SYNOPSIS

RECOMBINANT EXPRESSION OF HEAT SHOCK PROTEINS (HSPs) FROM *ARTEMIA FRANCISCANA* AND EXPLORATION OF THEIR *IN VITRO* CHAPERONE ACTIVITY

Artemia (brine shrimp) is a crustacean that inhabits hypersaline ecosystems like salterns. Under extreme environmental conditions, they reproduce through encysted embryos called cysts that can withstand extreme temperature, salinity and radiation. Small heat shock proteins (sHSPs) like ArHSP21, ArHSP22 and P26 are abundant in *Artemia* cysts, which confer resistance against environmental stresses.

Heat shock proteins (HSPs) are chaperone proteins that assist correct folding of nascent peptides. They also prevent aggregation and irreversible denaturation of cellular proteins and assist in the refolding of misfolded proteins. These proteins impart a degree of resistance to cells against stresses like heat, toxins, oxidative stress etc. The sHSPs are ATP independent HSPs with a monomeric molecular weight ranging from 12 kDa to 43 kDa. They are also known as holdases because they bind to misfolded proteins preventing further denaturation and hold them in a partially unfolded form on which other ATP dependent HSPs like HSP70 can act and actively refold.

Artemia samples were collected from the Thamaraikulam salterns near Nagercoil, Kanyakumari District, Tamil Nadu (8°06'N 77°29'E) and grown in lab. Cysts produced were used for total RNA isolation and cDNA synthesis. The collected species was identified as *Artemia franciscana* on the basis of COI, ITS-1 and P26 gene sequences homology and phylogenetic analysis. The results indicate that the native *Artemia parthenogenetica* that had previously inhabited this region was completely removed due to the introduction of invasive alien species *A. franciscana*, most probable from aquaculture facilities.

The HSP genes ArHSP21, ArHSP22, P26 and HSP70 from *A. franciscana* were PCR amplified, inserted to pQE30/31/32 expression vectors and transformed to M15(pREP4) *E. coli* cells. Among these, all except ArHSP22 were successfully expressed in *E. coli*. The IPTG concentration and incubation duration required for the expression of HSPs were optimised. The histidine tagged recombinant HSPs were purified using Ni-NTA agarose gel. Western blotting and LC/Q-TOF MS analysis of the purified proteins were carried out to confirm their identity. Native PAGE analysis was used to investigate the different oligomeric states of ArHSP21 and P26 using 8–16% gradient native-PAGE. P26 was found to exist predominantly as monomers, while a high proportion of ArHSP21 occurred as oligomers. A band corresponding to tetramers was seen in P26 and ArHSP21, while dimer band was seen only in P26. Different higher oligomeric forms were present in both proteins.

The *in silico* physico-chemical characterization of all four HSPs were carried out using bioinformatics tools like ExPASy ProtParam, PROSITE, Pfam, SOPMA and GOR4. Homology modelling of the HSPs was performed using MODWEB server and the quality of the models were assessed using RAMPAGE, ERRAT and VERIFY3D tools.

The recombinant HSPs studied were found to exhibit *in vivo* and *in vitro* chaperone activity. M15(pREP4) *E. coli* cells expressing recombinant HSPs showed better thermotolerance in comparison to M15 cells with empty pQE31 plasmid indicating *in vivo* protection by the expressed HSPs. On heat treatment at 45°C for 1 hr, cells with HSP70 gene showed the highest survival, followed by P26 and ArHSP21 clones. P26 and HSP70 clones were also tolerant to overnight incubation at 45°C.

The *in vitro* chaperone activity of recombinant HSPs were tested on industrially significant enzymes like α-amylase and chitinase, restriction enzyme *Sma*I and on citrate synthase, a model substrate protein for chaperone activity. The individual protective activity of the HSPs and also the combined activity of each sHSP along with HSP70 were also studied. Addition of different HSPs to α-amylase reaction mix heated to 50°C for 1 hr imparted protection to the enzyme, with P26 showing highest protection, followed by ArHSP21 and HSP70. The protection imparted was observed to improve in reaction mixtures having HSP70 in combination with sHSPs.

The recombinant HSPs were found to be capable of preventing the denaturation of citrate synthase (CS) during heat exposure to 47°C for 25 min. ArHSP21 was most efficient in retaining CS in its active form, followed by P26 and HSP70. The addition of HSP70 improved the protection to CS when added along with P26.

The effect of different concentrations of ArHSP21, P26 and HSP70 in protection of *Sma*I, a temperature sensitive restriction enzyme from thermal denaturation was demonstrated. P26 and ArHSP21 were found to preserve *Sma*I at 37°C for 2 hr, a temperature at which this enzyme usually get denatured, while HSP70 had no effect.

A novel chitinase with antifungal activity was identified in *Streptosporangium nondiastaticum* and the chitinase gene was cloned and expressed in M15(pREP4) *E. coli* cells. The effect of ArHSP21 and HSP70 in protecting this chitinase from thermal inactivation at 65°C for 30 min was assessed. Highest protection was observed with a combination of 8 μg/ml each of ArHSP21 and HSP70 (88.97% relative activity).

Enzymes are widely used today for industrial, diagnostic and research purposes. The temperature sensitive nature of enzymes imposes difficulty during long term storage (at sub-zero temperatures), transportation and in reactions involving high temperature. This study demonstrates the *in vitro* chaperone activity of HSPs in protecting enzymes and making them more stable at high temperatures. It was also observed that the chaperone activity of the sHSPs (ArHSP21 and P26) improved when used in combination with HSP70 indicating a synergistic action between these proteins. The incorporation of HSPs into enzyme mixtures for protecting them from high temperatures is an efficient strategy to be further explored. The concentration and composition of HSPs to be used must be optimized for each enzyme and reaction condition. These results open a new avenue to develop a novel stabilizer technology for thermolabile enzymes based on the *in vitro* chaperone properties of HSPs from *Artemia* sp. and offer enormous applications in modern enzyme industry.