# Functional analysis of SdNP; A protein of unknown function in *Setariadigitata*

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***Abstract*** - *Lymphatic filariasis, also known as elephantiasis, is a deleterious human disease caused by the parasitic nematode Wuchereriabancrofti. If left untreated, the infection can develop into elephantiasis which can only be managed with surgical excision. Studying the parasitology of W. bancrofti is extremely challenging because there are significant complications in procuring adult parasites from the lymphatic system. Therefore, cattle filarial parasite Setariadigitata was used as a model organism as it shares homologous counterparts with W. bancrofti and can be easily cultured in the laboratory.*

*A novel protein called SdNP (S. digitata novel protein) was identified from S. digitata that may play a significant role in pathogenesis. Recently, SiRNA inhibition studies showed that inhibiting SdNP expression severely impairs adult parasite's locomotion, consequently leading to death of the adult worm. The research work presented here describes in vitro characterization of SdNP. Built on Bioinformatic analysis, an enzyme coupled ATPase assay was used to detect the ATPase activity of the putative kinase motifs. Our results confirmed that SdNP is a phosphor-protein that can bind and hydrolyze ATP to ADP and inorganic Pi in a substrate-independent manner. In addition, native-PAGE and gel-filtration chromatography results showed that SdNP forms a stable tetramer in vitro. The fact that SdNP is unique to parasitic nematodes and is essential for the survival of adult worm suggests that functional analysis of SdNP could pave the way to design effective clade-specific drugs against filariasis.*

***Keywords-****Setariadigitata*, SdNP, ATPase activity

## I. INTRODUCTION

Lymphatic filariasis is a deleterious disease mainly caused by parasitic nematodes *Wuchereriabancrofti*, *Brugiamalayi* and *Brugiatimori*(Rodrigo, Dassanayake and Voronin, 2014; Perumal, Gunawardene and Dassanayake, 2015).Among these species of human lymphatic filarial parasites, 91% of the disease condition is caused by *W. bancrofti* (Mendoza *et al*., 2009). The complicated parasitology of *W. bancrofti* creates difficulties in treating lymphatic filariasis. The main limitations of the research concerning *W.bancrofti* include difficulty in accessing of adult parasite from the infected individuals and culturing of nocturnally periodic microfilariae *in vitro* (Perumal, Gunawardene and Dassanayake, 2015). Therefore, a model organism which closely resembles *W. bancrofti*is widely used to study the parasitology of *W. bancrofti*.

Amongst the various types of parasitic nematodes, *S. digitata*species are used as model organism as they are highly available in Sri Lanka and have similar morphological, histological and antigenic features that resemble *W. bancrofti*. In addition, the availability and accessibility of the worms are higher than human parasite (Rodrigo *et al*., 2014).Bioinformatic analysis supports that animal filarial parasite *S. digitata* shares a greater number of homologous genes with *W. bancrofti.*

Identification and characterization of parasitic nematode-specific gene can be a tool for the analysis of biology and biochemistry of parasitic nematodes. The uncharacterized novel, parasitic nematode-specific gene called *S.digitata* novel gene (*SdNP*) which shares 88% homology to *W. bancrofti*novel gene (*WbNP*) is chosen for analysis from all the identified and sequenced genes of *S. digitata.* SdNP was identified through cDNA library screening and open reading frame analysis. SdNP has significant homology to structurally and functionally annotated sequences of parasitic nematodes. The identified ORF code for SdNP (consists of 205 amino acids) which plays a major role in parasitism (Rodrigo, 2013) of the worm.

The parasitic nature and the biological properties of the nematode were investigated recently with the help of Bioinformatics. Sequence alignment of SdNP indicates that SdNP has a putative chain A sequence of muscle specific Twitchin kinase-like domain from *C. elegans*, putative calcium dependent protein kinase domain from *A. thaliana* and may contain several phosphorylation sites and calcium binding sites. Phylogenetic analysis indicated that *S. digitata* is closely related to the human filarial parasite *W. bancrofti*.

## II. METHODOLOGY

*A. Enzyme-coupled assay and ATP binding studies confirmed that SdNP can bind and hydrolyze ATP in vitro.*

Built on our sequence analysis results, we have investigated the function of the putative ATPase motif of SdNP. First, the ability of SdNP to bind with ATP was investigated using fluorescence spectrophotometry (Figure 1A). Since SdNP contains several conserved tryptophan residues, the fluorescent property of the amino acid is used to study interactions with ATP. Our results indicate that SdNP can bind ATP with micro molar affinity.

Next, the conversion of ATP to ADP by SdNP in substrate independent manner was monitored by using a coupled enzyme assay (Figure 1B). This assay specifically monitors the hydrolysis of the beta-gamma bond of ATP by quantifying the amount of ADP produced over time. ADP, the hydrolysis product, was detected and quantified by a series of coupled enzymatic reactions resulting in the oxidation of NADH to $NAD^+$. The decrease in NADH over time is proportional to the amount of ADP generated, and was monitored by the decrease in UV absorbance at 340 nm. The assay results confirmed that SdNP is an ATPase, which can hydrolyze ATP to ADP.

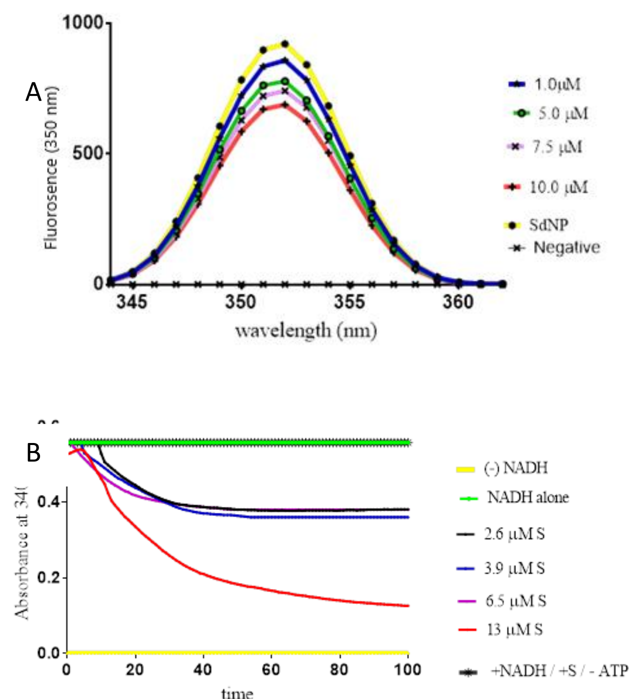


Figure 1: A. Fluorescence spectra (350 nm) for SdNP (0.65µM) binding to different concentrations of ATP (1.0 µM, 5.0 µM, 7.5 µM, 10.0 µM). B.SdNP can hydrolyze ATP in a substrate independent manner. Enzyme coupled ATPase assay was conducted with various concentrations of SdNP as a function of time to investigate the ATPase activity of SdNP

*B. Pro-Q Diamond phosphoprotein gel stain method confirmed that SdNP is a phosphoprotein.*

As mentioned, Bioinformatics analysis suggests that SdNP contains several phosphorylation sites. To check whether SdNP is a phosphoprotein, Pro-Q diamond phosphoprotein gel stain was used, which is forphosphorylated proteins only. The staining results of affinity-purified SdNP (from bacteria) and dephosphorylation of the protein using calf-intestinal phosphatase, followed by Pro-Q staining confirmed that SdNP is a phosphoprotein (Figure 2). Further experiments need to be carried out to investigate whether the observed ATPase activity of SdNP contributes to auto-phosphorylation of the protein.

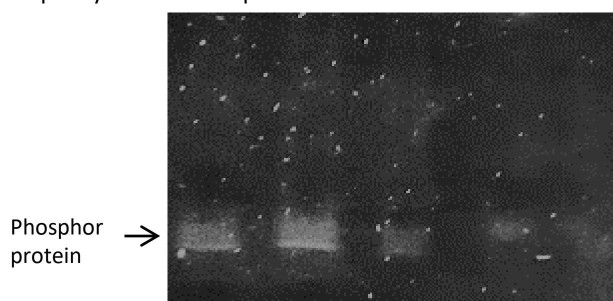


Figure 2: Pro-Q diamond stained gel image under UV transilluminator (lane 1 and 2- SdNP (untreated), lane 3 and 4- Alkaline phosphatase treated SdNP and lane 5- BSA)

*C. Electrophoretic Mobility Shift Assay (EMSA) and Size Exclusion Chromatography (SEC) confirmed that SdNP is an oligomer (approximately a tetramer) in vitro.*

SEC and EMSA are widely used techniques to analyze/separate a wide range of molecules of different sizes. We used the two techniques to independently examine the oligomerization state of SdNP. First, a 10% native gel was run to enhance separation of SdNP monomer and stable oligomers that may exist *in vitro*. We have observed a protein band in the lane containing native SdNP, close to the molecular weight marker, BSA, (~66 KDa) (Figure 3B). Denatured SdNP sample was used as a control to identify the location of the monomeric SdNP in the gel. Our native gel results demonstrate that SdNP forms a stable oligomer (approximately a tetramer) *in vitro*.

To confirm EMSA results, we carried out SEC analysis for SdNP (Figure 3A) along with BSA and *Taq* DNA polymerase as the molecular weight markers. In SEC, the high molecular weight proteins elute first with a short retention time. Considering the reciprocal relationship between the log value of molecular weight and retention

time, the molecular weight of native SdNP was calculated using the retention time of the molecular weight markers. Based on the calculated values, we confirmed that SdNP exists as a tetramer *in vitro*.

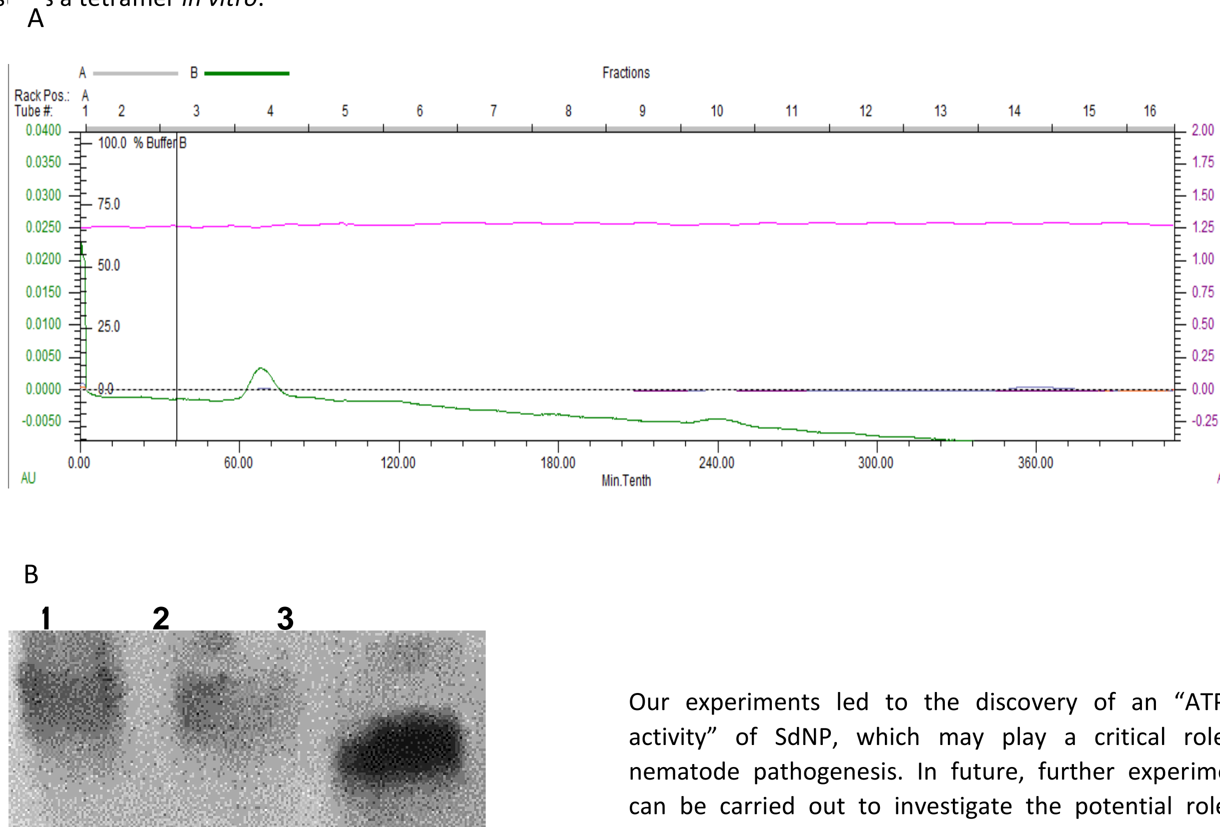


Figure 3: A. SEC analysis of SdNP. The calculated molecular weight of SdNP is ~90 kD. B. EMSA results (lane 1- SdNP incubated with ATP, lane 2- SdNP without ATP, lane 3- boiles BSA (~66 kDa)). The size of monomeric SdNP is ~23 kDa

## III. CONCLUSIONS AND FUTURE DIRECTIONS

Eradication of filarial diseases caused by filarial nematodes like *Wuchereriabancrofti*, *Brugiamalayi* and *Brugiatimori* will be extremely challenging with current technology. No vaccines are available, vector control programs have ended or are facing insect resistance, and drugs are largely ineffective against the worm's adult stage. Current drugs can only effectively eliminate the worm's larval stages, but their broad use also increases the likelihood of accelerated drug resistance. Therefore, biological validation of nematode proteins is of critical importance to develop effective drugs predominantly against worm's adult stage to completely eradicate lymphatic filariasis in human and livestock. Therefore, understanding their pathogenesis is of critical importance to irradiate such deleterious diseases. To address this issue, we have investigated the role of pathogenic protein SdNP, which is unique to parasitic nematodes.

Our experiments led to the discovery of an "ATPase activity" of SdNP, which may play a critical role in nematode pathogenesis. In future, further experiments can be carried out to investigate the potential role of ATPase activity in pathogenesis, which could pave the way to design effective pathogen specific drugs particularly against adult filarial worms. Furthermore, understanding the assembly characteristics and elucidating the structure of native SdNP will also expand our knowledge about the pathogenic nature of such proteins. An attempt is underway to elucidate the crystal structure of the SdNP bound to an ATP analog to elucidate the role of key amino acids involve in catalysis.

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