Cloning and characterization of a small heat shock protein cDNA clone of *Wuchereria bancrofti*

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A 426 bp cDNA encoding a predicted small heat shock protein (smHSP) was isolated from *Wuchereria bancrofti* lambda Zap L<sub>2</sub> cDNA expression library by immunoscreening with microfilaremic sera. The open reading frame of the cDNA clone encodes a predicted protein of 142 amino acids (aa), which had high sequence identity with other nematode smHSPs. The homologous regions conserved in several different nematode species reflect its importance in parasites that require mammalian host as a part of their development. SmHSPs and alpha-crystallin constitute a family of related molecular chaperons that exhibit striking variability in size ranging from 10 to 43 kDa. Here we describe the cloning and characterization of this cDNA clone encoding predicted 15.5 kDa smHSP of *W. bancrofti*.

The small heat shock proteins (smHSPs)<sup>1</sup> whose relationship to the alpha-crystallin family was first noted in *Drosophila*<sup>4</sup> and in *Caenorhabditis elegans*<sup>2</sup> have been found in all kingdoms of life and have an evolutionary history dating back to the common ancestors of all present-day living cells. Prokaryotic members of the superfamily are often in the 12 to 18 kDa range, but many eukaryotic homologues are significantly larger; smHSPs range over 12 to 40 kDa and generally exist as a large multimeric assembles in solution<sup>3-5</sup>. The first three-dimensional structure determined for a smHSP (HSP 16.5) from the thermophile *Methanococcus jannaschii*, revealed a spherical array of 24 subunits of a polypeptide consisting largely of ␤-sheets<sup>6</sup>. Several contrasting models have been proposed to account for the arrangement of subunits within the smHSP aggregate<sup>7</sup>. Cryoelectron microscopy of smHSPs/alpha-crystallin proteins from several sources has shown that oligomer structure ranges from well defined to variable, leading to the idea that structural plasticity elicits low specificity and permits binding of different target proteins<sup>8-10</sup>. Several molecules of denaturing proteins, present in an unstable molten globule state, interact with a single oligomer when chaperoning occurs. The proteins are protected from irreversible aggregation under stress, their activity may be preserved, and they either refold spontaneously or with the assistance of other chaperons upon release<sup>11-15</sup>. In multicellular organisms, smHSPs are among the most highly induced HSPs under stress conditions, and some smHSPs may also be subject to developmentally regulated expression in the absence of stress, as first noted in *Drosophila* studies<sup>16</sup>. In different organisms, the number of smHSPs is variable, for example, there are at least 20 in *Arabidopsis thaliana* (*Arabidopsis* database), 28 in human (NCBI), and 4 in *Drosophila melanogaster* (*Drosophila* database), 5 in mouse (NCBI), 10 in *C. elegans* (NCBI) and 5 in *Saccharomycarces cervisiae*<sup>17-19</sup>. The large HSPs have been implicated in major physiological processes such as cell division, transcription protein folding, transport and membrane function<sup>20-23</sup>. They can form large oligomeric complexes<sup>24-27</sup>, and have a role in thermotolerance in mammalian cells and *Drosophila*<sup>28,29</sup>, but not in yeast cells<sup>30</sup>. SmHSPs bind specifically to cytoskeleton elements such as actin and to intermediate filaments such as desmin, vimentin and glial fibrillar acidic protein<sup>30,31</sup>. It has also been reported that smHSPs modulate apoptosis through the Fa8/ApoI receptor<sup>32</sup> and are involved in cell growth and differentiation<sup>33</sup>.

In the present study, we report the identification, cloning and characterization of a cDNA from *Wuchereria bancrofti* L3 cDNA library, encoding for a predicted smHSP. *Brugia malayi*, *W. bancrofti* and *Brugia timori* are the causative agents of human lymphatic filariasis, which is a major public health problem in the tropical and subtropical world, with at least 120 million people infected and majority of them suffering with disease manifestations<sup>34</sup>. In India alone, around 25 million people harbour microfilariae in blood (microfilaremic), while 19 million people suffer from disease manifestations<sup>34</sup>. An approach of immunoscreening stage-specific cDNA libraries of *W. bancrofti* was undertaken to identify and clone the genes of parasite origin, which may have a potential in diagnosis and designing of drugs as a candidate for vaccine and in identification of novel proteins.

*W. bancrofti* L3 cDNA library (kindly provided by Steven Williams, Smith College, MA, USA) was immunoscreened with pooled microfilaricemic and endemic normal sera (prepared using 10 sera from each group) collected from Sevagram and surrounding villages in Maharashtra, India. Immunoscreening of the cDNA library was per-

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**Table 1.** Representative members of smHSP protein family

<table>
<thead>
<tr>
<th>Organism</th>
<th>Accession number in GenBank</th>
<th>Number of amino acids</th>
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<tbody>
<tr>
<td><em>Brugia pahangi</em></td>
<td>S57399</td>
<td>177</td>
</tr>
<tr>
<td><em>Acanthocheilonema viteae</em></td>
<td>S29691</td>
<td>179</td>
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<td><em>Onchocerca volvulus</em></td>
<td>S29693</td>
<td>165</td>
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<tr>
<td><em>Caenorhabditis elegans</em></td>
<td>NP_050165</td>
<td>149</td>
</tr>
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<td>AAC63387</td>
<td>150</td>
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<td><em>Bombyx mori</em></td>
<td>AAG30944</td>
<td>186</td>
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<td><em>Anopheles gambiae</em></td>
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<td>211</td>
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<td>AAC63387</td>
<td>206</td>
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<tr>
<td><em>Squalus acanthias</em></td>
<td>P02512</td>
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<tr>
<td><em>Poeoilioptis lucida</em></td>
<td>AAB46593</td>
<td>201</td>
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formed according to standard procedure\textsuperscript{35}. In brief, the recombinants were plated at approximately 800 plaques per 80 mm petri dish, grown at 42°C for 3 h and overlaid with nitrocellulose (Amersham, Biosciences, USA), impregnated with 1 mM isopropyl-\(\beta\)-thiogalactopyranoside and grown at 37°C for an additional 4 h. The filter was then removed, blocked with 1% bovine serum albumin (BSA) and reacted with optimally diluted (1:100) pooled serum that was earlier absorbed with \textit{Escherichia coli} phage lysate. The filter was then washed and incubated with alkaline phosphatase-conjugated goat anti-human immunoglobulin G followed by washing and further incubation with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate (Sigma Chemicals Co, USA).

Immunopositive plaques identified during primary screening were purified by secondary and tertiary screening using the same sera. cDNA clones reacting with microfilaricemic sera were randomly selected for sequencing and the insert length of each selected clone was examined by PCR with primers SP6 (5′ ATT TAG GTG ACA CTA TAG AA 3′) and T7 (5′ TAA TAC GAC TCA CTA TAG GGA 3′). The amplified PCR products were cloned directly into 3′ T overhangs of the pCR TOPO vector (Invitrogen, USA) and transformed into \textit{E. coli} DH5\(\alpha\) cells. The selected clones were sequenced by Dideoxy termination method on ABI 377 automated DNA sequencer (Bangalore Genei, India) and all sequences were then analysed for coding probability with DNA tools program\textsuperscript{36}. Initial comparison against the GeneBank proteins database was performed using the BLAST network server at the National Centre for Biotechnology Information\textsuperscript{37,38}. Multiple protein sequences were aligned using default settings of the web-based GeneBee software (http://www.genebee.msu.su/services/malign_reduced.html).

Protein sequences were aligned and the phylogenetic tree was constructed using Phylip program of ClustalW (version 1.86). Accession numbers of the smHSP sequences in the GenBank database used for comparison are listed in Table 1.

Immunological screening of recombinant clones of \textit{W. bancrofti} with microfilaricenic sera resulted in the identification of 20 recombinants. A single pass analysis revealed that 8 out of 20 clones were distinct. Five out of 8 recombinants showed high homology to transforming growth factor \(\beta\) (860 bp), 18S rRNA gene (1.75 kbp) and threodoxin peroxidase-2 (TPX-2, 400 bp) of \textit{Brugia malayi}, sulphur globule protein (426 bp) of \textit{Onchocerca volvulus} and to the smHSP (covered in the present communication) respectively.

The cDNA sequence obtained for \textit{W. bancrofti} smHSP was 426 nucleotides in length and encoded a predicted protein of 142 amino acids residues, with a predicted molecular weight of 15.5 kDa and isoelectric point 6.1. The initial database searches by BLAST suggested a 45\% identity and 53\% similarity with \textit{O. volvulus} smHSP (Figure 1). The amino acid sequences of 11 representative members of smHSPs of other nematode species as a revealed by a search of the GenBank database were aligned (Figure 2). It was found that all the amino acids constituting alpha-crystallin domain of smHSP, were conserved in \textit{W. bancrofti}-deduced amino acid sequences like other nematodes. The phylogenetic tree constructed from the sequence of \textit{W. bancrofti} smHSP and identified members of smHSPs indicated that the \textit{W. bancrofti} smHSP is residing in the same clade along with other closely related nematodes (Figure 3).

In conclusion, the cDNA clone obtained from immunoscreening of \textit{W. bancrofti} L3 cDNA library, encodes for

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<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
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<tbody>
<tr>
<td>Wb</td>
<td>LVDPGCRNSARATTMLAFPSLRWHNFPIEGRRTVGGLMNLRLLEDFED----SLQPF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ov</td>
<td>QTSPME--RFIVNLDDSTFDRSSRPLHSVAPY</td>
<td></td>
<td></td>
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\(70 \quad 80 \quad 90 \quad 100 \quad 110 \quad 120 \)

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<th></th>
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<tbody>
<tr>
<td>Wb</td>
<td>W-------ECSTTSKNSFGEIVDNKDSFGIRLVDVHFPRPEELSVKMDQGRFLFVEGHEER</td>
</tr>
<tr>
<td>Ov</td>
<td>WLHQPELNECNIG--NSLGEVINEOKDFAVRADVSHHPKELSVNVRDELVIEGHEER</td>
</tr>
</tbody>
</table>

\(40 \quad 50 \quad 60 \quad 70 \quad 80 \)

\(90 \quad 100 \quad 110 \quad 120 \quad 130 \quad 140 \)

Figure 1. Pairwise alignment of deduced amino acid sequence of \textit{Wuchereria bancrofti} smHSP with that of \textit{Onchocerca volvulus} smHSP (GenBank accession no. Ov S29693). Residues that are identical to \textit{W. bancrofti} smHSP are indicated by double dots and gaps are represented by (--) Wb, \textit{W. bancrofti}; Ov, \textit{O. volvulus}.
Figure 2. Multiple alignment of deduced amino acid sequence of *W. bancrofti* smHSP with sequences of other nematode species, using default settings of web-based GeneBee software (http://www.genebee.msu.su/services/malign_reduced.html). Residues that are identical to *W. bancrofti* smHSP are indicated by asterisks. Comparative similarities among different sequences are shown by dots and plus sign. Gaps introduced into sequences to optimize alignments are represented by (---). See Table 1 for sequence references.
smHSP protein with conserved domain of alpha-crystallin.

This is the first report on the presence of smHSP in human lymphatic filarial parasite, W. bancrofti.


Regeneration and mass multiplication of *Arachnis labrosa* (Lindl. ex Paxt.) Reichb: A rare and threatened orchid

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Asymbiotic seed germination of *Arachnis labrosa* was achieved by culturing immature embryos/seeds 16–18 weeks after pollination. Better germination was recorded on medium enriched with sucrose (3%), coconut water (15%) and α-naphthalenacetic acid (NAA) and N6-benzyl adenine (BA) (20.0 + 16.0 µM in combination). After 23–25 days of culture, ~ 81% germination was recorded on Mitra *et al.* medium followed by Murashige and Skoog (66%), and Knudson ‘C’ (55%). The protocorm-like bodies differentiated into multiple shoot buds after 20 days of culture on regeneration medium containing sucrose (3%), coconut water (15%), and NAA and BA (10.0 and 8.0 µM respectively, in combination). After maintaining for 10–12 weeks on regeneration medium, the rooted plants were transferred to potting mix and acclimatized before transfer to natural habitat.

ASYMBIOTIC seed germination has emerged as an important tool for propagating a large number of orchid species and hybrids. The orchid seed can also germinate in vitro prior to reaching maturity. The technique is variously referred to as ovule/embryo/green pod/green fruit culture, which ensures better germination frequency and favours the production of virus-free seedlings at a faster rate. The medium used for asymbiotic germination is more complex than that for symbiotic germination, as all organic and inorganic nutrients and sugars must be in a form readily available to the orchid, without the intermediary fungus. Wimber first formulated and described the shoot-tip-based procedure for mass and rapid clonal propagation of *Cymbidium* species. Incidentally, orchid has been the first floricultural crop to be successfully propagated through shoot-tip culture. This novel technique has generated tremendous interest among orchid growers and revolutionized orchid-based industries world over. *Arachnis labrosa* is a monopodial epiphytic orchid of the *Vanda* group. The flower is pale yellow with irregular dark brown marking and is also called spider orchid. In India, the distribution of this orchid is restricted to few patches in Arunachal Pradesh and Nagaland. In this communication we report the establishment of a feasible protocol of in vitro regeneration of plantlets and mass multiplication of *A. labrosa* by green pod culture.

*A. labrosa* cultures were initiated using immature embryos from green pods in different developmental stages (8–20 weeks after pollination) (WAP) at two week intervals. Seeds were scooped out from sterilized pods and cultured on different media like Knudson C, Mitra *et al.* and Murashige and Skoog (MS) containing (NAA) (0–30 µM) (BA) (0–24 µM) singly or in combination (Table 1), sucrose (0–3%; w/v) as organic carbon source and coconut water (0–15%; v/v). After autoclaving at 121°C and 1.05 kg cm⁻² for 20 min, pH of the media was adjusted to 5.6 using 0.1 N NaOH and HCl. Cultures were maintained at 25 ± 2°C under cool white fluorescent light at 40 µmol m⁻² s⁻¹ and 12/12 h photoperiod. Cultures were sub-cultured at 4–5 week intervals unless mentioned otherwise. Experiments were repeated at least thrice.

Protocorm-like bodies (PLBs) developed from the cultured immature embryos were maintained on the same basal initiation medium for further development and differentiation. For regeneration and culture multiplication, the differentiated PLBs were separated and cultured on different basal media containing various levels of IAA (0–33 µM), NAA (0–30 µM), BA (0–24 µM) and Kn (0–27 µM) singly or in combination, in conjunction with sucrose (3%) and coconut water (15%).

The tiny plantlets measuring 3–4 cm were transferred to perforated plastic pots of 10 cm diameter with potting mixture containing charcoal pieces, brick pieces, coconut

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