Cloning and expression of a small heat and salt tolerant protein (Hsp22) from *Chaetomium globosum*

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The present study reports molecular characterization of small heat shock protein gene in Indian isolates of *Chaetomium globosum*, *C. perlucidum*, *C. reflexum*, *C. cochlioides* and *C. cupreum*. Six isolates of *C. globosum* and other species showed a band of 630bp using specific primers. Amplified cDNA product of *C. globosum* (Cg 1) cloned and sequenced showed 603bp open reading frame encoding 200 amino-acids. The protein sequence had a molecular mass of 22 kDa and was therefore, named Hsp22. BlastX analysis revealed that the gene codes for a protein homologous to previously characterized Hsp22.4 gene from *C. globosum* (AAR36902.1, XP 001229241.1) and shared 95% identity in amino acid sequence. It also showed varying degree of similarities with small Hsp protein from *Neurospora* spp. (60%), *Myceliophthora* sp. (59%), *Glomerella* sp. (50%), *Hypocrea* sp. (52%), and *Fusarium* spp. (51%). This gene was further cloned into pET28a (+) and transformed *E. coli* BL21 cells were induced by IPTG, and the expressed protein of 30 kDa was analyzed by SDS-PAGE. The IPTG induced transformants displayed significantly greater resistance to NaCl and Na$_2$CO$_3$ stresses.

Keywords: *Chaetomium globosum*, Gene cloning, Small heat shock protein, Stress tolerance

Heat shock proteins (HSPs) are soluble and intracellular proteins which act as molecular chaperones by helping refold misfolded proteins and assisting in their elimination if they become irreparably damaged$^1$. HSPs also help to prevent accumulation of protein precursors, accelerating protein trafficking, and absorb complexes with unfolded proteins to maintain their transport ability. Heat shock response is found universally from bacteria to human, and Hsp genes are among the most evolutionarily conserved genes$^2$. Disruption of normal cellular processes can cause a rapid increase in the synthesis of a group of HSP family proteins$^3$. Synthesis of HSPs has been studied in Yeast$^4$ and filamentous fungi$^5$. HSPs can be classified into 3 categories according to their molecular weight: (i) high-molecular weight (69–120 kDa), (ii) medium-molecular weight (39–68 kDa), and (iii) low molecular weight (below 38 kDa)$^6$. Small HSPs are a group of homologous proteins of 15–30 kDa that contain an alpha-crystalline domain and protect other proteins during physiological stress.

*Chaetomium globosum*, an important biocontrol fungus, has ability to antagonize other fungi$^7$–$^{10}$. Antifungal properties of *C. globosum* have been related to production of many kinds of antibiotics and ergosterols to inhibit the growth of pathogens$^{11}$–$^{14}$. There are studies on physiology of *C. globosum*. However, its potential applications on bio-control mechanism are few. Ability of some of *Chaetomium* spp. to overcome extreme environments facilitates their presence in varied environment. In the present study a small heat shock protein gene was cloned and its expression in response to heat and salt stress has been investigated.

Materials and Methods

Strains and culture conditions—Pure cultures of *Chaetomium globosum* isolates with Indian Type Culture Collection (ITCC) accession no. Cg1(1627); Cg2(1620); Cg3(2401); Cg4(2034); Cg5(6215); Cg6(6211); Cg7(6214); Cg8(6218); Cg9 (6212); Cg10(6220); Cg11(6216); Cg12(6221); Cg13(6219); Cg14(6213); Cg15(6217) and one isolate each of *C. reflexum* (5002), *C. cupreum* (4600), *C. cochlioides* (3346) and *C. perlucidum* (6009) were isolated from different soil and leaf samples collected from different locations of India. Cultures were maintained
on potato dextrose agar (PDA) medium (potato infusion 200 g, dextrose 20 g, and water 1,000 ml) at 4 °C. Each Chaetomium isolate was grown in potato dextrose broth (PDB) at 28 °C with shaking for 72 h. The mycelium was then subjected to heat shock at 37 °C for 3 h. harvested by centrifugation (10 min at 10,000 g), washed twice with distilled water, and stored at -70 °C for RNA isolation. Total RNA was isolated from Chaetomium mycelium using trizol reagent (Invitrogen) and treated with DNase (RNase free, promega) at 37 °C for 30 min, then extracted by phenol and chloroform, and precipitated using alcohol. Single stranded cDNA was synthesized from 1 µg of total RNA at 42 °C for 60 min with 200 U of reverse transcriptase (superscript II, Invitrogen). The synthesized single stranded cDNA acted as template for amplifying HSP gene in Chaetomium spp.

Amplification of HSP gene in Chaetomium spp—HSP gene amplification in different Chaetomium spp., was carried out by left forward primer (HSPF: 5′-GACATGGATCC-3′) (Bam H I site is underlined) and reverse right primer (HSPR: 5′-TAATGCTCGAGTTGATAGCAACACGGCGCGC-3′) (Xho1 site is underlined) synthesized using NCBI data base (AY491980). Twenty µL reaction mixture contained 2 µL 10 × Taq PCR buffer; 0.4 µL dNTP (10 mmol/L); 0.5 µL primer HSPF (20 µmol/L), HSPR (20 µmol/L), 1 µL template cDNA, 2 U Taq polymerase, and dH₂O to make up volume 20 µL. PCR program was: 2 min at 94 °C; 20 s at 94 °C, 30 s at 64 °C, 90 s at 72 °C for 30 cycles. After the last cycle, the amplification was extended to 10 min at 72 °C. The amplified products were separated by agarose gel electrophoresis and fungal isolates showing HSP gene amplification were screened15.

Cloning of HSP gene and construction of expression vector—Chaetomium globosum isolate (Cg1) which showed HSP gene amplification was selected and its amplified product was cloned into pGEMT (Promega) vector following the method given in manual supplied with Promega catalog no. A1360. The cloned product was sequenced and transformed into E. Coli strain XLBlue. The recombinant pGEMT along with pET 28a+ expression vector were digested separately by Bam H1and Xho1. Digested product of recombinant pGEMT was gel eluted, purified and ligated into pET 28a(+) and finally transformed into E. coli XLBlue. Recombinant pET HSP were screened in Luria broth (LB) medium containing 50 mg/L ampicillin. The transformants were screened by colony PCR and double enzyme digestion using Bam H1 and Xho1.

Induced expression of recombinant vector pET-HSP in BL21—E. coli BL21, an expression host was transformed with recombinant vectors pET-HSP, isolated from transformed host XLBlue. Overnight (16 h) grown culture of transformed E. coli BL21 was used for inoculating fresh 100 mL of LB when the optical density at 600 nm (OD₆₀₀) reached 0.5, expression of the fusion protein was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM and incubated at 37 °C for 1 h, 2 h, 3 h, 4 h and 5 h. The collected cells were added with 1 × loading buffer, boiled for 5 min, and centrifuged 10 min at 8000 rpm. Thereafter, the supernatants were loaded onto the slab gel of 12% SDS-PAGE. Gel was stained with Coomassie Brilliant Blue G-250. The E. coli BL21 pET-HSP showing expression of fusion protein were selected for further studies.

Assay of recombinant E. coli under heat shock stress—To determine heat tolerance of HSP gene in vivo, wild type BL21, pET BL21 and HSP-pET BL21 were given heat stress. E. coli cultures were incubated at 37 °C until cells reached mid log phase (OD₆₀₀=0.5) before induction with 1 mM IPTG for an additional 2 h. Concentration of these cultures was identified to OD₆₀₀=0.5 and then diluted serially (1:10, 1:100, and 1:1000, respectively). Five micro liters of each sample was spotted onto Yeast Tryptophan (YT) plates with or without 1 mM IPTG and incubated at 37 °C for 15 h, 2 h, 3 h, 4 h and 5 h. The collected cells were added with 1 × loading buffer, boiled for 5 min, and centrifuged 8000 rpm. Thereafter, the supernatants were loaded onto the slab gel of 12% SDS-PAGE. Gel was stained with Coomassie Brilliant Blue G-250. The E. coli BL21 pET-HSP showing expression of fusion protein were selected for further studies.

Bioinformatic analysis of HSP gene—Multiple alignment of amino acid sequence of HSPs gene from different organisms was conducted. Small heat shock protein of Chaetomium globosum sample (Cg1) and from other fungi viz., C. globosum (AAR36902), Neurospora crassa (XP_332056.1), Metarhizium anisopliae, (ABD49719.1) Fusarium lichenicola (ACP18865.1), Neurospora tetrasperma (EEO52865.1), Hypocrea lixii (AAX55622.1), Hypocrea virens (ABO32163.1) and Glomerella graminicola (EFQ28752.1A) etc., were aligned by Clustal X program. Phylogenetic tree was drawn by Mega 4 program (http://www.megasoftware.net; MEGAV.4)
overnight (16 h) in LB medium at 37 °C. For stress tolerance, LB was amended with 1M and 5M NaCl and 6%, 8% and 10% Na₂CO₃. Experiment was run in triplicate in two sets. All LB flasks were inoculated with overnight grown culture of transformant (OD 600 of cells reached 0.4-0.6), then one set of flasks was induced with IPTG to final concentration of 1.0 mmol/L, while other set was left uninduced and then incubated at 37 °C for 1 h. After treatments, stressed cells were centrifuged at 8,000 g for 2 min, resuspended in 10 ml sterile water, and 100 µL of each sample was spread on LA medium (supplemented with 50 mg/L Kanamycin), and incubated at 37 °C for 12 h. Plates were scanned using a digital camera at 400 dpi resolution. Survival rates of two samples (IPTG induced transformed BL21 HSP cells and uninduced transformed BL21 HSP cells) were compared for the HSP gene stress tolerance.

Results

Amplification of HSP gene in Chaetomium spp—Reverse-transcriptase-PCR using RNA extracted from 15 isolates of C. globosum showed an amplicon of 630bp in 6 isolates viz., Cg1, Cg4, Cg5, Cg6, Cg7 and Cg9. Amplification was also observed in 4 other species of Chaetomium viz., C. cochliloides, C. reflexum, C. perlucidum and C. cupreum using HSPF:5’-GACATGGATCCATGTCCTTCTTCGTGGCCTT-3’ and HSPR: 5’-TAATGCTCGAGTTAGTTGATAGCAACACCGGC-3’ primers (Fig. 1).

Cloning and sequencing of HSP gene—PCR product of 630bp from C. globosum isolate Cg1 was gel eluted and cloned into pGEMT (Promega) vector and sequenced. The sequenced product was 739bp long. After removing sequences of vector, a product of 603bp was obtained. Double enzyme digestion of recombinant vector pGEMT–HSP also confirmed a 603bp product. Out of 30 white BL21 colonies, 20 were selected and used for colony PCR. Seventeen colonies showed 630bp sequence amplification indicating the presence of desired sequence in transformed BL21. Double digestion of vector isolated from two positive white colonies released a fragment of approximately 603bp which further confirmed that recombinant vectors named as pET 17-HSP and pET21-HSP had desired fragment.

Expression of pET-HSP recombinant protein—HSP protein was successfully expressed only in E. coli BL21 pET-HSP17, while no protein was produced in E. coli BL21 pET-HSP21 which suggests that target gene is inserted correctly into pET-HSP17only. The molecular mass of IPTG induced (+IPTG) fusion protein in E. coli BL21 pET-HSP17 was about 30 kD, which was absent in uninduced (-IPTG) E. coli BL21 pET-HSP17 (Fig. 2).
Thermotolerance of BL21 transformant expressing HSP fusion protein—There was not much difference in growth among the three different types of BL21 cultures without heat shock (37 °C), whether or not IPTG induction was given (Fig. 3). However, when different types of BL21 cultures without IPTG induction were given 50 °C heat treatment, all of them showed reduced growth, and there was no growth at 65 °C. However, IPTG induction supported better growth to BL21 pET-HSP in comparison to other two cultures under heat stress, particularly at 65 °C. All these results showed that the expressed HSP fusion protein provides thermo tolerance to the transformed BL21.

Stress tolerance of HSP in BL21—It was observed that there was no difference in survival rates between +IPTG and –IPTG transformants under nonstress conditions as they had identical growth rates. However, under NaCl and Na₂CO₃ stress conditions, there was difference in survival rates between the transformants (BL21 HSP) with IPTG and transformants without IPTG (control). At 6 and 8% (w/v) Na₂CO₃ stress, the survival rate of the transformed BL21 after IPTG induction was 1430 and 323 cells whereas it was 85 and 56 cells of control (without IPTG) respectively. This survival rate was approximately 17 and 6 times respectively higher to control (Fig. 4). At 10% (w/v) Na₂CO₃ stress, nearly all control cells failed to survive, while few (12 cell) IPTG induced transformants survived (not shown in figure). At 1 M and 5 M NaCl stress conditions, IPTG induced transformants showed survival of average 131 and 105 cells while, uninduced showed only 2 and zero cells survival respectively. These results demonstrated that the expression of HSP gene confers Na₂CO₃ and NaCl tolerance to BL21 transformant.
Bioinformatic analysis of HSP gene—Further phylogenetic tree drawn by neighbor joining of amino acid sequences showed that our Cg1 heat shock protein formed a cluster with C. globosum along with Myceliophthora and Nerospora spp. (Fig. 4). The number of amino acids in HSP protein were 228 in Neurospora crassa (OR74A), 214 aa in Fusarium oxysporum (FO5176), 202 aa in Neurospora tetrasperma (FGSC 2508), 214 aa in Hypocrea lixii, 210 aa in Fusarium lichenicola, 202 aa in Chaetomium globosum (AAR36902), 222 aa Metarhizium, 183 aa in Myceliophthora, 213 aa in Glomerella graminicola and 200 aa in our sample of C. globosum (Cg1). The above description reveals that there is large variation in amino acid number of small HSP in different fungi, however, 54 amino acids were conserved among them (Fig. 5).

Discussion
Using the BLAST X program of the NCBI website, the deduced amino acid sequence of HSP of C. globosum showed more than 50% identity and similarity with known HSP sequences of other fungi. About 55 amino acids of HSP protein were conserved among different fungi however; multiple sequence alignment indicates that there was overall variability in amino acid sequences of HSP protein of different fungi. Molecular phylogenetic tree by Neighbour joining of amino acid sequences of the HSP sequences of different fungi showed 75% similarity. The tree was further divided in two major groups i.e., group I and II. Our Cg1 isolate was present in group I and showed 95% similarity with existing C. globosum (AAR3690). Both were sub grouped in cluster ‘a’ of group I. Cluster ‘a’ showed 82%
similarity with sub cluster ‘b’ (Myceliophthora) and 78% with subcluster ‘c’ (Sordaria, Neurospora) of group I. The group II consisted of Golmerella, Hypocrea, Metarhizium and Fusarium and separated from group I by 25% dissimilarity. Previous research showed that a small Hsp gene from Xenopus laevis, Hsp30, is intronless, consistent with our result, that the HSP gene from C. globosum is also intronless. Through multiple sequence alignment, it was found that the conserved region of HSPs is not continuous, consisting of a non-conserved middle region and a highly conserved alpha crystalline domain (Fig. 5). Plesofsky et al.\textsuperscript{17} also reported that the Hsp30 gene consisted of conserved sequence is involved in structural organization, as well as nonconserved regions that may perform similar roles in each protein. The conserved alpha-crystalline domain can be divided into N-terminal and C-terminal subdomains that interact strongly with one another. Usui\textsuperscript{18} constructed N-terminal truncation mutants of Hsp from Sulfolobus tokodaii strain 7. Results revealed that the mutants exhibited reduced chaperone activity. Alpha-crystalline domain is predicted to be consisted of two hydrophobic beta-sheet motifs, separated by a hydrophilic region of variable length\textsuperscript{19}.

This study showed that the HSP gene is strongly induced by heat shock in some isolates of C. globosum and other Chaetomium spp., suggesting that it may play a role in heat tolerance. The induced expression analysis also showed that Hsp was synthesized on induction with IPTG under in vivo conditions in HSP transformants. HSP transformed BL21 cultures showed survival at 65 °C, which were otherwise harmful to untransformed cultures of BL21. In E. coli, a similar function to inhibit protein denaturation has also been seen earlier\textsuperscript{20}. Our observations on enhanced thermotolerance of HSP transformed BL21 indicated expression of HSP gene in host, which conferred protective function against damage of protein. Liu et al.\textsuperscript{21} also showed that OsHsp 90 buffered many bacterial proteins from precipitation under different heat shock conditions and, thus maintained the growth of E. coli cells expressing OsHsp 90 on exposure to heat.

Many studies have shown that the expression of small Hsp genes confers tolerance not only to heat but also to other abiotic stresses such as cold, drought, salinity, oxidative stress and ethanol\textsuperscript{22-25}, naphthalene, insecticides, heavy metals, or desiccation\textsuperscript{26}. Other molecular chaperones such as Hsp67, Hsp68, and Hsp70 are also known to respond to environmental stresses\textsuperscript{27-31}. It has been suggested that they contribute to thermotolerance\textsuperscript{28}, and some of them (Hsp22, Hsp68, and Hsp70) affect longevity\textsuperscript{32}. In confirmation to these studies our studies also showed that the small HSP characterized in present study conferred higher NaCl and Na\textsubscript{2}CO\textsubscript{3} tolerance in IPTG induced BL21 transformants, suggesting that small heat shock proteins act as molecular chaperone to prevent aggregation and precipitation of proteins and protecting them from different stresses. Treweek et al.\textsuperscript{33} showed that the alpha-crystalline domain is believed to play a role in subunit–subunit interaction and contains a putative chaperone-binding region, acting as molecular chaperones to prevent aggregation and precipitation of proteins. The combination of a conserved alpha crystalline domain probably modulates the properties of HSP as a stress-protective and structural oligomeric protein. Although details of the chaperone activity and the molecular mechanism of the Hsp-mediated stress resistance are largely unknown, it is possible that these genes may also affect developmental processes.

Results of this study indicated that HSP22 protein has contributed to abiotic stress tolerance that may explain the capacity of some C. globosum strains to overcome the adverse environmental conditions facilitating their presence in varied geographical locations, and potential as biocontrol agents.

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References


