High-Level Expression of the Brugia malayi Protein Kinase, Bm-MPK 1, in Insect SF9 Cells

Arti Rana

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ABSTRACT

Lymphatic Filariasis (elephantiasis) is a neglected tropical disease caused by the filarial nematodes *Brugia malayi* (*B. malayi*), *Brugia timori*, and *Wuchereria Bancrofti* (1,8). These parasites are present in over 83 counties in the tropics and sub-tropics where more than 1.4 billion people are at risk of infection and 130 million people are presently infected (1,7). Previous work from our lab has led to the identification and expression of a *B. malayi* stress-activated protein kinase, Bm-MPK1 (a human p38/*C. elegans* PMK-1 ortholog). Bm-MPK1 plays an important role in the parasites’ protection against oxidative stress (1), and as such is a potential therapeutic drug target for the treatment of Lymphatic Filariasis. Bm-MPK1 was successfully expressed in mammalian HEK-293 F cells yielding levels suitable for characterization of the enzyme. However, these cells did not produce adequate levels of recombinant protein for crystallography and high throughput screening purposes. In order to produce high levels of recombinant Bm-MPK1, I have established a novel SF9 cell/Baculovirus expression system. A synthetic Bm-MPK1 was successfully subcloned into a pFASTBAC plasmid, which was then transposed into DH10Bac cells to generate a Bm-MPK1/pFASTBAC-based baculovirus. Using this system, I have demonstrated that recombinant Bm-MPK1 protein kinase is highly expressed in infected SF9 insect cells. Similar to mammalian cell expression, I demonstrated that Bm-MPK1 can be activated in SF9 infected cells by treatment with sodium arsenate. An excellent yield of recombinant Bm-MPK1 was obtained with approximately 3.25 mg of protein recovered from a 100 mL culture.
High-level expression of the *Brugia malayi* protein kinase, Bm-MPK1, in Insect SF9 cells

by

Arti Rana

A Master’s Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science, Concentration in Pharmaceutical Biochemistry

January 2014
HIGH-LEVEL EXPRESSION OF THE *BRUGIA MALAYI* PROTEIN KINASE, Bm-MPK1, IN INSECT SF9 CELLS

A THESIS

Submitted in partial fulfillment of the requirements
For the degree of Master of Science

By
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Fall 2013
ACKNOWLEDGMENTS

I would like to thank Dr. John J. Siekierka for making me a part of his research group and for providing the tools I needed for the study. I am really grateful for his guidance, support, and mentoring throughout my research study.

I would like to thank Dr. Ueli Gubler for designing this project and teaching me various lab techniques to complete the project. I am extremely thankful for his continuous guidance and encouragement throughout the project.

I would like to thank Dr. Ronald Goldberg for his support in the lab and for ordering all the supplies needed throughout the study.

I would like to thank Dr. Jim Dyer and Dr. Nina Goodey for being a part of my thesis review committee members.

I would like to thank my friend and my colleague, Agnieszka Chojnowski for her contribution to this project as well as her continuous support, understanding and help throughout the study.

I would like to thank Monika Prorok and Tamara Kreiss for their support and contribution to this project.

I would like to thank the Department of Chemistry and Biochemistry as well as the Sokol Institute for Pharmaceutical Life Sciences for providing me the resources and foundation for this research.

Lastly, I would like to thank my parents for believing in me and for supporting me throughout my study. I would specially like to thank Vaibhavi Rana, Rajan Rana, and Vinny Mirto for their incredible understanding and support.
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Abstract</td>
</tr>
<tr>
<td>Thesis Signature Page</td>
</tr>
<tr>
<td>Title Page</td>
</tr>
<tr>
<td>Acknowledgements</td>
</tr>
<tr>
<td>Table of Contents</td>
</tr>
<tr>
<td>List of Figures</td>
</tr>
<tr>
<td>Introduction</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Materials and Methods</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>I. Generation of pFASTBAC plasmid containing a Bm-MPK1 Insert</td>
</tr>
<tr>
<td>II. Generation of a Recombinant Bm-MPK1/pFASTBAC-based Baculovirus</td>
</tr>
<tr>
<td>III. Transfection of SF9 cells with Bm-MPK1/pFASTBAC-based Baculovirus</td>
</tr>
<tr>
<td>Expression Vectors</td>
</tr>
<tr>
<td>IV. Bm-MPK1/pFASTBAC-Baculovirus Suspension Culture Titration Assay</td>
</tr>
<tr>
<td>V. Infection of SF9 cells with Bm-MPK1/pFASTBAC-Baculovirus</td>
</tr>
<tr>
<td>V. a Determination of virus dilution</td>
</tr>
<tr>
<td>V. b SF9 cell Infection for production of Bm-MPK1</td>
</tr>
<tr>
<td>V. c Stress Treatments of infected SF9 Cells</td>
</tr>
<tr>
<td>V. d Cell Lysis using IPER®</td>
</tr>
</tbody>
</table>
VI. Purification of GST-tagged Bm-MPK1

VII. Western Blot and SDS-PAGE analysis of Bm-MPK1 protein

VIII. IMAP assay

IX. Activation of Bm-MPK1 by upstream kinase MKK6

Results

Generation of Vectors

Expression and purification of recombinant Bm-MPK1

Enzymatic activity of GST-Bm-MPK1

Protein yield

Discussion

Figures

References
LIST OF FIGURES

Figure 1. Nucleotide sequence of SF9Bm-MPK1

Figure 2. Generation of recombinant baculovirus and gene expression with the Bac-To-Bac expression system

Figure 3. DNA Gel Electrophoresis analysis of pFASTBAC plasmid containing a Bm-MPK1 Insert

Figure 4. Identification of correct transposition via DNA Gel Electrophoresis analysis

Figure 5. Verification of Bm-MPK1 Insert via SfiI restriction enzyme digestion

Figure 6. Transfection of SF9 cells with Bm-MPK1/pFASTBAC-based Baculovirus

Figure 7. Production of GST-Bm-MPK1 from SF9 Insect cells

Figure 8. Purification of GST-Bm-MPK1 from SF9 Insect cells

Figure 9. Dual phosphorylation of the Bm-MPK1 TGY domain by Sodium Arsenate, Copper Sulfate, and Heat Induced Oxidative stress

Figure 10. Enzymatic activity of activated Bm-MPK1 using an IMAP assay

Figure 11. Enzymatic activity of MKK6-activated Bm-MPK1
INTRODUCTION

Lymphatic Filariasis (elephantiasis) is an infectious disease caused by thread-like nematode worms, *Brugia malayi* (*B. malayi*), *Brugia timori*, and *Wuchereria Bancrofti* (1, 6). These parasites are endemic in over 83 countries in the tropics and subtropics where more than 1.4 billion people are at risk of infection and approximately 130 million people are currently infected (6). Elephantiasis has a major impact on society because it leads to long-term and permanent disability (6, 7, 8). The life cycle of the filarial parasites has three different stages: microfilaria (mf), infective larva (La or L3) and adults. *B. malayi* parasites are transmitted by the *Mansonía* species of mosquito (6). The cycle begins when a mosquito takes a blood meal from an infected person and ingests mfs, which subsequently develop into L3 infectious larvae in the mosquito. When the infected mosquitos take another blood meal, they introduce L3 into the host. L3s molt and develop into L4 larvae, which migrate to the lymphatic vessels and develop into adult worms. Adult worms can live from 8-15 years in the lymphatic system of the host (6). During this time, adult female worms produce millions of mfs, which are introduced into the blood stream living up to 14-70 days (9).

The current drugs used to treat lymphatic filariasis are diethylcarbamazine citrate (DEC), albendazole, and Ivermectin. These drugs can effectively remove 99% of mf from the blood; however, they fail to kill adult worms (9). To interrupt filarial transmission quickly and to eliminate the disease, it is necessary to find new therapeutic drug targets that kill both mf and adult worms. The sequencing of the *B. malayi* genome
should help to provide a better understanding of filarial parasite biology and potential drug targets.

Recent studies in our lab have led to the identification of a potential drug target in *B. malayi*. A *B. malayi* p38/PMK-1 ortholog, Bm-MPK1, was identified and shown to be critical for protective responses against reactive oxygen species (ROS) produced by macrophages, neutrophils, eosinophils, and basophil granulocytes during infection (1). ROS promotes protein modification, lipid oxidation, and nucleic acid damage (1). Human stress activated protein kinase p38, PMK-1 from *C. elegans*, and the Bm-MPK1 are members of the mitogen-activated protein kinase (MAPK) superfamily. They exhibit the characteristic 12-domain protein structure as well as the highly conserved TGY activation-motif in domain VIII (1, 13).

MAP kinases play an important role in cellular responses to external stress signals including osmotic stress, viral infection, UV, heat, and inflammatory cytokines (1, 2). In humans, p38 is activated via dual phosphorylation of TGY motif by the upstream kinases, MKK3 and MKK6 (13). This results in conformational changes, which modifies the configuration of N-terminal and C-terminal domains leading to an increased interaction with substrate that enhances enzymatic activity (13). Activated p38 leads to the phosphorylation and activation of a variety of protein kinases and transcription factors (1, 13). In addition, PMK-1 found in free-living nematode *C. elegans*, is similarly activated by dual phosphorylation of TGY motif by an upstream kinase SEK-1. The activation of PMK-1 leads to the phosphorylation of a transcription
factor, SKN-1 resulting in translocation of SKN-1 into intestinal nuclei where it regulates the expression of a variety of antioxidant and phase II detoxification genes (1). It has been shown that the filarial parasite *B. malayi* has a similar stress-activated signaling pathway where Bm-MPK1 is activated by dual phosphorylation of an upstream kinase, Bm-SEK-1; however, the downstream kinase SKN-1 is absent in *B. malayi*. We believe that activation of Bm-MPK1 plays a similar role in activating various transcription factors.

It has been previously demonstrated in our lab that known p38 inhibitors inhibit Bm-MPK1 and compromise the ability of *B. malayi* to counteract oxidative stress validating Bm-MPK1 as a potential anti-parasitic target. To that end, the Celgene Corporation is currently sponsoring a drug discovery effort in our lab. In order to screen large numbers of protein kinase inhibitors for activity against Bm-MPK1 and to generate enough Bm-MPK1 for determining a crystal structure for the kinase, large quantities of Bm-MPK1 are required. To support the efforts, a highly efficient expression system is required for the production of recombinant enzyme. MAP kinases produced in bacterial systems are usually inactive because they are not phosphorylated at their specific activating threonine and tyrosine residues (3). Expression of MAP kinases in mammalian systems such as human embryonic kidney cells (HEK 293T & HEK 293F) has the advantage that the kinases can be activated by stressing the cells with sodium arsenate (1, 3). Although mammalian expression systems produce enough recombinant Bm-MPK1 for studying this enzyme at the biochemical level, they are not adequate for producing high level of recombinant proteins for high throughput screening.
In order to increase the production of recombinant, active, Bm-MPK1, I established the SF9 cell/Baculovirus expression system in the lab. The SF9 cell/Baculovirus expression system is widely used to express heterogeneous genes in cultured insect cells and yields high-level protein expression (13). Baculoviruses are double-stranded, circular, supercoiled DNA molecules in a rod-shaped capsid originating from arthropods (12). The two most common isolates used for foreign gene expression are known as AcMNPV (*Autographa californica* multiple nuclear polyhedrosis virus) and BmNPV (*Bombyx mori* (silkworm) nuclear polyhedrosis virus). The virus replication occurs in three stages [Early phase (virus synthesis phase), Late phase (viral structural phase), and Very Late phase (viral occlusion protein phase)] leading to both lytic and occluded life cycles.

SF9 cells are a clonal isolate of the *Spodoptera frugiperda* cell line IPLB-Sf21-AE, and are extensively used for Baculovirus Expression Vector Systems (BEVS). These cells were originally derived from ovarian tissue of the fall armyworm (12). The polyhedrin gene within the wild-type viral genome can be replaced with an exogeneous open reading frame, allowing a recombinant baculovirus to be used as an expression vector for vitro infection of insect cells. The recombinant genes are placed under the transcriptional control of the strong polyhedrin promoter, which is active during the very late stage of infection. Thus, recombinant product is expressed in place of the naturally occurring polyhedrin protein. When SF9 cells are infected with recombinant baculovirus, cellular structure begins to change in the early phase (30 min -6 h) of infection. Normal cellular functions decline and early-phase viral proteins become evident. Normal cellular
functions terminate in the late phase (6 h-24 h) of infection. At this point cells stop dividing and budding virus production increases dramatically. During the very late stage of infection (20-36 h after infection), budding virus production stops and the assembly, production, and expression of recombinant gene product begins.

The SF9 cells/Baculovirus system is a very useful eukaryotic protein expression system for recombinant protein expression, with the advantages of biological safety, ease of scale up, high levels of recombinant gene expression and the full complement of eukaryotic posttranslational modifications. The main goal of my project was to develop the SF9 cells/Baculovirus protein expression system for the high-level production of parasitic protein kinases for high-level inhibitor screening and crystallization studies.
MATERIALS & METHODS

I. Generation of a pFASTBAC plasmid containing a Bm-MPK1 insert

The SF9Bm-MPK1 gene (Figure 1) was chemically synthesized and cloned into pUC57 vector (Genscript, Piscataway, NJ). The SF9Bm-MPK1 gene was excised from pUC57 and sub-cloned into Vector “S”, a modified pFASTBAC plasmid containing an N-terminal GST-tag and two different Sfil sites flanking the open reading frame to be expressed. Vectors of this kind allow the easy parallel expression evaluation of ORFs (open reading frames) as well as easy transfer of DNA sequences into proper vectors for different expression hosts with a single subcloning step. The SF9Bm-MPK1 gene is flanked by the Sfil site A (5’-GGCCCCtcagcGGCC-3’) on its 5’ end and by the Sfil B site (5’- GGCCgggtcGGCC-3’) on its 3’-end, allowing easy, directional subcloning with only one restriction cut.

The lyophilized pUC57/SF9Bm-MPK1 plasmid was reconstituted in water (80 µg/µl) as per manufacturer’s instructions (Genscript). The pUC57/SF9Bm-MPK1 plasmid (2 µg) and Vector “S” were digested in a 50 µl volume with 2 µl of Sfil restriction enzyme (10 units/µl) and 5 µl of 10X Sure/Cut Buffer M for Restriction Enzyme (Roche Molecular Biochemicals, RMB). The reaction was incubated in a 50°C water bath for 90 min. The digested plasmids were loaded onto a 1.5% agarose gel in 1x TAE buffer along with λHindIII1OX HaeIII DNA marker (310-23,130 base pairs) to obtain a Bm-MPK1/sfil insert and Vector S/sfil fragment. The bands were excised with a
sterile razor blade and purified using the QIAquick gel extraction kit protocol (QIAGEN).

The purified Bm-MPK1/sfi1 was ligated with Vector S/sfi1 via a DNA ligation reaction using T4 ligase (New England Biolabs, NEB) and 10x ligase buffer (NEB) in a 20 μl reaction, which was incubated overnight in a 16°C water bath. The insert to vector molar ratio was 5:1. A “No Insert control” tube was set up as a control. The ligation reaction (10 μl) was transformed into DH5α chemically competent E.Coli cells (70 μl) (Invitrogen). The transformation mixture was kept on ice for 30 min and was heat shocked at 37°C for 45 seconds. Immediately after this step, the transformation mixture was moved back to ice for 2 min. Then, 500 μl of SOC medium was added at room temperature. The transformation mixture was incubated at 37°C on a shaker (sideways) at 240 rpm for 1 h. Transformation mixture was plated onto pre-warmed LB agar plates containing 100 μg/mL ampicillin and incubated (upside down) overnight at 37°C.

From the LB plate, six single colonies assumed to contain the Bm-MPK1/pFASTBAC expression vector construct were inoculated separately in 2 mL of LB Broth with 100 μg/mL ampicillin in 15 mL conical centrifuge tubes. The cultures were incubated overnight in 37°C incubator with shaking at 240 rpm. The plasmid was purified using Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge protocol (QIAGEN) as per manufacturer’s instructions. The Bm-MPK1/pFASTBAC expression vector DNA was eluted in 50 μl of EB buffer. The concentration of the purified DNAs was measured using a NanoDrop spectrophotometer.
(Thermo-Scientific, Wilmington, DE). Each purified vector DNA (8 μl) was digested with 1 μl of SfiI restriction enzyme and 10X Sure/Cut Buffer M in a 10 μl reaction which was incubated for 1 h in 50°C water bath. The reaction was run on 1.5% agarose gel in 1x TAE buffer along with a λHindIII/OX HaeIII DNA marker (310-23,130 base pairs) to verify the correct Bm-MPK1 ligation into the pFASTBAC plasmid.

II. Generation of a Recombinant Bm-MPK1/pFASTBAC-based Baculovirus

In order to generate recombinant Bm-MPK1/pFASTBAC-based Baculovirus, one plasmid construct (34.2 ng/μl) was transformed into the DH10Bac competent cells. These cells contain the bacmid with a mini-attTn7 target site and the helper plasmid (12). The helper plasmid provides the transposition proteins, which help the mini-Tn7 element on the pFASTBAC plasmid to transpose to the mini-attTn7 target site on the bacmid (12). Approximately 100 ng of Bm-MPK1/pFASTBAC expression vector DNA was added to 50 μl of commercial high efficiency DH10Bac competent E.coli cells (Invitrogen). The transposition mixture was kept on ice for 30 min and moved to a 42°C water bath for 45 seconds for heat-shock. Instantly, the transposition mixture was placed on ice and 900 μl of SOC medium was added after 2 min incubation. The tube was sealed with parafilm and placed in a 37°C incubator for 3-4 h on a shaker at 200-240 rpm for recovery. The transposition mixture was diluted 10^2 and 10^3 times after the 4 h incubation. From both diluted samples, 100 μl were plated onto pre-warmed LB plates containing KAN+GENT+TET+IPTG+X-GAL (standard LB with 50 μg/mL KAN, 10 μg/mL TET (in EtOH) and 7 μg/mL Gentamycin, 40 μg/mL IPTG, and 100 μg/mL Bluo-Gal) and
incubated for 48 h at 37°C. Plates were subsequently transferred into the cold room to
obtain a better blue-white color distinction. Ten recombinant white colonies were
separately inoculated in 1 mL LB +KTG media into 1.5 mL eppendorf tubes. These 1
mL cultures were grown overnight at 37°C on a shaker at 200-240 rpm (turning
overhead). The Bm-MPK1/pFASTBAC-Baculovirus (BACMID DNA or recombinant
bac virus) was purified using the Plasmid DNA Purification QIAprep Spin Miniprep Kit
and a Microcentrifuge protocol (QIAGEN).

A two-step Polymerase Chain Reaction (initial denaturation step at 94°C for 6
min; annealing at 94°C for 30 seconds followed by extension at 68°C for 6 min; 30
cycles) was performed on purified recombinant bacmid DNA (from each of 10 isolates)
to verify the correct transposition. The M13-F and M13-R primers were designed and
synthesized at Hoffmann-La Roche. The sequence for M13-F (Forward Primer) is “ccc
agt cac gac gtt gta aaa eg” and for M13-R (Reverse Primer) is “AGC GGA TAA CAA
TTT CAC ACA GG”. The melting temperatures for forward and reverse primers were
73°C and 70°C, respectively. For each reaction, 3 µl of purified bacmid DNA was mixed
with 12.5 µl of Taq PCR Master Mix (QIAGEN), 7.5 µl distilled water, 1 µl forward
primer, and 1 µl reverse primer in 200 µl PCR tubes. The PCR reactions were run on a
1.5% agarose gel in 1x TAE buffer to identify correctly transposed clones, where a 3.7 kb
insert is expected (a 2.3 kb pFASTBAC-1 insert combined with cloned and transposed
1.1 kb Bm-MPK1 plus 0.3 kb GST tag). One positive clone was chosen to generate Bm-
MPK1/pFASTBAC based Baculovirus DNA. The DNA from 25 mL culture was
purified using the QIAprep Spin Miniprep Kit and a Microcentrifuge procedure
Prior to purification, 3 mL of culture was used to prepare 1 mL aliquots of 15% glycerol stocks for long term storage at -80°C. The lysate derived from the 25 mL culture was divided into four parts and run over four QIAprep spin columns. Each of the four columns was eluted in 50 µl EB buffer. The concentration of purified bacmid DNA was measured using the NanoDrop spectrophotometer.

For subsequent DNA purifications, the Bm-MPK1/pFASTBAC- Baculovirus cells were inoculated into 50 mL LB broth containing TKG and incubated overnight in a 37°C incubator with a rotator speed of 200-240 rpm. The DNA was purified using PureYield™ Plasmid Midiprep system as per manufacturer’s instructions (Promega). Three elutions were collected using 500 µl of nuclease free water. To again verify the presence of a 1.1 kb Bm-MPK1 insert, an SfiI restriction enzyme digestion was performed on 1 µg of purified Bm-MPK1/pFASTBAC-Baculovirus DNA in 20 µl final volumes. The mixture was incubated at 50°C for 1 h and run on a 0.5% agarose gel parallel to undigested BACMID DNA, BACMID DNA from the previous QIAGEN prep and DNA markers.

III. Transfection of SF9 Insect cells with Bm-MPK1/pFASTBAC-based Baculovirus Expression Vector

SF9 Insect cells (Invitrogen) were used for transfection with the Bm-MPK1/pFASTBAC-based Baculovirus expression vector to express Bm-MPK1. Cells were grown in suspension and were seeded at 0.8-1 x 10^6 cells/mL in SF-900-II SFM
media (Gibco, Life Technologies) with 2% FCS plus 1x penicillin/streptomycin. They were passaged every 2-3 days when the cell density reached 6-8 \times 10^6 \text{cells/mL} with the cell viability being greater than 96%. Later in the study, media was substituted with SF-900-III SFM (Gibco by Life Technologies) due to changes in the manufacturer’s yeast extract component. Cells were grown at 27\degree C in a non-humidified environment with shaking at 120-140 rpm and maintained in 150 mL or 250 mL non-pyrogenic sterile plastic storage bottles (Corning Incorporated, Corning, NY) with tightly closed caps. The titer range from 1-8 \times 10^6 \text{cells/mL} with a viability of 96-100% was used for experiments. The trypan blue exclusion method with manual hemocytometer cell counting was used to determine cell titers and viability.

Cells were split to 1 \times 10^6 \text{cells/mL} 3-4 days before transfection and grown until the cell density reached 4-6 \times 10^6 \text{cells/mL}. On the day of transfection, the cell density was 5 \times 10^6 \text{cells/mL} with 99% cell viability. Using this culture, 1 \times 10^6 \text{cells} (200 \mu l) per each 35 mm well in sterile 6 Well Cell Culture Cluster plate (Corning Incorporated, Corning, NY) were seeded in 2 mL SF-900-II SFM media containing 2% FCS and 0.5x pen/strep. Cells were incubated at 27\degree C for 1 h to allow attachment. To transfect two wells with Bm-MPK1/pFASTBAC-Baculovirus vector DNA, 2 \mu g of plasmid was diluted in SF-900-II SFM media without FCS to obtain a final volume of 200 \mu l. In a separate tube, 12 \mu l of Cellfectin 2000 (Invitrogen) was mixed with SF-900-II SFM without FCS in a final volume of 200 \mu l. The diluted plasmid DNA was transferred to the diluted Cellfectin to form the DNA-Cellfectin complexes. The contents were mixed gently by tapping the tube. In addition, 6 \mu l of Cellfectin 2000 was added into 100 \mu l of
SF-900-II SFM without FCS, which was mixed with another 100 μl of SF-900-II media without FCS to represent the NO DNA control. These tubes were incubated at room temperature for 45 min. After incubation, SF-900-II SFM media without FCS was added to the DNA-Cellfectin mix and to the No DNA control to a final volume of 2 mL and 1 mL, respectively.

After cells attached, media from each well was removed. Cells were washed gently with 2 mL of fresh SF-900-II media without FCS. Then, 1 mL of diluted Cellfectin-DNA complex mixture was added to two wells. The No DNA mixture was added into a third well. A GFP virus (2 \( \times \) \( 10^9 \) PFU/mL stock) was added to two wells as a control. The GFP virus stock was diluted by adding 5 μl and 0.5 μl virus to 1000 μl of complete media to obtain an approximate MOI (multiplicity of infection) of 1 (200x) and 0.1 (2000x), respectively. From these diluted samples, 100 μl for each sample was added onto another two wells. The plate was placed at 27°C for 4 h. The DNA-Cellfectin mixture and NO DNA mixture were removed from the wells after 4 h incubation and replaced with 2 mL of pre-warmed SF900-II SFM media with 2% FCS and 1x pen/strep. The plate was placed back into the 27°C incubator for 72 h in a humidified container (plastic container with a few layers of water-saturated paper towels on the bottom). The sixth unused well was used as a reference well for healthy looking cells. After 72 h, transfected vs. un-transfected cells were observed under a microscope. The supernatant (media containing virus) from the two wells transfected with Bm-MPK1/pFASTBAC-baculovirus DNA was removed (3-4 mL) and pooled together into 15 mL conical centrifuge tube. The supernatant was sterile-filtered using 0.22 μm filter unit.
(MILLEX®- GS, Millipore Corporation) for further virus amplification. The collected 4mL supernatant was considered passage 1 (P1) and stored at 4°C. Collected GFP transfected cells and no DNA-transfected cells were stored at 4°C.

To generate passage 2 (P2), 3.5 mL of virus from P1 was added to 15 mL SF9 cells at 2 \times 10^6 cells/mL. Cells were grown at 27°C for 4 days. The cell density and cell viability were determined every 24 h. The cell viability decreased from 93.2% on day 1 to 79.7% on day 4 (Figure 6). On day 4, 12 mL from P2 was added to 100 mL actively growing SF9 cells at 3 \times 10^6 cells/mL to generate passage 3 (P3). Cells were grown at 27°C for 3-4 days. When cell viability was 75-80%, the cells were spun at 4000 rpm for 10 min. The supernatant (100 mL) was collected and transferred into a new bottle. FCS was added to obtain a final concentration of 10% to increase the virus stability for long-term storage. Collected virus stock was stored at 4°C. The tubes were covered with aluminum foil to protect the virus from light.

**IV. Bm-MPK1/pFASTBAC-Baculovirus Suspension culture Titration Assay**

When baculovirus production processes in suspension culture are scaled up, titration of the viral stocks used is important to ensure reproducible infections via controlled MOIs. A suspension culture titration method was therefore used to measure Bm-MPK1/pFASTBAC-Baculovirus titer (5). In this method, cessation of cell growth is correlated to the viral titer used for cell infection. For this assay, 20 mL SF9 cell cultures at a known initial cell density (ICD) of 1 \times 10^6 cells/mL were infected with different
virus dilutions. Three cultures received 1 mL virus aliquots of $10^2$, $10^4$ and $10^6$ fold diluted GFP standard virus (known initial titer = $2 \times 10^9$ pfu/mL). The Bm-MPK1/pFASTBAC-based Baculovirus was diluted at $10^3$, $10^4$, and $10^5$ fold in SF-900 II SFM media with 2% FCS + 1% pen/strep in 5 mL blue-capped tubes. From each dilution, 1 mL diluted virus was added to three 20 mL cultures. The cells were incubated on a shaker (120 rpm) at 27°C for 3 days. The Peak Cell Density (PCD) values in each culture were determined by hemocytometer count after a 3 day culture period. Based on reference 5, the correlation between MOI and resulting cell densities was described as log PCD/ICD = -0.16 log MOI + 0.1. Using this equation and the standard GFP virus dilutions, a Bm-MPK1 virus titer was estimated.

V. Infection of SF9 cells with Bm-MPK1/pFASTBAC-Baculovirus

V. a Determination of virus dilution

One initial experiment was performed to determine the correct virus dilutions and cell harvest times for use with the Bm-MPK1/pFASTBAC-Baculovirus. For this purpose, 100 mL SF9 cells were grown to 3-4 x $10^6$ cells/mL. In three 250 mL flasks, cells were then seeded at 2.5 x $10^6$ cells/mL in SF-900-II SFM media with 2% FCS +1 % pen/strep in a final volume of 40 mL. Subsequently, 4 mL, 400 $\mu$L and 40 $\mu$L of Bm-MPK1/pFASTBAC-Baculovirus were added to obtain final virus dilution of 10X in flask 1, 100X in flask 2, and 1000X in flasks 3, respectively. For the non-infected control, cells were seeded at 2.5 x $10^6$ cells/mL in final volume of 20 mL. Cells in each
of the four flasks were mixed well by pipetting. An aliquot was collected to determine the Initial Cell Density (ICD) value. All flasks were incubated at 27°C for 4 days on a shaker (120 rpm). The Trypan blue exclusion method with hemocytometer counting was used to obtain cell density and cell viability each day. From each flask, 5 mL aliquots were collected on days 2, 3, and 4 and the cells were spun out at 4000 rpm for 10 min and saved for later analysis.

V. b  SF9 cells Infection for production of Bm-MPK1 protein

For Bm-MPK1 production, 100 mL SF9 cells seeded at 2.5 x 10^6 cells/mL in a 1L plastic bottle were infected with 10 mL of Bm-MPK1/pFASTBAC-Baculovirus to obtain 10X final virus dilution. Cells were grown at 27°C for 3-4 days on the shaker until the cell viability dropped to 75-85%. Infected cells were harvested by spinning at 800 x g for 5 min. Cell pellets were stored at -80°C for later analysis.

V. c  Stress Treatments of the SF9 cells by Sodium arsenate, Copper sulfate, or Heat Shock

For all stress treatments, SF9 cells were first infected with Bm-MPK1/pFASTBAC-Baculovirus as stated in above section V.a. When the cell viability dropped to between 75-85%, cells were stressed by several different regimens. For the sodium arsenate and copper sulfate treatment, cells were stressed 4 h prior to harvests at a final concentration of 400 μM. For the heat shock treatment, cells were incubated at
37°C and 43°C for 1 h and 4 h prior to harvesting the cells. Some infected cells were harvested prior to the stress treatments as control. As a further control, the same stresses were performed on uninfected SF9 cells. Afterwards, cultures were spun at 800 x g for 5 min to collect the pellets.

V. d Cell Lysis using IPER®

The Insect cell pellets were washed twice with 10 mL of cold 1x PBS (Phosphate Buffered Saline) and centrifuged at 800 x g for 5 min. The insect cells were lysed with 1 mL of I-PER® (Insect Cell Protein Extraction Reagent; Thermo Scientific) with 1:100 Halt™ Protease Inhibitor Cocktail (Thermo-Fisher Scientific) for every 5 x 10⁶ – 2 x 10⁷ cells. Resuspended cells were incubated on ice for 10 min. The lysis solution was then centrifuged at 15,000 x g for 15 min at 4°C. The cell lysate was transferred into new 15 mL conical centrifuge tubes.

VI. Purification of GST-tagged Bm-MPK1

GST-tagged Bm-MPK1 from infected SF9 insect cells was purified using Thermo Scientific Pierce® Glutathione Agarose in a Gravity-Flow Column at 4°C. The column was packed with 16 mL of Pierce-Glutathione Agarose and equilibrated using 10 bed volumes of 1x PBS. The equilibrated resin was removed from the column to mix with crude extract. The mixture was incubated for either 3 h or overnight on a rotator in the cold room. After, the mixture was loaded onto the column to collect flow through which
was reloaded to allow for more complete binding. The resin was washed four times with 5 mL 1x PBS, followed by a fifth 5 mL wash with buffer containing 25 mM HEPES, 150 mM NaCl or 500 mM NaCl, 1 mM DTT, 10% glycerol, and 1:100 Halt™ Protease Inhibitor Cocktail. Then, proteins were eluted in 50-100 mL of the above buffer with added 10 mM reduced L-glutathione (Sigma-Aldrich) by gravity flow. Protein concentrations in the different fractions were measured using the standard relation 1 mg/ml = 1A280 with the NanoDrop spectrophotometer. In order to concentrate the proteins, eluted fractions were spun for 30-45 minutes at 4000 rpm using Amicon® Ultra-15 mL (30,000 Nominal Normal Weight Limit, [NMWL]) or 0.5 mL (10,000 NMWL) Centrifugal Filters (Merck Milipore Ltd).

VII. Western Blot and SDS-PAGE of GST-Bm-MPK1 protein

The GST-Bm-MPK1 protein collected from Bm-MPK1/pFASTBAC-Baculovirus infected SF9 cells were analyzed by SDS-PAGE and Western blot. Gel samples were prepared in 20 µL volume using 65% of protein samples and 35% master-mix (25% NuPAGE SDS sample buffer (Invitrogen) and 10% of 500 mM DTT reducing agent). Samples were then placed in a 90°C heat block for 5 min. After the heating step, the samples were quickly spun using a tabletop centrifuge. Samples were loaded onto 10-well NuPAGE 4-12% Bis-Tris gel (Invitrogen) along with molecular weight markers; Novex sharp pre-stained protein standard (Invitrogen) for SDS-PAGE and MagicMark™ XP Western Standard (Invitrogen) for Western blots. Gels were run at 200-V for 40 min in cold MOPS SDS running buffer (Invitrogen). Gels were stained with staining solution
(0.05% Coomassie Blue, 10% acetic acid and 44% methanol) for 1 h, fixed for 30 min in fixing solution (50% methanol and 10% acetic acid), and destained for 1 h in 5% acetic acid. Later in the study, gels were stained using a commercial reagent, InstantBlue™ (Expedeon).

For Western blots, proteins separated on gels were electro-transferred onto polyvinylidene fluoride (PVDF) membranes in NuPAGE transfer buffer (Invitrogen) using a TE77XP Semi-Dry Blotter (Hoefer, Holliston, MA) at 54 mA per blot for 1 h. Membranes were then placed into 5% non-fat dry milk (blocking solution) in TBS-T (Tris-buffered saline and 0.05% Tween® 20) for 1 h to block non-specific binding sites. Membranes were then incubated in primary antibody (1:1000 goat anti-GST polyclonal IgG antibody, GE Healthcare) diluted in 10 mL of TBS-T containing 10 mg BSA and 0.01% sodium azide for 1 h at room temperature or overnight at 4°C. Membranes were washed three times with TBS-T buffer for 5 min before the 30 min incubation with alkaline phosphatase-coupled secondary antibody. The secondary antibody solution was prepared by adding 2 μl of mouse anti-goat IgG-AP, 1:5000 (Santa Cruz Biotechnology, Santa Cruz, CA) into 10 mL of TBS-T. The membranes were washed twice with TBS-T and once with distilled water for 5 min. Finally, they were developed using 2 mL of Western Blue® Stabilized Substrate for Alkaline Phosphatase (Promega). Water was used to terminate the reaction after bands became visible. Additional western blots were probed with a primary antibody specific for the phosphorylated kinase (phospho-p38 rabbit polyclonal IgG antibody (1:2000, Santa Cruz Biotechnologies); Secondary
antibody: alkaline phosphatase linked anti-rabbit IgG (1:2500, Promega)) to detect stress activated GST-Bm-MPK1 protein from cells treated with arsenate, copper or heat.

VIII. IMAP assay

In order to measure the enzymatic activity of Bm-MPK1 produced in SF9 cells, an IMAP FP (Immobilized metal ion affinity-based fluorescence polarization) assay (Molecular Devices) was performed using the manufacture’s procedures. The IMAP assay was initiated with the incubation of Bm-MPK1 enzyme with fluorescent peptide substrate, FAM-p38tide (120 nM) in the presence of ATP (100 μM). The kinase reaction was incubated for 1 h to form phosphorylated fluorescent peptide product. Nanoparticles (1:1000 dilution) containing a coupled trivalent metal ion were added to the reaction. These nanoparticles bind to the phosphorylated peptide product decreasing the rotational speed of the peptide, resulting in a higher fluorescence polarization signal. The Bm-MPK1 kinase activity was detected by high-affinity interactions of nanoparticles with phospho groups on either serines, threonines, or tyrosine. The assay was read using a Synergy 2 Microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 528 nm.

IX. Activation of Bm-MPK1 by upstream kinase MKK6

Purified Bm-MPK1 was activated by 1 μl (0.30 μg/μl) of the p38 upstream kinase MKK6 in a 1 mL reaction containing 1 mM DTT, 10 μM ATP, 5X assay buffer and
distilled water. In order to optimize the activation procedure three different volumes of Bm-MPK1 were used, 50 μl, 100 μl and 250 μl. A negative control was performed for each condition without MKK6. The reactions were incubated at 30°C for 30 min. The activity was evaluated using IMAP assay as described above.
RESULTS

Generation of Vectors

In order to produce recombinant Bm-MPK1 in SF9 cells, a synthetic open reading frame encoding Bm-MPK1 was successfully subcloned into a pFASTBAC plasmid using DNA ligation and bacterial transformation. The purified plasmid DNAs from transformed colonies were digested with SfiI restriction enzyme and analyzed using DNA gel electrophoresis. The gel data showed two bands for each of six colonies analyzed (Figure 3). The higher molecular weight band migrating between 6557 and 4361 bp indicate pFASTBAC (~5kb) and the lower molecular weight bands between 1353 and 1078 bp show the Bm-MPK1, 1.1 kb insert. This result verifies the successful Bm-MPK1 ligation with the pFASTBAC vector. The concentrations of the purified plasmid DNA from six colonies were 25.6 ng/μl, 17.7 ng/μl, 34.2 ng/μl, 21.6 ng/μl, 18.0 ng/μl, and 20.6 ng/μl. The negative control plate did not yield any colonies.

The Bm-MPK1/pFASTBAC-based Baculovirus was successfully generated in DH10Bac competent cells via site-specific transposition (4, 12). The gel data (Figure 4) shows that 7 out of 10 colonies contain a 3.7 kb band verifying the correct generation of the recombinant Bm-MPK1/pFASTBAC recombinant bac virus (0.3 kb GST, 1.1 kb Bm-MPK1, and 2.3 kb pFASTBAC fragments totaling a 3.7 kb band as expected). Isolate #9 (Figure 4, lane 10) was chosen for amplification and purification of bacmid DNA. Using the QIAGEN Miniprep kit, 28.075 ng/μl of bacmid DNA was obtained. Using Pure
Yield™ Plasmid Midiprep system (Promega), a total of 141.63 μg of bacmid DNA (362.1 ng/μl, 210 ng/μl, and 155 ng/μl) was collected.

The bacmid DNA from both preps were digested with SfiI and analyzed on 0.5% agarose gel parallel to undigested bacmid DNA (Figure 5). The bacmid DNA purified using the QIAGEN procedure was obtained at a very low yield only (lanes 2 and 6), making a meaningful analysis difficult. For the Promega prep DNA, SfiI digestion clearly showed the release of a correctly sized Bm-MPK1-insert at 1.1 kb (lane 3), further supporting the results from the PCR analysis shown in figure 4.

**Expression and purification of recombinant Bm-MPK1**

The protein kinase, Bm-MPK1 was successfully expressed in Insect SF9 cells as a GST-tagged recombinant protein. The optimal virus dilution conditions for SF9 cell infection were determined by analyzing cells infected with 10X, 100X, and 1000X virus dilutions and harvested on days 2, 3 and 4. The western blot results show that at all three viral concentrations lead to similar expression levels of Bm-MPK1 protein with the expected molecular weight 67 kDa (Bm-MPK1-40kDa + GST-tag, 27kDa) (Figure 7A). However, SDS-PAGE analysis shows that protein bands obtained after infection with a 10X virus dilution are stronger compared to bands for the other two virus concentrations (Figure 7B). Since the recombinant Bm-MPK1 virus titer was unknown prior to this experiment, 10-fold virus dilution and day 3 was used for further expression and harvesting. Later, a Bm-MPK1 virus titer of about 2 x 10⁹ pfu/mL was estimated using
suspension culture titration assay. Using this titer, the actual MOI used for experiments was 4.

For purification of GST-Bm-MPK1 protein, cell lysates from infected SF9 cells were purified using Thermo Scientific Pierce® Glutathione Agarose in a Gravity-Flow Column. The column fractions were analyzed by SDS-PAGE and Western blot showing the successful expression and purification of GST-tagged Bm-MPK1 from SF9 cells (Figures 8A -8B). The purification procedure gave an about 95-99% pure protein preparation (Figure 8B). Recombinant Bm-MPK1 was successfully expressed and purified under arsenate, copper, and heat stress conditions (Figure 9B).

**Enzymatic activity of Bm-MPK1**

The enzymatic activity of recombinant Bm-MPK1 was evaluated using an Immobilized metal ion affinity based fluorescence polarization (IMAP) assay. Similar to the mammalian expression system, purified recombinant Bm-MPK1 from infected but unstressed SF9 cells did not show any kinase activity. To determine if recombinant Bm-MPK1 can be activated, infected SF9 cells were stressed with 400 μM sodium arsenate, 400 μM copper sulfate, or heat shock treatments at 37°C and 43°C. I have demonstrated that sodium arsenate treatment leads to the activation of recombinant Bm-MPK1 produced in infected SF9 cells (Figure 10). There was no activity for copper sulfate treatment, as the activity looks similar to unstressed Bm-MPK1. The heat shock 37°C treatment does not fully activate the recombinant Bm-MPK1 from infected SF9 cells...
The infected SF9 cells stressed at 43°C die (data not shown). In addition, the inactive recombinant Bm-MPK1 was significantly activated using the upstream activator MKK6 (Figure 11).

A western blot was performed to confirm the activation of Bm-MPK1 as evidenced by the dual phosphorylation of the highly conserved TGY active site. The blot analysis with the anti-phospho p38 antibody shows a 67 kDa band (Figure 9A) for each stress condition but with various intensities. The Bm-MPK1 band intensity for Arsenate stress (9A-lane 7), and 37°C (9A-lane 5) heat shock is very strong indicating that the protein Bm-MPK1 is phosphorylated at the TGY domain. The Bm-MPK1 fractions collected from copper stressed SF9 cells results in a band pattern (9A-lane 8) similar to unstressed cells (9C-lane 4) indicating low or no phosphorylation. The same protein samples were run on a western and probed with anti-GST antibody showing two bands with various intensity for 37°C heat shock and sodium arsenate treatment. These findings indicate that about 90% and 60% of the kinase proteins are activated (higher band) for arsenate treatment and 37°C heat shock treatments, respectively. These results are also supported by the IMAP assay results.

**Bm-MPK1 protein yield**

An excellent yield of recombinant Bm-MPK1 was obtained with approximately 3.25 mg of protein from a 100 mL culture. Recombinant Bm-MPK1 was eluted in 100 mL of elution buffer containing 25 mM HEPES, 10 mM reduced L-glutathione, 500 mM
NaCl, 1 mM DTT, 10% glycerol, and 1:100 Halt\textsuperscript{TM} Protease Inhibitor Cocktail by gravity flow. The protein concentration was 0.2 mg/mL in collected elutions. Proteins were concentrated approximately 15 fold. The concentration reading for concentrated elution (6.5 mL) was approximately 0.5 mg/mL.
DISCUSSION

Previous work in our lab has demonstrated that Bm-MPK1 can be inhibited by known p38 inhibitors compromising *B. malayi* anti-oxidative stress responses and leading to parasite death. In view of this, Bm-MPK1 is a potential anti-parasitic drug target for the treatment of lymphatic filariasis and other filarial diseases. In order to fully exploit the potential of Bm-MPK1 as a drug target, large quantities of Bm-MPK1 are required for drug screening campaigns and structural determination. To achieve this goal, I have established the SF9 cell/Baculovirus expression system for high-level production of recombinant Bm-MPK1. Illustrated a highly efficient SF9 cell/Baculovirus expression system for high-level production of recombinant Bm-MPK1.

The SF9 cell/Baculovirus expression vector system has many advantages compared to other eukaryotic systems such as the mammalian HEK 293-Freestyle cells for expressing heterologous proteins. The major advantage of the insect cell expression is that once Baculovirus containing an open reading frame infects SF9 cells and viral stocks are made, large quantities of SF9 cells can be grown and infected in one step. In contrast, mammalian expression systems such as HEK cells can only be transiently transfected with expression plasmids requiring time consuming plasmid preparation. The SF9 cell/Baculovirus system also generally produces much more recombinant protein than mammalian systems. Estimates have been made indicating that up to 30% of total SF9 cellular protein can be recombinant(4). My typical yields of Bm-MPK1 using SF9 cell/Baculovirus systems are \( \sim 400 \mu g \) per 20 mL culture, whereas for mammalian cell
expression, a similar yield would require 100-300 mL of culture. Another advantage of BAC system is that insect cells provide a full complement of eukaryotic posttranslational modifications thereby combining high expression levels with protein modification capabilities clearly superior to *E. Coli* systems.

In summary, I have successfully cloned a synthetic Bm-MPK1 open reading frame into a pFASTBAC vector and generated a Bm-MPK1/pFASTBAC-based Baculovirus via transposition. I have successfully expressed recombinant Bm-MPK1 in SF9 insect cells. Bm-MPK1 was successfully activated in cells by reactive oxygen species generated by sodium arsenate treatment and in vitro with the human upstream activator, MKK6. This expression system will be an important tool for producing high-levels of proteins supporting screening for potential enzyme inhibitors and for crystallography.
Figure 1: Nucleotide Sequence of SF9Bm-MPK1. The gene sequence was chemically synthesized at GenScript (Piscataway, NJ). The sequence is 1139 base pairs long and contains Sfil restriction enzyme sites at each end. The sequence for the 5'-end Sfil A site is GGCCTCAGCGCCGCA (highlighted in yellow) and for the 3'-end Sfil B site is GGCCGGGTCGGGCC (highlighted in green).
Figure 2: Generation of recombinant baculoviruses and gene expression with the Bac-To-Bac expression system. This approach for generating a recombinant baculovirus uses site-specific transposition with Tn7 to insert foreign genes into bacmid DNA propagated in *E. coli*. The Bm-MPK1 protein kinase ORF was cloned into a pFASTBAC vector to produce a Bm-MPK1/pFASTBAC plasmid vector, which was then transformed into DH10Bac competent cells. These cells contain the bacmid with a mini-attTn7 target site and the helper plasmid (12). The helper plasmid provides the transposition proteins recognizing the mini-Tn7 elements on the pFASTBAC plasmid and the bacmid (12).
Figure 3: DNA Gel Electrophoresis analysis of pFASTBAC plasmid containing a Bm-MPK1 Insert. λHindIII/HaeIII DNA markers (lane 1 & 8), Bm-MPK1/pFASTBAC plasmid construct (lanes 2-7) generated via DNA ligation and transformation. Plasmid DNA (7 μl) was added with loading dye (3 μl) and loaded on 1.5% agarose gel in 1x TAE buffer. EtBr was used to stain the gel. Black arrow indicates 5 kb pFASTBAC plasmid insert. Red arrow indicates 1.1 kb Bm-MPK1 insert.
Figure 4: Identification of correct transposition via DNA Gel Electrophoresis analysis: \(\lambda\)HindIII\(\Omega\) X HaeIII DNA markers (lane 1 & 13), Bm-MPK1/pFASTBAC-based Baculovirus (lanes 2-11), No template DNA control (lane 12). This gel shows the analysis of the PCR products generated to demonstrate correct transposition, using BAC virus DNA from different isolates as template. Red arrow indicates 3.7 kb Bm-MPK1/pFASTBAC-based Baculovirus construct.
Figure 5: Verification of Bm-MPK1 inserts in purified viral DNA isolates via SfiI restriction digestion: λHindIIIØX HaeIII DNA markers (lane 1 & 5), Bm-MPK1/pFASTBAC-based Baculovirus – QIAGEN (Digested- lane 2, Undigested – lane 6), Bm-MPK1/pFASTBAC-based Baculovirus- PROMEGA (Digested- lane 3, Undigested – lanes 7 & 8), BmJNK/pFASTBAC-based Baculovirus from another project- PROMEGA (Digested- lane 4, Undigested- lanes 9 & 10). Red arrow indicates the presence of 1.1 kb Bm-MPK1 insert
Figure 6: Comparison between uninfected and infected SF9 cells. (A) Uninfected SF9 cells. (B) SF9 cells infected with Bm-MPK1/pFASTBAC-based Baculovirus, Day 3 after infection. Cells were observed using a light microscope and phase contrast at a 100X magnification. Images were taken using an OptixCam Microscope camera.
Figure 7: Production of GST-Bm-MPK1 from SF9 Insect cells.  (A) Western Blot of GST-Bm-MPK1 developed using anti-GST goat polyclonal IgG antibody (1: 1000). MagicMark™ XP Western Protein Standard (lane 1), 10x- Day 2 (lane 2), 10x- Day 3 (lane 3), 10x- Day 4 (lane 4), 100x- Day 2 (lane 5), 100x- Day 3 (lane 6), 100x- Day 4 (lane 7), 1000x- Day 3 (lane 8), 1000x- Day 4 (lane 9), and Negative Control (lane 10). (B) Coomasie Blue stained 4-12% Bis-Tris gel. 10x- Day 2 (lane 1), 10x- Day 3 (lane 2), 10x- Day 4 (lane 3), 100x- Day 2 (lane 4), 100x- Day 3 (lane 5), 100x- Day 4 (lane 6), 1000x- Day 3 (lane 7), 1000x- Day 4 (lane 8), Negative Control (lane 9), and Novex® Sharp Standard (lane 10).
Figure 8: Purification of GST-Bm-MPK1 from SF9 Insect cells. Molecular weight markers; MagicMark™ XP Western Protein Standard & Novex® Sharp Standard (lane 1), Crude Extract (lane 2), Flow through #2 (lane 3), Wash 1 (lane 4), Wash 5 (lane 5), Eluent-1 (lane 6), Eluent-2 (lane 7), Eluent-3 (lane 8), Eluent-4 (lane 9), and Eluent-5 (lane 10) [A] Western blot of GST-Bm-MPK1 developed using anti-GST goat polyclonal IgG antibody (1:1000). [B] Coomassie Blue stained 4-12% Bis-Tris gel
Figure 9 Dual Phosphorylation of Bm-MPK1 in Crude Extracts. [A] Western blot developed using phopho-p38 rabbit polyclonal IgG antibody (1:2000, Santa Cruz Biotechnologies). [B] Western blot developed using anti-GST goat polyclonal IgG antibody (1:1000). MagicMark™ XP Western Protein Standard (lane 1), Bm-MPK1 from mammalian cells- Positive Control (lane 2), Uninfected SF9 cells-Negative Control (lane 3), Infected SF9 cells/Inactivated Bm-MPK1 (lane 4), activated Bm-MPK1 at 37°C (lane 5), activated Bm-MPK1 at 43°C (lane 6), activated Bm-MPK1 with 400 μM Arsenate (lane 7), activated Bm-MPK1 with μM Copper sulfate (lane 8), Uninfected SF9 cells stressed with 400 μM Arsenate (lane 9), and Uninfected SF9 cells stressed with 400 μM Copper sulfate. Each stress treatment was performed for 4 hours with a buffer containing 500 mM NaCl.
Figure 10: Enzymatic activity of recombinant Bm-MPK1 using IMAP assay: The recombinant Bm-MPK1 from infected SF9 cells was activated by stressing the cells with various treatments 4 hours prior to harvest. The blue diamonds represent unstressed Bm-MPK1. Purple X represent activated Bm-MPK1 by 400 μM copper sulfate. Red squares represent Bm-MPK1 activated by 37°C heat shock. Green triangles represent activated Bm-MPK1 by 400 μM sodium arsenate. The assay was read using a Synergy 2 Microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 528 nm.
Figure 11: Activation of recombinant Bm-MPK1 using MKK6: Purified Bm-MPK1 was incubated with 0.30 μg of p38 upstream kinase MKK6 in a 1 mL reaction with 1 mM DTT, 10 μM ATP, 5X assay buffer and distilled water. The reaction was incubated for 30 minutes at 27°C. The reaction (10 μl) was serially diluted to check the activity using IMAP assay. The activity was read at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Blue diamonds represent inactive Bm-MPK1. Red square represents activated Bm-MPK1 by MKK6.
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12. Guide to Baculovirus Expression Vector Systems (BEVS) and Insect Cell Culture Techniques (Invitrogen)