by the rWSP, but not by Escherichia coli LPS

Pretreatment of proteinase-K drastically abrogated the transcription of the proinflammatory cytokines induced by the rWSP (**Figure 41**). The highly expression of IL-1β, IL-6 and TNF-α mRNA in the RAW 264.7 cells in response to 9.0 μg/ml rWSP was relatively decreased 34.5, 6.5 and 4.1 folds, respectively with the treatment of proteinase K. Furthermore, the treatment effect was rWSP-dose dependent. We found that the transcription of IL-1β, and TNF-α in response to 1.0 μg/ml rWSP was inhibited by pretreatment of proteinase K 15.0 and 3.1 folds, respectively. Proteinase K treatment had no effect on the activity of LPS.

Boiling had less influence to the IL-1β, IL-6, and TNF-α transcription, when 9.0 μg/ml rWSP was used to stimulate. In contrast, denaturing rWSP by boiling had profound effect on the transcription of IL-1β, and TNF-α induced by 1.0 μg/ml rWSP. Induction of the IL-1β mRNA expression by 1.0 μg/ml rWSP was influenced by boiling with relative decrease of 14.1 folds, while it had effect in response to 9.0 μg/ml rWSP of 1.8 folds. IL-6 mRNA expression induced by 9.0 μg/ml rWSP was influenced by boiling with relative inhibition of the transcription of 1.4 folds. There was no such boiling effect on the TNF-α transcription in response to 9.0 μg/ml rWSP; however we found 3.1 fold decreases in the TNF-α mRNA expression with boiling of 1.0 μg/ml rWSP.

The proinflammatory activity of the rWSP was slightly decreased by the treatment of polymyxin B. However, the inhibitory effect was independent to rWSP concentration. Polymyxin B inhibited the transcription of IL-1β induced by 9.0 and 1.0 μg/ml rWSP (3.4 and 3.8 folds decrease), respectively while the transcription of TNF-α was inhibited (1.3 and 1.5 folds decrease), respectively.

A


Stimulation with 9 μg/ml rWSP
IL-1β
Fold increase relative to control
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
rWSP
Boiled
rWSPPK
rWSPPB
PKC
PBC
LPS
LPSPK
LPSPB
IL-6
Fold increase relative to control
30000
25000
20000
15000
10000
5000
0
rWSP
Boiled
rWSPPK
PKC
LPS
LPSPK
TNF-α
Fold increase relative to control
160
140
120
100
80
60
40
20
0
rWSP
Boiled
rWSPPK
rWSPPB
PKC
PBC
LPS
LPSPK
LPSPB

B Stimulation with 1 μg/ml rWSP

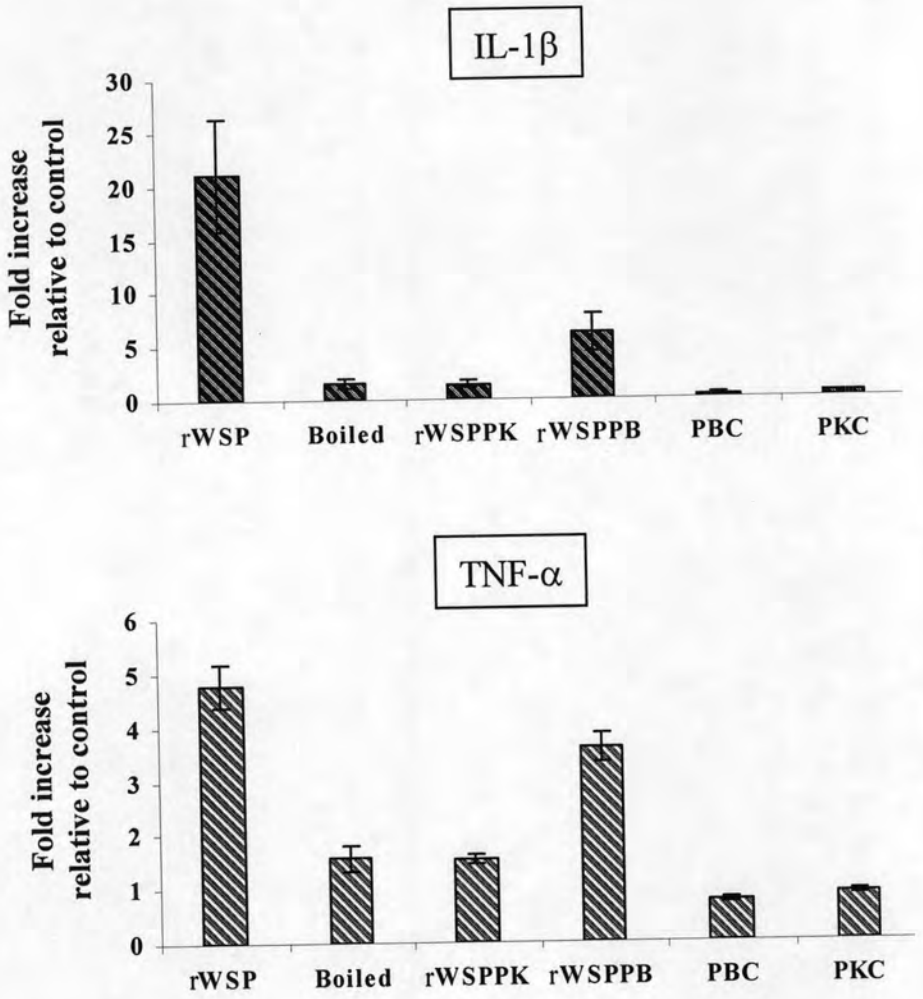


Figure 41 Proinflammatory cytokine responses to rWSP of *Brugia malayi Wolbachia*, but not to *Escherichia coli* LPS in the RAW 264.7 macrophage cell line. The cells were incubated with 9 μg/ml rWSP (A) or with 1 μg/ml rWSP (B), boiled rWSP (Boiled), rWSP + proteinase K (WSPPK), rWSP + polymyxin B (WSPPB), proteinase K (PKC), polymyxin B (PBC), 0.1 μg/ml LPS (LPS), 0.1 μg/ml LPS + proteinase K (LPSPK), and 0.1 μg/ml LPS + polymyxin B (LPSPB) for 3 h. The data represent the mean fold increases of cytokine transcripts relative to negative control ± SD.