

THE ROLE OF SMALL HEAT SHOCK PROTEINS OF THE
THERMOACIDOPHILIC ARCHAEON *THERMOPLASMA VOLCANIUM* IN
THE STRESS RESPONSE

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THERMOACIDOPHILIC ARCHAEON *THERMOPLASMA VOLCANIUM*
IN THE STRESS RESPONSE**

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ABSTRACT

THE ROLE OF SMALL HEAT SHOCK PROTEINS OF THE THERMOACIDOPHILIC ARCHAEON, *THERMOPLASMA VOLCANIUM* IN THE STRESS RESPONSE

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In this study, possible involvement of the small heat shock proteins (sHsps) from a thermoacidophilic archaeon, *Thermoplasma (Tp) volcanium* in the stress response was investigated. Our results showed that heterologous, high level expression of TVN0775/sHsp gene in *E.coli* increased its thermotolerance at 53°C for two hours. But, the second sHsp of the *Tp. volcanium*, TVN0984/sHsp was not effective in improvement of the thermal resistance of the mesophilic bacterium (*i. e., E.coli*).

The expression of the TVN0775/sHsp and TVN0984/sHsp genes increased about 3 fold after heat-shock at 65°C, as revealed by Real-Time PCR analysis. Although expression of the both genes was induced at 70°C, TVN0984/sHsp gene expression was increased higher (about 5 fold) than that of the TVN0775/sHsp gene expression (about 1.5 fold).

Tp. volcanium cells were exposed to high pH (pH: 3.5, pH: 4.0, pH: 4.5, pH: 5.0), and the change in the sHsp genes' expression profile were analyzed. The results showed that TVN0775/sHsp gene expression was more sensitive to increased pH

than TVN0984/sHsp gene expression. The TVN0775/sHsp gene transcription induced at most 2.5 fold at pH 4.0 and the gene expression either reduced or did not change at higher pH values (*i.e.*, pH 4.5 and 5.0). On the other hand, TVN0984/sHsp gene expression did not change at pH 4.0 but significantly reduced at higher pH values.

The effect of oxidative stress on the expression of TVN0775 and TVN0984 genes was investigated by treatment of *Tp. volcanium* cells with 0.01 mM, 0.02 mM, 0.03 mM and 0.05 mM H₂O₂. For both sHsp genes, transcription was induced at lower concentrations of H₂O₂ (0.01 mM and 0.02 mM). At higher concentrations of H₂O₂ expression of both genes' transcription either did not change or down regulated.

Lastly, in this study we have purified the recombinant TVN0775/sHsp, as an N-terminal 6x his-tag fusion to homogeneity on Ni-NTA affinity column. Purified protein samples were used in the chaperone activity assays using bovine glutamate dehydrogenase enzyme (boGDH) as substrate. We have found that the recovery of glutamate dehydrogenase activity at 45°C, 50°C and 53°C in the presence of the *Tp. volcanium* sHsps was higher than that of spontaneous refolding. Also, TVN0775/sHsp increased the recovery of the boGDH enzyme that was denatured at 2.5 M GdnHCl concentrations for 30 min.

Keywords: Archaea, stress, small heat shock proteins (sHsps), *Thermoplasma volcanium*.

ÖZ

THERMOASİDOFİLİK BİR ARKEA OLAN *THERMOPLASMA VOLCANIUM*'UN KÜÇÜK ISI ŞOKU PROTEİNLERİNİN STRES TEPKİSİNDEKİ ROLÜ

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Bu çalışmada termoasidofilik bir arkea olan, *Thermoplasma volcanium*'dan elde edilen küçük ısı şoku proteinlerinin stres tepkisindeki rolleri araştırılmıştır. Elde ettiğimiz sonuçlar, *E.coli*' de heterolog olarak yüksek düzeyde anlatımı yapılan TVN0775 geninin bu bakterinin 53°C' de, iki saat boyunca sıcaklığa dayanıklılığını arttırdığını göstermiştir. Ancak *Tp. volcanium*' un ikinci küçük ısı şoku proteinini kodlayan TVN0984 geni, mezofilik bakteri (*E.coli*) nin ısıya dayanıklılığını arttırmada etkili olmamıştır.

TVN0775 ve TVN0984 gen anlatımlarının, 65°C' de ısı şokundan sonra 3 kat arttığı, Gerçek Zamanlı Polimeraz Zincir Reaksiyonu (RT-PCR) ile gösterilmiştir. Her iki genin de anlatımı 70°C' de uyarılırken, TVN0984 geninin anlatımındaki artış (yaklaşık 5 kat), TVN0775 (yaklaşık 1,5 kat), geninden daha fazla olmuştur.

Tp. volcanium hücreleri yüksek pH'ya (pH: 3.5, pH: 4.0, pH: 4.5, pH: 5.0) maruz bırakılmışlar ve küçük ısı şoku proteinlerinin gen anlatımındaki değişim profilleri incelenmiştir. Sonuçlar TVN0775 gen anlatımının, yüksek pH derecesine, TVN0984 gen anlatımından daha duyarlı olduğunu göstermiştir. TVN0775 gen

transkripsiyonu, pH: 4.0' te en fazla 2.5 kat uyarılmış ve daha yüksek pH değerlerinde (pH: 4.5 ve pH: 5.0) ise ya azalmış ya da değişmemiştir. Diğer yandan, TVN0984 gen anlatımı pH: 4.0'te değişmemiş ancak daha yüksek pH değerlerinde önemli ölçüde azalmıştır.

Oksidatif stresin TVN0775 ve TVN0984 genlerinin anlatımına etkisi, *Tp. volcanium* hücrelerine, 0.01 mM, 0.02 mM, 0.03 mM ve 0.05 mM H₂O₂ uygulanarak incelenmiştir. Düşük H₂O₂ derişimlerinde (0.01 mM ve 0.02 mM), her iki küçük ısı şoku protein geninin de transkripsiyonu uyarılmıştır. Daha yüksek H₂O₂ derişimlerinde ise her iki genin transkripsiyonu da ya değişmemiş ya da azalmıştır.

Bu çalışmada son olarak, rekombinant TVN0775/sHsp'ni, N-ucundaki 6X Histidin-etiketi taşıyan bir füzyon proteini olarak Ni-NTA afinite kolon ile homojen olarak saflaştırılmıştır. Saflaştırılmış protein örnekleri, substrat olarak sığırdan elde edilen glutamat dehidrojenaz enziminin kullanıldığı şaperon aktivite deneylerinde kullanılmıştır. *Tp. volcanium* kaynaklı küçük ısı şoku protein varlığında 45°C, 50°C ve 53°C' lerde, glutamat dehidrojenaz enzimin aktivitesini yeniden kazanımının; kendiliğinden katlanmadaki kazanımına göre daha fazla olduğu görülmüştür. TVN0775 küçük ısı şoku proteini, 30 dakika süresince 2.5 M derişimindeki GdnHCl ile denatürlenmiş boGDH enziminin aktivitesinin yeniden kazanımını da arttırmıştır.

Anahtar Kelimeler: Arkeabakteri, stres, küçük ısı şoku proteinleri, *Thermoplasma volcanium*.

To my family,

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CHAPTER 1

INTRODUCTION

1.1 Protein Folding

Proteins can be present in three different conformations in cells; fully folded, unfolded and molten globule form. Molten globule structure can be defined as a collapsed molecule with highly mobile aromatic side chains and also this form of the protein has native-like secondary structure (Hartl and Martin, 1992).

When the proteins are treated with denaturing agents, such as guanidine hydrochloride, urea or heat, their structures are disturbed. This non-native conformations can lead to formation of inclusion bodies which are inactive and large protein aggregates. Denatured proteins can be refolded on their own under *in vitro* conditions after suppressing the effect of denaturing agents. It can be said that proteins can fold spontaneously without energy input or cellular protein folding machinery. But of course, the protein folding in experimental conditions is not as same as the cellular protein folding *in vivo* (Seckler and Jaenicke, 1992). Also, newly synthesized polypeptides can fold (tertiary structure) and assemble (quaternary structure) by themselves *in vivo*, without external help (Hartl and Martin, 1992). It can be speculated that protein folding is an autonomous process under proper conditions, which can be take place spontaneously. During protein folding, polypeptides bind each other non-covalently. Hydrophobic surfaces of proteins provide this interaction. In this process to avoid wrong domain pairing, aggregation or protein renaturation specific enzymes or polypeptide chain binding proteins actively participate folding *in vivo*.

Misfolded proteins are either degraded in the cell or returned to the initial folding states, called *de novo* protein folding. In these mechanisms, proteolysis is achieved by proteases and refolding is succeeded by molecular chaperones. If they could not be refolded by chaperones, they are degraded by proteases.

Cells are exposed many stresses during their life among these heat shock is the most studied one. If we compare the heat shock condition with optimal condition; under normal conditions, the speed of transcription and translation is quite high and the spontaneous folding rates are higher than the chaperone mediated folding. But, under the heat shock, the positions of the mechanisms change; while the rate of transcription and translation decreases, spontaneous folding of proteins also slows down, but proteolytic degradation of unfolded proteins and chaperone activities increase. The decline in transcription and translation rate is related with RNA polymerase subunits and the translation factor, Elongation Factor-G (EF-G), which are sensitive to heat (Narberhaus, 2002).

Newly synthesized (nascent) protein has three major pathways to go through:

- It can be degraded by proteolysis.
- It can be aggregated.
- It can be folded. Folding process can be performed by also two ways; proteins can be spontaneously folded or they can be folded by the assistance of molecular chaperones.

1.2 Molecular Chaperones and Heat Shock Proteins

The role of molecular chaperones are related with the modulation of protein conformation and they can be defined as, "a family of proteins that mediate the correct assembly of other polypeptides, but are not themselves components of the final functional structure" (Ellis R.J.,1993).

Chaperones play important roles in diverse cellular processes. The primary function of molecular chaperones can be accepted as to facilitate the folding or refolding of proteins by suppressing their aggregation or misfolding. Most chaperones bind to hydrophobic surfaces of newly synthesized or nonnative proteins and thereby stabilize them to prevent their aggregation. Some of the chaperones fulfill their roles by using ATP. Chaperones also play central roles in the translocation of proteins across intracellular membranes into organelles and also in proteolytic degradation or refolding of unstable proteins (Fink A.L., 1999).

Molecular chaperones consist of members of highly conserved families of proteins, many of which are **heat shock proteins** (Hsps) or stress proteins. The general chaperone classes are composed of small heat shock proteins (sHsps), Hsp40, Hsp60, Hsp70 and Hsp90. The heat shock proteins Hsp40, Hsp60 and Hsp70 are common in chaperone systems in eukaryotes and these proteins in bacteria are called DnaJ, GroEL and DnaK, respectively. The activities of Hsp60 and Hsp70 are modulated by binding to and hydrolysis of ATP (Narberhaus, 2002).

Hsp70 family is the most highly conserved and the largest of all the heat shock protein families. They bind to target proteins to help their folding, transport, and repair. Moreover, Hsp70 helps some proteins by stabilizing them in an unfolded intermediate state for targeting and translocation to cellular compartments, including the endoplasmic reticulum, mitochondria and chloroplast. Hsp70 also has a crucial role in the subsequent folding of peptides after import (Hartl et. al., 1990).

Under stress conditions, the synthesis of Hsp70 is stimulated and it protects the cell from damage. Hsp70 migrates to the nucleus from cytoplasm and it binds to pre-ribosomes and other protein complexes to help them by preventing denaturation (Lindquist, S., 1986).

Members of the Hsp70 proteins constitute a large family and found in different compartments of cells. Some of them are; Hsc70 (or Hsp73), the constitutive cytosolic member; Hsp70 (or Hsp72), the stress-induced cytosolic form; Bip (or Grp78) (in mammalian cells), the ER form; and mHsp70 (or mito-Hsp70, or Grp75), the mitochondrial form. Ssa1–4 and Kar2 are the other Hsp70 proteins in yeast which are the homologs of Hsc70 and Bip. The *E. coli* form of Hsp70 is known as DnaK and DnaK needs two cofactors (DnaJ, 41 kDa; GrpE, 20 kDa) for folding function (Fink, 1999).

Heat shock protein 70 is composed of two functional subunits; first is the most conserved, N-terminal domain which contains a high affinity ATP-binding site (ATPase domain, 44 kDa) and less conserved C-terminal domain (27 kDa) which is responsible for binding substrate proteins and polypeptides.

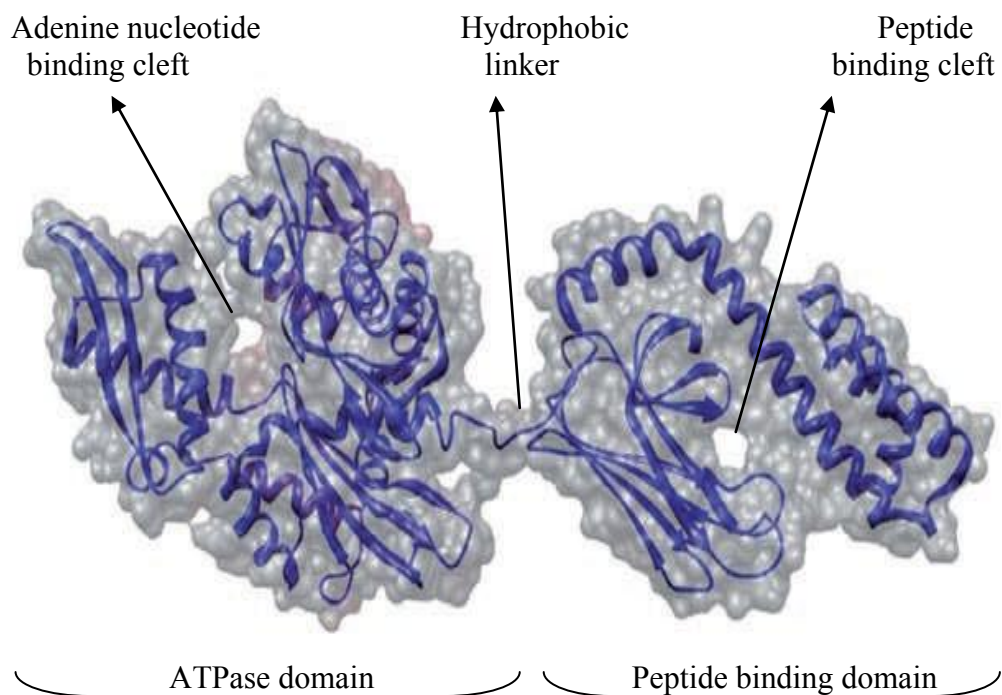


Figure 1.1 The Hsp70 (ADP bound form). Hydrophobic linker which is flexible serves as a bridge between the ATPase domain and peptide-binding domain (Kampinga and Craig, 2010).

In *E.coli*, target peptides (substrates) bound to DnaK by their hydrophobic regions of about five amino acids. Binding and releasing of substrate is regulated by the co-chaperones DnaJ and GrpE (nucleotide exchange factor) by ATP hydrolysis.

Chaperone function of the Hsp70 system is explained in the following scheme (Figure 1.2).

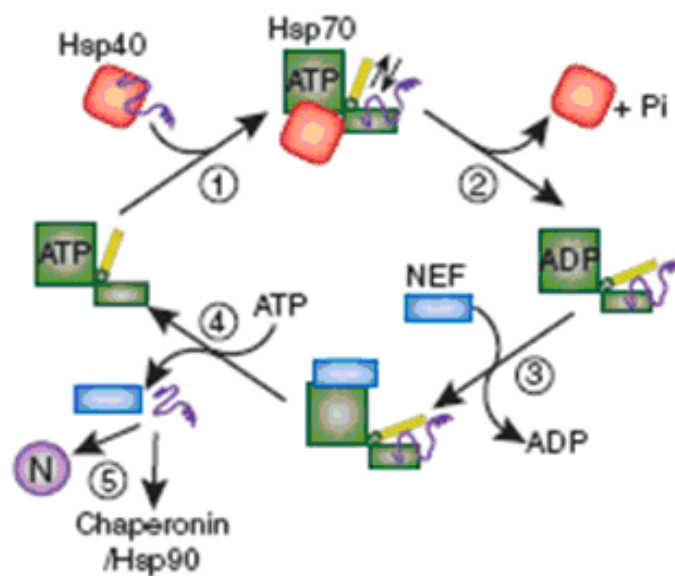


Figure 1.2 “Hsp70 reaction cycle. NEF, nucleotide-exchange factor (GrpE in case of *E.coli* DnaK; Bag, HspBP1 and Hsp110 in case of eukaryotic cytosolic Hsp70). (1) Hsp40-mediated delivery of substrate to ATP-bound Hsp70. (2) Hydrolysis of ATP to ADP, accelerated by Hsp40, results in closing of the α -helical lid and tight binding of substrate by Hsp70. Hsp40 dissociates from Hsp70. (3) Dissociation of ADP catalyzed by NEF. (4) Opening of the α -helical lid, induced by ATP binding, results in substrate release. (5) Released substrate either folds to native state (N), is transferred to downstream chaperones or rebinds to Hsp70” (from Hartl and Hartl, 2009).

The general properties of Hsp70 proteins:

- (a) They have a highly conserved amino-terminal ATP-binding site followed by a more variable carboxy-terminal substrate-binding side;
- (b) They contact with partially denatured or unfolded polypeptides.
- (c) They need ATP energy for releasing the bound substrates but not for binding of unfolded proteins or peptides. (F. U. Hartl and J. Martin, 1992).

Hsp60 protein families are called “**Chaperonins**” which are multi-subunit complexes. In their central cavities, they capture the denatured proteins and they increased the efficiency of protein folding reactions by using ATP.

Chaperonins are big cylindrical complexes. GroEL and TCP-1 are the mostly known members of chaperonin family. GroEL found in the bacterial cytosol, mitochondria and also chloroplasts (stroma) of plants (Rubisco subunit binding protein, RBP). GroEL has a double ring structure with two seven-membered identical subunits, each containing a central cavity. Each subunit (57 kDa) consists of three domains: the equatorial, the intermediate and the apical. ATP binding domain is the **apical domain** and the cochaperonin GroES binds to apical domain and it has role in enlarges the central cavity of GroEL. The second domain is **intermediate domain** which joins the apical domain with the large **equatorial domain**. Equatorial domain has the nucleotide-binding site (Bigotti and Clarke, 2008).

GroES has also seven subunits and the molecular weight of each is 10 kDa. GroES complexes is seen as a dome. GroES promotes the ATP binding and hydrolysis by GroEL and is necessary for the release and folding of many GroEL substrates (Hendrick and Hartl, 1993).

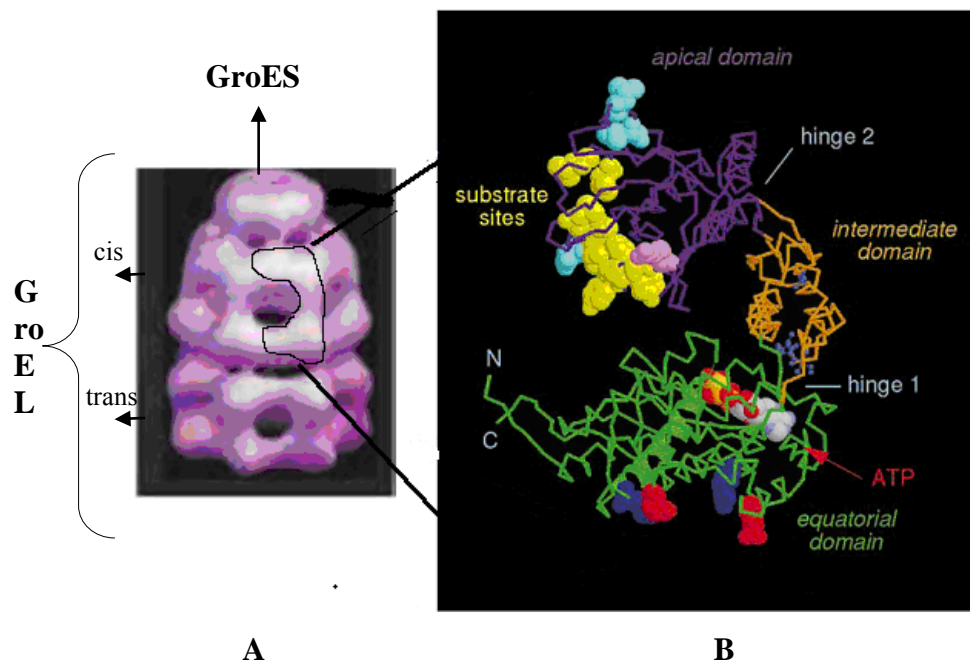


Figure 1.3 Cryoelectron microscopy picture of GroEL/GroES complex (**A**) and subunits of GroEL (**B**) (Figures are from Mogk et. al., 2002 and Ranson, et. al.,1998).

TCP-1 (970-kDa) is specific to the archaeal and eukaryotic cytosol. It is heterooligomeric ring complex (TRiC; also known as chaperonin containing TCP-1 [CCT]) composed of eight or nine subunits. TCP-1 ring complex appears to function independently of a small co-chaperonin protein such as GroES.

The chaperonins mediate folding of a subset of newly synthesized polypeptides by using ATP. Folding occurs in the central cavity of GroEL where the polypeptide substrate is transiently enclosed by GroES. These conformational changes are directed by ATP binding.

Chaperonins have two types; group I and group II. Group I are found in bacteria, mitochondria and plastids and group II are found in the eukaryotic cytoplasm and in archaea (eg., TCP-1). In hyperthermophilic archaea, chaperonins have great importance in suppressing protein denaturation. In terms of structure, group II

chaperonins look like to group I s. Although both of them are composed of double-ring, they have different subunit numbers; members of group I are consist of seven subunits and members of group II are formed by generally eight subunits. However, there are nine subunits only in a few archaea. There is a major difference between the two groups of chaperonins related with their structures. In the group II proteins, a helical protrusion of approximately 30 residues forms a lid on the cavity which is responsible for opening and closing steps of the reaction cycle. In the group I chaperonins, this is achieved by the co-chaperonin GroES and its homologues (Large et. al., 2009).

GroEL from prokaryotes and the ribulose biphosphate carboxylase (Rubisco)-binding protein of chloroplasts can be accepted as the important examples of chaperonin proteins. Hsp60 is an oligomeric complex with two stacked rings of seven 60-kD subunits each and they are well known with their functions in protein assembly reactions. Although the Hsp70s function by monomers or dimers, the Hsp60 proteins are functional as large oligomers (Hartl and Walter, 1990).

As it was mentioned, Hsp60 is responsible for folding and assembly, it is also responsible for the export of certain proteins from the matrix to the inter membrane space of mitochondria (Parsell and Linqvist, 1993).

Chaperonins bind to unfolded or partially folded substrate proteins and assist their folding under conditions where they do not refold spontaneously. This requires binding and usually hydrolysis of ATP (Figure 1.4).

The basic role of **Hsp90 family** proteins is in protein folding of other cellular proteins. They help to other proteins but not form a complex with them. In about all cells, there are many Hsp90 family proteins under non-stressful conditions to play role in secretory pathways and intracellular transport. Under stress conditions, the synthesis of these proteins accelerated and so cellular metabolism may be redirected to increase the stress tolerance (Linqvist and Craig, 1988).

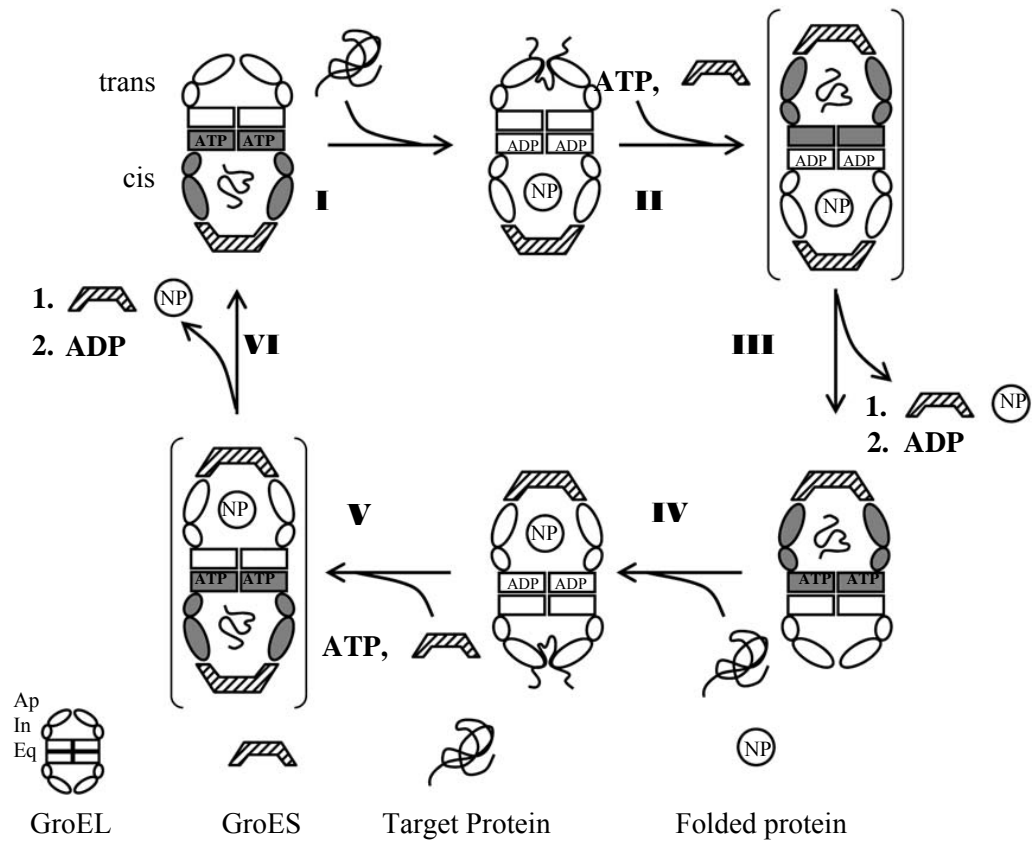


Figure 1.4 The chaperonin mediated protein folding in *E. coli*. GroEL/GroES complex; different conformational states of ATP and ADP containing forms of GroEL are shown by grey. Chaperonin GroEL: **Ap**, **In** and **Eq**, apical, intermediate and equatorial domains, respectively. **GroES**, cochaperonin; **NP**, refolded target protein. Steps of the cycle: Target protein binds to hydrophobic centers of Ap domains on the open trans ring of GroEL (**I**). On the other hand, the target protein in the cis ring is refolded by ATP hydrolysis. ADP promotes the binding of ATP to the trans ring and so the hydrophobic properties of Ap domains declines and the target is released (**II**). By binding the GroES, trans state turns into the cis and the target is encapsulated by hydrophilic cavity. Then, first GroES and the refolded target are fast released and then ADP from the neighboring ring is left (**III**). After that, the first three steps are repeated respectively by beginning from the cis ring and finally protein folding occurs and ADP, GroES and folded protein release (**IV,V,VI**) (Melnikov and Rotanova, Molecular Chaperones, 2010).

Hsp90 chaperones work with Hsp70 proteins to stabilize newly synthesized proteins and also they participate in the assembly/dis-assembly of multiprotein complexes. The subunit of chaperone Hsp90 is composed of three domains: N-terminal ATP binding (**N domain**, ~25 kDa); central (**M domain**, ~35 kDa), including the catalytic loop with an arginine residue, which is important for ATP hydrolysis; and C-terminal (**C domain**, ~20 kDa). Hsp90 proteins create “open” V-like dimers during the interaction of C domains. By binding ATP, subunits approach each other and the interaction between N domains compose a closed form of a chaperone. The Hsp90 regulation occurs with some co-chaperones by binding to different domains of Hsp90. For instance, the Hop protein interacts with the C domain, Cdc37 with the N domain, and Aha1 with the M domain (Melnikov and Rotanova, 2010).

Hsp100 family proteins contain two conserved domains which are about 200 aminoacids and have ATP binding sites. Under heat shock, its structure is seen as a ring. This high molecular weight protein is produced in many eukaryotic cells as a response to high temperature. They have only been studied in detail in higher organisms (in mammals). Well known members of this family: ClpB (from *E.coli*) and Hsp104 (from yeast). The Hsp104 associates with the nucleolus under heat shock conditions. Hsp104 and its homologs are protein disaggregating machines for cells. ClpB is the member of bacteria-originated Hsp100 chaperone, and Hsp104 and Hsp101 have the orthologs function in the yeast and plant cells. Protein disaggregation is achieved by a bichaperone system composed of Hsp70 and Hsp100.

Hsp100 chaperones belong to a superfamily of AAA+ proteins (ATPases associated with a variety of cellular activities). They are called as mechanoenzymes which use ATP energy for the conformational remodeling of bound protein targets that is mediated by their unfolding (the “unfoldase” activity of Hsp100 chaperones). Hsp104 has a symmetric hexameric molecular model and Clp/Hsp100 proteins assemble into ring structures. Hsp104 has a large central

cavity. Domains of Hsp100 family proteins are N-terminal domain (**N domain**) followed by a nucleotide-binding domain (**NBD-1**), a middle domain (**M domain**) and a second nucleotide-binding domain (**NBD-2**) (Doyle and Wickner, 2008).

Molecular chaperones can also be grouped according to their roles. If the main group accepted as “folding chaperones”, there are another three groups which help folding chaperones in folding process (Baneyx and Mujacic, 2004). These are:

1. Co-chaperones
2. Disaggregating chaperones
3. Holding chaperones

For example, DnaJ and GrpE, are the co-chaperones of DnaK. Firstly, they stimulate its reactivation, then, recognize the substrate and finally they release the chaperone, DnaK, from the substrate – chaperone complex. On the other hand, GroES can be thought as the co-chaperone of GroEL and it helps to GroEL in the chaperone – releasing step.

Another group of assistant chaperones are holding chaperones, as we understand from its name, they have role in the stabilization of unfolded or partially folded proteins. In some literature, small heat shock protein family was defined as holding chaperones (Baneyx and Mujacic, 2004).

E. coli IbpA and IbpB are well studied sHsps. The molecular weight of these homologous proteins is 16 kDa. “Ibp” refers to the inclusion body (Ib)-associated proteins which were determined that they are primary proteins associated with the inclusion bodies in recombinant *E. coli*. If we want to describe the folding and holding chaperone mechanism, basically; IbpB catches the unfolded proteins and hold them as they are. When the stress is relieved, folding chaperones come and take unfolded proteins from holding chaperone and folding process is completed, successfully (Baneyx and Mujacic, 2004).

Although they are called “stress proteins” or “heat shock proteins”, these proteins are not only induced under stress or heat shock conditions. They are also stimulated as response to other stresses such as, anoxia (oxygen deficiency), ethanol, and certain heavy metal ions. Effect of such these inducers can be various in different organisms. Heat shock proteins help organisms to decrease the destructive effects of such stress conditions.

All heat shock proteins and genes have the following features:

1. They are found in all kinds of organisms but their expression features can show variation according to cells or organisms; they are tissue specific and tissue-specificity is also related with the type of the stressors.
2. The strength of stress is also important for the expression levels of Hsps.

Depending on optimum growth temperatures of the organisms expression of Hsps are stimulated at different temperatures. For example, the Hsps of *Drosophila melanogaster* are induced at 33-37°C because their normal growth temperature is lower than this temperature. Arctic fishes grow at 0°C and their Hsps are induced at 5-10°C and also in mammalian cells, the induction occurs at the temperature that exceeds the normal body temperature (>37°C). On the other hand, thermophilic organisms grow at 50°C and their stress proteins are induced at about 60°C (Lindquist and Craig, 1988).

1.2.1 Small Heat Shock Proteins

Small heat shock proteins are the most widespread but least conserved group among the family of heat shock proteins. The primary function of sHsps is that holding the proteins in a folding-competent state (Lee et al.,1997). In addition to

having role in molecular chaperone function, they also play role in regulation of cytoskeletal proteins, especially in actin polymerization (Gusev et. al., 2002).

sHsps are ubiquitous which means that they can be members are found in all kinds of organisms including archaea, bacteria and eukarya but the numbers differ in different organisms (Narberhaus, 2002). These proteins are expressed in high amount under cellular stress conditions like other Hsps (Laksanalamai et. al., 2001).

Small heat shock proteins are different from other heat shock protein families with these properties :

1. They are ATP-independent.

While other chaperones need ATP to be functional, sHsps do not require energy for their activity. (Jakob et. al., 1993).

2. To be active, they need flexible assembly and reassembly of oligomeric complex structures.

There are several lines of evidences that sHsps are prone to oligomerization to acquire their chaperone activity. This oligomeric complex can be small or large. For instance, the study of structural analysis in bacterial sHsps have shown that their active oligomer can be composed of 2 to 32 subunits. The oligomeric conformation of a sHsp can change under the stress condition. These structure-related data were obtained from the studies with size exclusion chromatography, electron microscopy (EM) and X-ray crystallography. Particularly by using EM, many different quaternary structures of sHsps have been monitored (Haslbeck et. al., 1999; Shearstone and Baneyx, 1999).

As a result of structural analysis, diameter of particles and composition of subunits can vary in different organisms and also structure of sHsps can display a

spheric model with a central cavity which is consist of different numbers of subunits.

3. They have a large substrate-binding capacity.

Although small heat shock proteins are low molecular weighted, small molecules, they can form dynamic oligomeric structures ranging from 9 to 50 subunits. Unlike other chaperone families, sHsps can bind several nonnative proteins per oligomeric complex. In an example study, Hsp26 was tested in terms of its substrate binding efficiency (Haslbeck et. al., 1999). Hsp26 was treated with increasing amounts of citrate synthase (CS) enzyme and analysed on SDS–PAGE and the amounts of Hsp26 and CS were identified by densitometry. Finally it was found that the formation of the large Hsp26–CS complex is a highly cooperative process.

Substrate binding and disaggregation studies showed that, sHsp can prevent the aggregation of proteins which are exposed to physical (thermal) or chemical denaturants and this protecting activity is concentration-dependent.

The sHsps are ubiquitous, but this does not mean that all organisms would have them without any exception or they are not indispensable even though they have crucial role in cell survival under stress conditions. So, they could not find in some studied organisms: *Haemophilus influenzae* Rd KW20, *Helicobacter pylori* 26695 and *Neisseria meningitis* MC58 which are only some to mention (Narberhaus F., 2002).

The presence of sHsps is dependent on genome size, life-style, and heat conditions faced by the organism(Narberhaus F., 2002)

1.2.1.1 Structure of Small Heat Shock Proteins

Almost all investigated sHsps have large oligomeric structures. Oligomer generally consists of numbers of subunits ranging from 9 to 24 and these large complexes vary in size between 200-800 kDa. Basically, the oligomeric structure consists of dimers. The dimer is accepted as the minimal functional unit of a sHsp. General view of a small heat shock protein under X-ray crystallography looks like a hollow sphere and the outside diameters of sHsps is between 100-180 Å. By using electron microscopy in addition to X-ray crystallography, three dimensional structures of some family members have been analyzed; i.e., Hsp16.5 from *Methanococcus jannaschii* (Kim et al., 1998), Hsp16.3 from *Mycobacterium tuberculosis* (Yang et al., 1999), Hsp16.9 from wheat (Montfort et al., 2001) Hsp26 from *Saccharomyces cerevisiae*. All these sHsps form hollow, ball-like structures, but oligomer subunit numbers of them are different; Hsp16.5 and Hsp26 are composed of 24 subunits, while Hsp16.3 and Hsp16.9 have 12 subunits.

The monomeric molecular weight of sHsp ranges between 12-43 kDa. Although their sequence and oligomeric compositions are highly variable, the sHsps protect their general structural organization; conserved α -crystallin domain is preceded by a poorly conserved amino-terminal (N – terminal) domain and followed by a short and flexible carboxy terminal (C-terminal) (Figure 1.5). C-terminal domain is related to stabilization of oligomeric structure and function of sHsps (Leroux et al. 1997). The sequence and length of N-terminal region is significantly varied in distinct organisms, for example, this domain is composed of 24 residues in *C. Elegans* Hsp12.2, and 247 residues in *S. cerevisiae* Hsp42.0. Hydrophobic nature of N-terminal domain may influence subunit oligomerization and chaperone activity. All three domains are necessary for oligomerization of sHsps (MacRae T.H., 2000) .

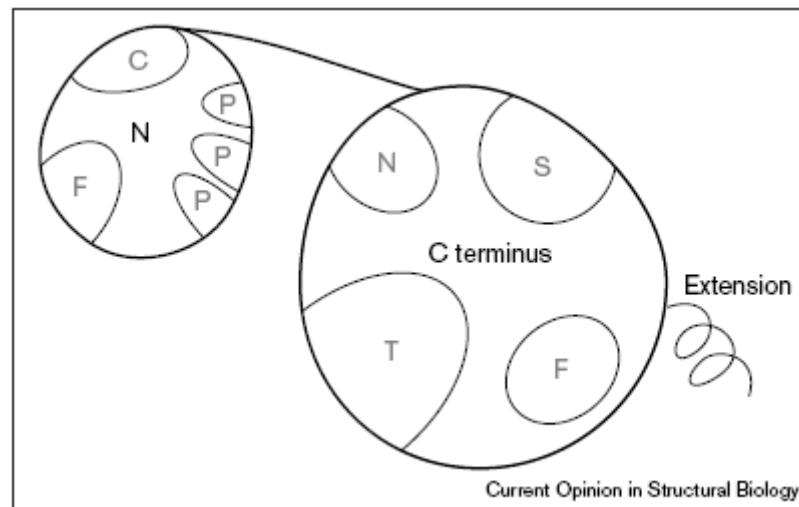


Figure 1.5 A model for interactive sites on sHsps. “The sHsp molecule has subunit sites (S) which are related with dimer formation and has role in the assembly of the functional high molecular weight complexes (C) including N terminus and are phosphorylation - sensitive. The phosphorylation sites (P) on the N terminus of sHsp have great importance for complex formation, actin dynamics and chaperone activity, although this can be different among members of the sHsp family. Although adjacent domains in a large complex may be involved, sites for target substrates (T), nucleotides (N) and cytoskeletal proteins (F) appear to be present on the C-terminal core α -crystallin domain. An other site for cytoskeletal proteins (F) may be present on the N terminus” (Clark and Muchowski, 2000).

In spite of a relatively low sequence homology among sHsps, they were classified together based on a conserved domain, referred as **α -crystallin**, which is named after the α -crystallin of the vertebrate eye lens (Caspers et al. 1995). The α -crystallin domain is the signature structure of sHsps and contains about 80 - 100 aminoacid residues (Figure1.6). The α -crystallin domain consists of two hydrophobic β -sheet motifs, separated by a hydrophilic region which is variable in length. The compact β -sheet sandwich is recognize by similarity to the immunoglobulin-like fold (Montfort et. al., 2001).

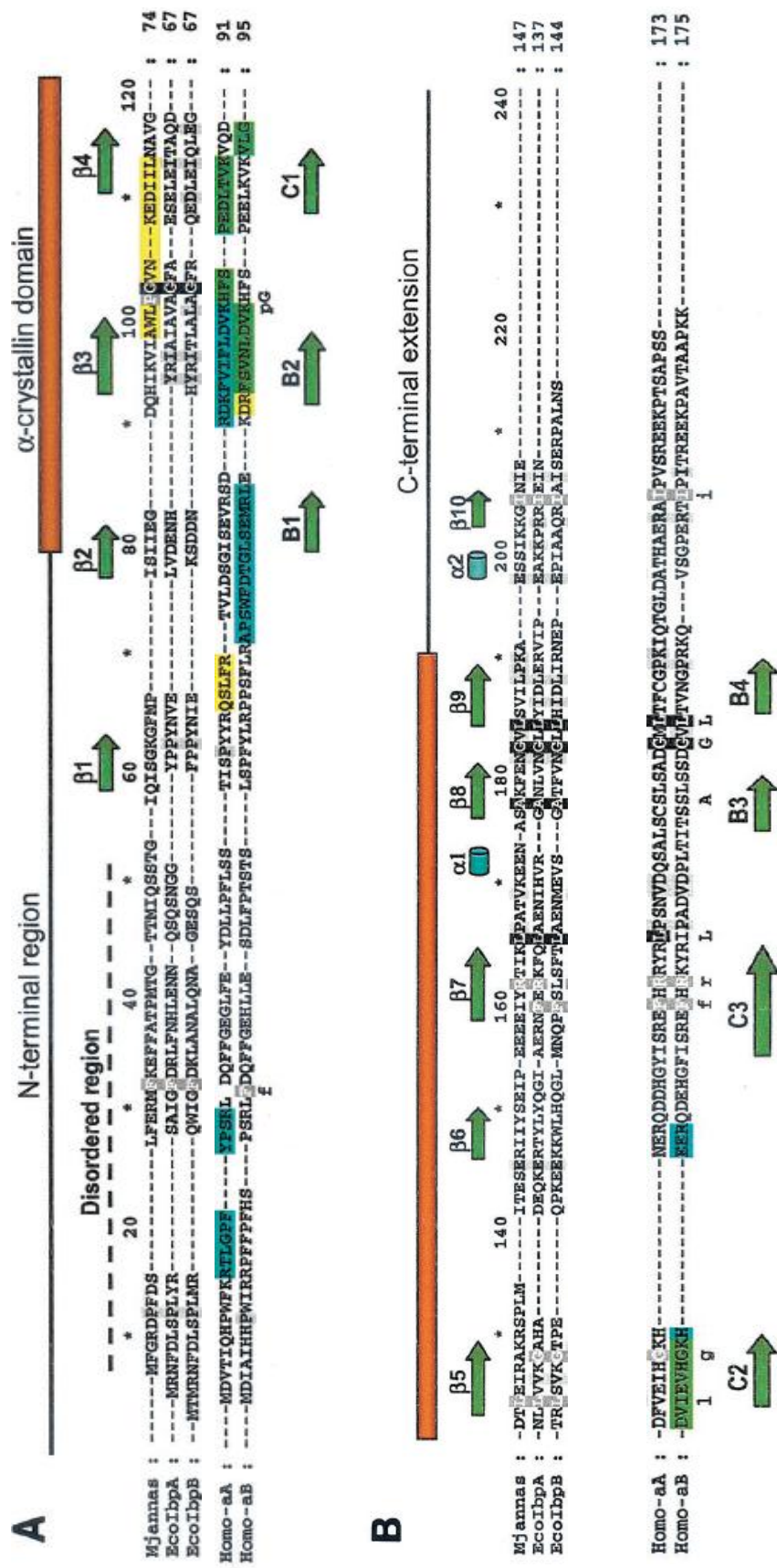


Figure 1.6 Amino acid alignment of bacterial and archaeal α-crystallins. Mjannas, *M. jannaschii*; Eco, *E. coli*; Homo, Human αA- and α B-crystallins. (from Narberhaus, 2002)

Alpha crystallin is the main protein of vertebrate eye lens and exists in mixed complexes with Alpha β -crystallin. In most vertebrate lenses the molar ratio of α -A and α -B subunits is three to one. In eye lens, α -crystallin has role in maintenance of lens transparency and by preventing the aggregation of denatured proteins functions as a molecular chaperone (Parsell and Linnik, 1993). Mutations and knockouts of α -crystallin genes led to development of cataracts in experimental animal and this results indicate physiological importance of α -crystallin as a chaperone (Sun and MacRae, 2005).

The Hsp16.5 from *Methanococcus jannaschii* (Kim et. al., 1998) is the first characterized archaeal sHsp and the general crystal structure of a sHsp has been first time studied in this archaeal species. The analysis of the crystal structure of MjHsp (Kim et. al., 1998) revealed that the 24-mer complex of the MjHsp is a sphere with hollow of octahedral symmetry and with eight trigonal and six square 'windows'. The diameter of the outer face of the sphere is 120Å and the inner face is 65Å. The monomeric folding unit of the 24 mer complex is a composite β -sandwich. Subunits of the MjHsp16.5 oligomer make extensive contacts with each other (Figure 1.7).

The structure of MjHsp and characteristics of its subunits were examined by Bova et. al. at different temperatures (50°C, 65°C, 68°C and 72°C) (Bova et. al., 2002). The effect of temperature on the secondary, tertiary and the oligomeric structure of MjHsp were analyzed by using fluorescence resonance energy transfer, circular dichroism, and size exclusion chromatography with online multi-angle light scattering, absorbance, and refractive index detectors. There were important changes in the secondary and tertiary structure of MjHsp at 25°C, 37°C and 70°C. It was suggested that this change is due to subunit exchange at different temperatures and that the process was reversible.

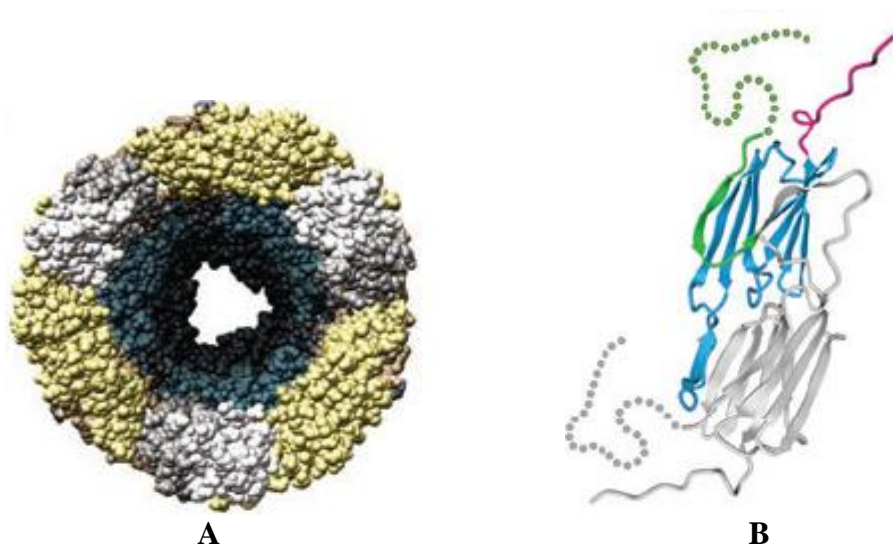


Figure 1.7 The quaternary structure of *M. jannaschii* Hsp16.5, consists of 24 identical subunits which form a tetrahedral hollow sphere (**A**). The three functionally important regions; Green, N-terminal region; blue, α -crystallin domain; pink, C-terminal extension; gray sHsp in the dimer (**B**). (from Haslbeck et. al., 2005)

The structure of an other archaeal sHsp, *T. sHsp* (sHsp from, *Thermococcus sp.* Strain KS-1) was analyzed by Usui et. al. (Usui et. al., 2001) by using size exclusion chromatography-multiangle laser light scattering (SEC-MALLS) and electron microscopy. The molecular weight of the oligomeric complex was about 478.6 kDa and that of monomeric unit was about 19,995 Da. Thus they suggest that the *T. sHsp* was composed of 24 mer like MjHsp. The diameter of the sphere was about 14 nm.

In 2002, Laksanalamai et. al. have investigated the relationship between the polydisperse structure and the function of Pfu-sHsp (from *Pyrococcus furiosus*). The deletion mutations in the domains of Pfu-sHsp indicated the importance of carboxy terminus in subunit assembly and the amino terminal deletion did not have any effect on assembly of subunits. The molecular weight calculations based on size exclusion chromatography and dynamic light scattering implied that dimeric molecular weight of Pfu-sHsp was 24 kDa.

Two sHsps, Hsp19.7 and Hsp14.0, exist in thermoacidophilic crenarchaeon, *Sulfolobus tokodaii* strain 7 (Usui et al., 2004). Both sHsps were spherical oligomers. Electron microscopic analysis revealed that StHsp19.7 exists as an unusual filamentous structure with the width of 13-14 nm. This is the first report on filament formation of a sHsp. StHsp14.0 appeared very similar to homologous MjHsp; it exists as a spherical particle of 11–13 nm and composed of 24 subunits. The molecular weight of a subunit was measured as about 140 kDa.

The N-terminal truncation mutations of StHsp14.0 indicated that this domain is important for the temperature-dependent complex dissociation and molecular chaperone activity (Usui et.al., 2004). The C-terminal (especially consensus IXI/V motif) truncation mutations implied that this domain is responsible for maintenance of secondary structure and surface hydrophobicity that are necessary for the function of sHsp (Saji et. al., 2008).

Strict anaerobic, sulphur metabolizing archaeon *Archaeoglobus fulgidus* has two sHsps; Hsp20.1 and Hsp20.2 (Haslbeck et. al., 2008). Structural analysis of Hsp20.2 by transmission electron microscopy showed that this sHsp is composed of 24-mer spherical complexes with octahedral symmetry.

1.2.1.2 Substrates of Small Heat Shock Proteins

Small heat shock proteins can be associate with different molecules during their functions. These are:

1. Proteins

Peptides or oligomeric proteins without sequence or structure specificity can be a substrate for sHsps. By binding of a target protein molecule to one of the subunits of sHsps, it can be protected from denaturation (Haslbeck et. al.,1999). Also,

sHsps protect cells from different stress factors and apoptosis due to their strong interaction with many different proteins.

2. Cytoskeletal Elements

Small heat shock proteins can interact with the cytoskeletal elements - microfilaments, intermediate filaments and microtubules - of eukaryotic cells for their regulation or stabilization. In an example study which supports this idea, a sHsp from *Artemia franciscana* embryos, p26 associated with tubulin. This study demonstrated that p26 suppressed the denaturation of tubulin, so it provided *Artemia* cells resistance against stress (Day et. al., 2003).

3. Membranes

Small heat shock proteins can also associate with membranes under stress conditions to sustain the maintenance of them. In a related study with the *Mycobacterium tuberculosis* Hsp16.3 showed that, Hsp16.3 helped bacterium for the protection from the stress condition (reactive oxygen species) by interacting with the lipid layers of the membrane (Abulimiti et. al., 2003).

4. sHsp substrates in nucleus

An other important substrate for sHsps is nucleus. They interact each other under abnormal conditions and sHsp tries to decrease the harmful effects of stress on nucleus by regulating molecular processes or protecting proteins. An example is - that the sHsp from bovine lens, α -crystallin showed a nuclear function by binding to DNA under stress conditions (Singh et. al., 1998).

1.2.1.3 Action Mechanism of sHsps as a Molecular Chaperone

Under heat shock condition, the oligomeric structure of sHsp is distorted. The complex is disassociated into dimeric forms to act as a molecular chaperone. sHsps binds to heat denatured substrate proteins by dimers at the hydrophobic

sites. So formation of insoluble aggregates is prevented. The sHsp/substrate complexes may subsequently involved in refolding process by the Hsp70 or DnaK with the help of co-chaperones (Figure 1.8).

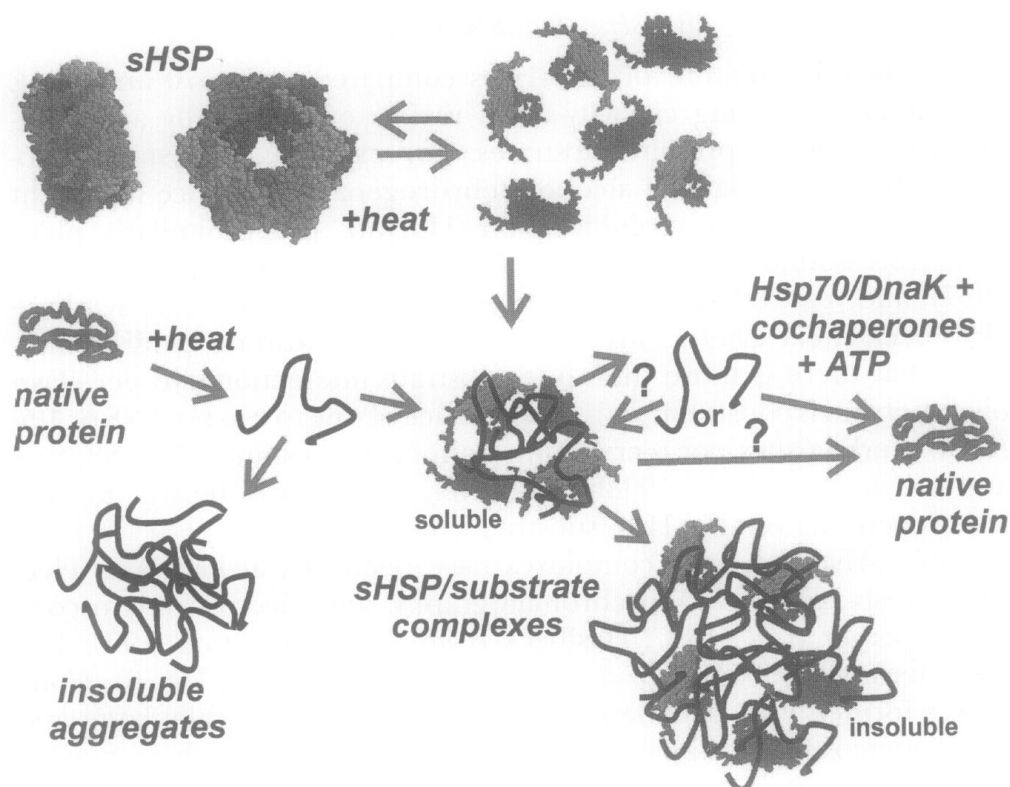


Figure 1.8 A model for molecular chaperone activity of sHsps (TaHsp16.9 as the model) (from Montfort et. al., 2001).

Two main jobs suggested for the sHsps in the cells are; a molecular chaperone activity and thermolerance element of cells.

1.2.1.3.1 Chaperone Activity of Small Heat Shock Proteins

Small heat shock proteins perform their chaperone activity by preventing proteins from aggregation under various stress conditions. This function of the archaeal sHsp, MjHsp16.5, was studied by using single chain monellin (SCM) as substrate (Kim et. al., 19998). This protein is stable at 60°C but not at 80°C. When

MjHsp16.5 was incubated with SCM at 80°C for 20 min. at 1:1 ratio, both proteins remained soluble, indicating efficient heat protection action of MjHsp16.5. Porcine heart citrate synthase (CS) was another substrate used in this study. Citrate synthase enzyme was alone aggregated at 40°C – 45°C.. MjHsp16.5 suppressed the precipitation of CS at 40°C. A molar ratio of 1:40 (CS:MjHsp16.5) protein was required to inhibit thermal aggregation at 40°C but 1:1 ratio (SCM:MjHsp16.5) was necessary for heat protection at 80°C determined at 280 nm.

In a similar study, when *T. sHsp* was added to CS, aggregation of the CS was prevented, although protection efficiency at 45°C was lower than that of MjHsp16.5 (Kim et. al., 1998). The protein folding activity of *T. sHsp* was shown by recovery level of the acid denatured green fluorescent protein (GFP) in the presence of excessive *T. sHsp* at high temperature (Muchowski et. al., 1998).

Heat protection activity of the Pfu-sHsp was studied with glutamate dehydrogenase (GDH) enzyme (from bovine liver). Its optimal activity temperature is 25°C and it was inactivated at 56°C. By adding Pfu-sHsp into the assay mix, GDH has remained in solution but was inactive. Thus, the enzyme was maintained in solution but not protected from denaturation (Laksanalamai et al., 2001). The sHsp from *Sulfolobus tokadaii*, StHsp14.0, prevented the thermal aggregation of 3-isopropylmalate dehydrogenase (IPMDH) (from *Thermus thermophilus*) at 87°C at a 24:1 ratio of StHsp14.0 monomer to IPMDH monomer. On the other hand, the second sHsp of *S. tokadaii*, StHsp19.7, was ineffective in suppressing aggregation of the model enzymes (Usui et al., 2004).

The Hsp20.2 (from *A. fulgidus*) was ineffective chaperone at low temperatures. Excess of Hsp 20.2 was required to prevent the aggregation of heat denatured at 43°C and chemically (by guanidinium chloride) denatured CS enzyme (from pig heart) (Haslbeck et. al., 2008).

Chaperone activity of Mb-sHsp (sHsp from *M. burtonii*, a cold adapted archaeon) was studied with a model enzyme, bovine glutamate dehydrogenase at a temperature range between 40 - 60°C. It was found that Mb-sHsp protected the boGDH activity up to 70% at 52°C. Also, the cooperative chaperone function of Mb-sHsp with Mb-Cpn (Chaperonin) was assessed with substrate enzyme at 42°C for 2 h and as a result, the retained boGDH activity tended to increase.

The SsHsp14.1 (sHsp from *Sulfolobus solfataricus*), protected aggregation of lipase B (CALB) (from *Candida Antarctica*) at 50°C and 60°C (Wang et. al., 2010).

1.2.1.3.2 Thermotolerance Activity of Small Heat Shock Proteins

Heat shock proteins have an important role in the induction of thermotolerance. Thermotolerance is the resistance of cells or organisms against the temperature ranges different from optimum growth temperatures of them. Organisms can resist to these extreme temperatures by their specific proteins, called heat shock proteins. Some organisms are not intrinsically resistant to extreme temperatures. To increase their thermal resistance heat shock proteins are employed. Synthesis of stress proteins correlates with acquired tolerance, “*a phenomena in which a mild conditioning temperature confers tolerance, at the cellular and organismal levels, to subsequent temperatures that would otherwise be lethal*”. Heat shock proteins confer tolerance by protecting proteins and protein complexes from denaturation and aggregation (Sanders,1993).

In addition to sHsps, Hsp104, Hsp70 and Hsp60 (chaperonin), proteins are involved in acquired thermotolerance. Furthermore, the time course of thermotolerance is related with induction of stress proteins, and inhibition of their synthesis blocks the response. Also, other stressors which induce stress proteins,

such as arsenite, cadmium, and ethanol, induce thermotolerance, too (Sanders,1993).

Several experiments on the thermotolerance have been performed in cultured cells or in many organisms , such as, *E.coli*, fish, rodent, mammalian embryonic systems...etc (Sanders B. M.,1993). As a result of these works, many organisms died quickly after the growth temperatures of these organisms were drastically increased.

The gene coding for MjHsp16.5 was cloned into the pET21a vector and transferred into the *E. coli* BL21 (DE3) strain, for expression. When extract from recombinant cell was exposed to high temperature at about 100°C, it stayed soluble but host cell extract began to precipitate at 60°C. This proved that expressed MjHsp in *E. coli* can conserve *E. coli* proteins or it can induce the expression of undetermined *E. coli* proteins that are capable of protecting other *E. coli* proteins from heat denaturation. This study showed that, purified MjHsp16.5 could protect *E. coli* cell extract from thermal aggregation. For this, MjHsp16.5 and *E. coli* BL21 (DE3) had been incubated with the plasmid pSJS1240 at a 2:1 or 1:1 ratio at 80°C and *E. coli* cell proteins remained 72% and 94% soluble, respectively (Kim et. al., 1998).

In another example, to test the thermoresistance activity of *T. sHsp*, it was expressed in *E. coli*, by the help of the vector, pET23a and thermotolerance after treatment at 75°C for 30 minutes was determined. It was found that thermal denaturation of cellular proteins of *E. coli* was prevented (Usui et. al., 2001).

Over expression of archaeal sHsps increased the thermotolerance of mesophilic bacteria by preventing protein aggregation at high temperatures. The *T. sHsp* constitutes about 0.1 % of total soluble protein in *Thermococcus sp. KS-1* cells at 93°C, due to increased gene expression. So it was suggested that *T. sHsp* played a role only in the thermotolerance of *T. KS-1* (Usui et. al., 2001).

Survivability of *E. coli* over expressing Pfu-sHsp was enhanced about 50 fold after 120 min. at 50°C (Laksanalamai et. al., 2001). It was suggested that Pfu-sHsp enhanced the thermo resistance of *E. coli* by inhibiting the aggregation of heat-tolerant proteins, since most of the proteins in the *E. coli* lysate remained soluble up to 40 min. at 105°C.

Archaeal sHsps were used to improve performance of a number of mesophilic enzymes under denaturation conditions (*i.e.*, heat and alkaline). Recombinant Pfu-sHsp protected the activities of Taq DNA polymerase, *HindIII* restriction enzyme and lysozyme at high temperatures (60°C -97°C) (Laksanalamai et al., 2006).

1.2.1.4 Biotechnological Importance and Disease Relation of Small Heat Shock Proteins

Because of the important functions in thermostability, disaggregation, and proteolysis inhibition, researchers believed that sHsps have to be understand well and so they are under investigation. Researchers have also speculated that these proteins can be the promising elements for nanobiotechnology, proteomics, bioproduction and bioseparation.

Small heat shock proteins are important in biotechnological applications for protein quality or quantity control. Biotechnological applications of sHsps can be grouped under the four general titles (Han et.al., 2008):

1. Cytoplasmic or periplasmic engineering for enhanced protein production.

In an example study, IbpA and IbpB were tested for their effect in recombinant protein production in *E.coli* cells. It was demonstrated that tested sHsps positively affected the protein production by protecting recombinant proteins from degradation (Han et. al., 2004).

2. Stabilization of proteins and peptides in biochip technology.

In the assays of protein-chip technology, there are two problems: one of them is related with immobilization of proteins on surfaces and the other one is related with limited shelf life of proteins or enzymes. To increase functionality of proteins in chip technology, nowadays, sHsps are thought to be the solution. In a study, Taq polymerase, used in polymerase chain reaction (PCR) at high temperatures was included in the reaction mixture with sHsp, which was purified from hyperthermophilic archaeon *Pyrococcus furiosus* (Pfu-sHsp). Thereby, the Pfu-sHsps has been effective for suppressing enzymatic aggregation and intracellular precipitation of Taq polymerase at elevated temperatures (Laksanalamai et al., 2006).

3. Refolding process for preventing aggregation or precipitation

In the study of Kohler et. al. (2000), a bioreactor which involves the complete GroEL/GroES machinery for protein folding was developed. This molecular chaperone-assisted protein folding bioreactor could be improved by using small heat shock proteins which overcome protein aggregation.

4. Proteolysis inhibition in proteomics

(Han et. al., 2005), used sHsps, IbpA and IbpB from *Escherichia coli* and Hsp26 from *Saccharomyces cerevisiae* for the prevention of proteolytic degradation. Proteolytic degradation is one of the major problems in two-dimensional gel electrophoresis (2-DE). Protease inhibitors can also be used in this system for the same purpose. However, using sHsps provides advantages to the system: they are extremely effective in the proteolysis inhibition, allowing the further detection of protein. Also, sHsps are not very expensive and production and purification of sHsps in recombinant *E. coli* cells are easy.

On the other hand, sHsps are due to their mutations implicated in some human diseases (Sun and MacRae, 2005). For example, it showed that missense mutation in α A-crystallin gene was found to be the cause of cataract development

(Litt et. al., 1998). Mutated small heat shock proteins also cause some disorders, such as desmin related myopathy (DRM), a human cardiovascular disease. The mutation associated with this disease is the substitution of glycine for arginine (R120G) in the core domain of α B-crystallin in human (Vicart et. al., 1998).

Protein aggregation, which is the significant cause of many diseases, occurs after partial protein unfolding and hydrophobic surface exposure, enhancing attractive forces among protein molecules. Until today, aggregation has been related with sickle cell disease, prion disease, cystic fibrosis, hepatic cirrhosis, cataract formation and neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease. (Clark and Muchowski, 2000).

It was also reviewed that not only protein misfolding, unfolding or aggregation of lens crystallins, but also over production of α B-crystallin can cause some diseases, such as, human brain tumors, multiple sclerosis, viral infection, ischemia, renal cell carcinoma and microbial diseases related with *Mycobacterium tuberculosis*. Under such pathological conditions, the amount of sHsps increases and this upregulation process can be thought as an alarm something wrong. For this reason sHsps could be new targets for drug design (Clark and Muchowski, 2000).

1.2.1.5 Archaeal Small Heat Shock Proteins

Several archaeal sHsps have been characterized up to now. These are the sHsps from up to now, *Methanococcus jannaschii*, *Thermococcus* sp. Strain KS-1, *Sulfolobus tokodaii* strain 7, *Pyrococcus furiosus*, *Archaeoglobus fulgidus*, *Methanococcoides burtonii* and *Sulfolobus solfataricus*.

Researchers firstly studied about the sHsp from *Methanococcus jannaschii*, MjHsp16.5 is a 16.5 kDa protein and the optimum growth temperature of this

organism is about 88°C. To understand the effects of MjHsp16.5 on thermal protection of *E.coli* cells and inhibition of thermal aggregation on different protein substrates were tested (Kim et. al., 1998).

The second analyzed archaeal sHsp is from an other hyperthermophilic organism (the growth temperature is between 85-90°C), *Thermococcus sp.* Strain KS-1 (*T. sHsp*) and the molecular weight of it is 20 kDa (Usui et. al., 2001). It is a spherical oligomer which is also composed of 24-mer and it was proved that it can protect some proteins from thermal aggregation at high temperature and promote the refolding of denatured green fluorescent protein in vitro. But there is no evidence about the thermal protection roles of this sHsp.

For the genomic analysis of *T. sHsp*, after the total DNA of *T. KS-1* had been extracted, it was amplified, cloned and sequenced. In PCR amplification, primers which were designed based on the aminoacid sequence of the sHsp from *Pyrococcus horikoshii* OT3 were used. The amplified DNA fragment was subcloned into the pT7Blue T vector and sequenced. By the PCR product as a probe, southern blot hybridization was done to see the place of sHsp in the genome. After N and C-terminal and adapter DNA amplification by PCR, full-length sequence of sHsp was obtained.

StHsp14.0 is an sHsp from the archaeon, *Sulfolobus tokodaii* strain 7. The optimum growth temperature of *S. tokodaii* is 80°C . There is no evidence about the thermal protection and protein refolding activity of StHsp14.0. To test the inhibition of thermal aggregation role of sHsp, the enzyme 3-isopropylmalate dehydrogenase was observed at 87°C.

Pyrococcus furiosus is another archaea which lives at about 100°C. The sHsp, Pfu-sHsp from *P. furiosus* was identified as a 20 kDa protein and heat inducible (Laksanalamai et. al., 2001). On the other hand, aggregation prevention effect of

Pfu-sHsp was demonstrated using bovine glutamate dehydrogenase enzyme as the substrate.

Another model organism, *Methanococcoides burtonii*, is a eurypsychrophilic archaeon which is isolated from Ace Lake in Antarctica where the temperature is less than 2°C. Mb-sHsp, a small heat shock protein which is an α B-crystallin homolog, was cloned, and it was shown that it had a role in molecular chaperone system in vivo (Laksanalamai, 2008). In this study, researchers cloned and expressed the genes of α subunit of chaperonin (Mb-Cpn), in addition to Mb-sHsp, in *E. coli* Rosetta strain to understand the functions of cold adapted molecular chaperones. Also, the binding capability of Mb-sHsp to native or denatured substrate was tested by surface plasmon resonance in the spectroscopy and it was found that the interaction between the Mb-sHsp and the denatured substrate was strong, while there was no interaction with the native form.

Sulfolobus solfataricus is an hyperthermophilic archaeon, normally grows at about 80°C. The sHsp of this organism (SsHSP14.1) was successfully cloned, expressed in *E.coli* and purified (Wang et. al., 2010). *In vivo* chaperone function of SsHSP14.1 for preventing aggregation of proteins during heating was investigated. The purified SsHSP14.1 protein was able to prevent *Candida antarctica* lipase B from aggregation for up to 60 min. at 80°C. Moreover, the SsHSP14.1 enhanced thermostability of bromelain extending its half-life at 55°C.

1.3 Stress and Induction of Stress Response

Stress is an unwanted situation for all organisms because it damages to cells. It is caused by a sudden change, called stressor (stress factor), in the environment that the organisms normally live in. Stressors can be of physical (temperature elevation) or chemical origin (increase or decrease in pH, salinity or oxygen availability). Also, oxidative stress (H₂O₂ exposure), ultraviolet (UV) light and

high metal and pollutant concentrations can be considered as other abnormal conditions for cells. All these stressors cause an increase in the amount of damaged or abnormal proteins in the cell.

All life forms show different stress tolerance to different stress factors. This related to type of organism or exposure time to the stressor. In general, organisms can be alive under mild stress conditions, but they can not tolerate the hard stress conditions that can be lethal for them (Lindquist, 1986) .

1.4 Stress Response In Archaea

Although most of the archaea can live in normal conditions, many of them live in extreme conditions, such as salt lakes or submarine volcanic habitats, so they are called “extremophiles”. Extremophiles are divided into different subgroups; hyperthermophilic (live at 80°C - 122°C), acidophilic (optimal pH value is about 3 or lower) or thermoacidophilic (live at 70°C - 80°C and pH range is between 2 and 3) archaea. Since they live in such unusual conditions, stress proteins should be of great importance for archaeal species.

Since the 1980s, the stress response in archaea has been studied, but firstly in 1991, an archaeal stress gene (hsp70) was cloned and sequenced (Macario et. al., 2004).

There are heat shock proteins or chaperones in all archaeal organisms, but not all of them are found in all archaea. For example, yet the chaperonin genes are expressed in all type of archaeal members, the co-chaperones are not conserved in archaea. For instance, bacterial co-chaperones, NAC and trigger factor and the eukaryal co-chaperones, BAG, Hop, and Hip, are not present in archaea, with the probable exception of the NAC alpha subunit. On the other hand, Hsp70 (DnaK)

and its co-chaperones (GrpE and Hsp40 (DnaJ)) are common in many bacteria and eukaryotes but not in archaea, they are not as widespread as being in them.

Prefoldin proteins are another group of the stress proteins. After eukaryotic prefoldins had been discovered, but their occurrence in bacteria has never been suggested. In archaea whose genomes were completely sequenced, prefoldins have been as two subunits. Since the eukaryotic prefoldin subunits show length variation, their sizes show high similarity in all archaeal species (Large et. al., 2009). Prefoldin is a hexameric complex which binds newly synthesized or unfolded proteins and transfers them to chaperonins for folding process.

As a result we can say that the most studied archaeal heat shock protein or chaperone is Hsp60 (chaperonin) and the least studied ones are Hsp90 and Hsp100 because archaeal species are generally lack of them.

1.5 Model Organism: *Thermoplasma volcanium*

Thermoplasma volcanium is one of the species of the kingdom of *Thermoplasmatales* and the genus of *Thermoplasma*. The other species of this genus is *Thermoplasma acidophilum*.

According to the endosymbiosis hypothesis, the archaeal genus, *Thermoplasma* is different from other counterparts, because they are thought that they can be the origin of eukaryotic nuclei. They can live both in aerobic or anaerobic conditions (Kawashima et. al., 2000).

Thermoplasma volcanium is a thermoacidophilic archaeobacterium which live in shallow marine vents and solfataric fields. The best conditions for their viability and growth are pH 2.0 and 60°C. Although these conditions are optimum they can

also live in the temperature range of 33-67°C and pH range is 0.5 – 4 for them. They sustain their life as a heterotroph (Schäfer et. al., 1999).

By the comprehensive genomic study of *Thermoplasma* species, although they do not have cell wall, these organisms can adapt to higher temperatures and acidic conditions because of tetraether lipid content in their cytoplasmic membrane (Hans-Joachim Fedi Freisleben, 1999). On the other hand, characteristics of genomic organization and the optimum growth temperature of *Thermoplasma volcanium* have shown a crucial parallelism. In terms of organization of DNA structure; although *Tp. volcanium* doesn't have any genes of DNA topoisomerase I, it has DNA topoisomerase IV subunits (Kawashima et. al., 2000). The complete genomic sequence of this organism (1,584,804 bp) was determined (Minezaki et. al., 2003).

1.6 Aim of This Study

In our study, we have aimed to analyze two archaeal small heat shock proteins in the stress response of *Thermoplasma volcanium*.

Firstly, we have tested the effect of overexpression of the *Tp. volcanium* sHsps on the thermo resistance of the mesophilic host organism, *E.coli*.

Then, differential expressions of the two sHsp-genes were evaluated under different stress conditions by Real-Time PCR

Finally, chaperone activity of one of the recombinant sHsp was assessed using bovine glutamate dehydrogenase enzyme as the substrate.

CHAPTER 2

MATERIALS and METHODS

2.1 Materials

2.1.1 Chemicals, Kits and Enzymes

All used chemicals were molecular biology grade and in the highest purity available. Agarose (low melting point gel), Ammonium chloride (NH_4Cl), Ammonium Per Sulfate, ampicillin, ethidium bromide (Et-Br), Ethylenediaminetetraacetate (EDTA), guanidine hydrochloride (GdnHCl), tetramethylethylenediamine (Temed), lysozyme, glutamate dehydrogenase (from bovine liver), alpha ketoglutarate (α -KG) and β -Nicotinamide Adenine Dinucleotide, reduced form (β -NADH) were purchased from Sigma Chemical Company, Spruce St. St. Louis, Missouri, USA.

Agar was purchased from Acumedia, Baltimore, USA. Hydrogen peroxide (H_2O_2 , 30%) was from Applichem, Darmstadt, Germany. Absolute ethanol was from Reidel de Häen. Tris(hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), sodium chloride (NaCl), sodium hydroxide (NaOH), β -mercaptaethanol, sodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogenfosfat (NaH_2PO_4), α -D-Glucose, potassium dihydrogen phosphate (KH_2PO_4), magnesium sulfate (MgSO_4) and tryptone were purchased from Merck; Darmstadt, Germany. Powder imidazole was purchased from Fluka Chemie AG, Switzerland. Yeast extract was from Difco, Detroit, USA.

Agarose MS (molecular screening agarose), M-MULV Reverse Transcriptase, M-MULV buffer, Transcriptor First Strand cDNA Synthesis Kit, LightCycler ® FastStart DNA Master SYBR Green I kit and LightCycler ® FastStart DNA

Master PLUS SYBR Green I kit were purchased from Roche Diagnostics Switzerland.

Primers used in RT-PCR experiments were synthesized by TIB MOLBIOL GmbH, Berlin, Germany.

Restriction endonucleases *Sall* and *PstI*, and their buffers were purchased from Fermentas UAB, Vilnius, Lithuania.

QIAexpressionist Kit, RNeasy Mini Kit, QIA Express Kit were purchased from QIAGEN Inc. Valencia, USA.

Wizard® Plus SV Minipreps DNA Purification System Kit was purchased from Promega Corporation, Madison, WI, USA.

2.1.2 Buffers and Solutions

Compositions of buffers and solutions used in this study were given in Appendix A.

All buffers and solutions made up in ultra-pure water and sterilized after preparation by an autoclave (ALP Co. Ltd., Tokyo, Japan).

2.1.3 Molecular Size Marker and Ladders

Molecular size markers for DNA (λ DNA/ EcoRI+HindIII Marker, GeneRuler™ and O'GeneRuler™ 50 bp DNA Ladders), RNA (RiboRuler™ Low Range RNA Ladder) and protein (PageRuler™ Prestained Protein Ladder) were from Fermentas UAB, Vilnius, Lithuania. Images of these markers were given in Appendix B.

2.2 Strains and Mediums

2.2.1 Archaeal and Bacterial Strains

Thermoplasma volcanium GSS1 (strain type 4299) was purchased from DSMZ (Deutsche Sammlung von Microorganismen und Zellkulturen, GmbH, Braunschweig, Germany), was used as the source organism in this work.

Recombinant *E. coli* pQE-31/775 strain, previously constructed in our laboratory, was used for isolation of TVN0775/sHsp.

2.2.2 Growth and Culture Conditions

Thermoplasma volcanium cells were grown in volcanium medium (Robb, 1995). That was supplemented with glucose and yeast extract (Oxoid, Difco). The final glucose and yeast extract concentrations in the medium was 0.5% and 0.1% respectively. The growing cultures were maintained by weekly transfer into fresh medium.

Active cultures of recombinant *E.coli* pQE-31/775 cells were grown on LB agar medium (see Appendix A), containing ampicillin (100 µg/ml) and incubated at 37°C overnight. The culture plates were stored on 4°C - 8°C and renewed once a month, routinely.

2.3 Methods

2.3.1 Plasmid DNA Isolation from Recombinant *E.coli* pQE-31/775 Cells

To check for presence of the expression plasmid harboring TVN0775/sHsp gene plasmid DNA was isolated from *E.coli* pQE-31/775 cells.

Putative recombinant colonies were collected depending on blue/white colony selection. Cells from the white colonies were inoculated into 10 ml Luria Bertanii (LB) broth medium containing ampicillin (1 %) were incubated in a shaker incubator at 37°C, 207 rpm (Heidolph Unimax 1010 Shaking Incubator, Heidolph Instruments GmbH, Kelheim, Germany) for a night. The next day, isolation protocol of the kit, Wizard® Plus SV Minipreps DNA Purification System (Promega Corporation, Madison, WI, USA) was followed. Following the protocol, sample tubes were harvested by centrifugation (IEC Clinical Centrifuge, Damon/IEC Division, USA) for about 10000 x g for 15 minutes. Taken samples were put into ice and cells were resuspended by adding 250µl Cell Resuspension Solution and mixing via vortex. In lysing step, cell lysis solution used as same amount as resuspension solution and mixed again but now by hands slowly until the cell suspension cleared. After 4 minutes of incubation at room temperature, alkaline protease solution was added and inverted the tubes for 4-5 times for the inactivation of endonucleases and other proteins at room temperature for 5 min. . Other proteins mentioned here were released during the lysing of the bacterial cells which can adversely affect the quality of the isolated DNA. At the following step of the procedure, samples were incubated in ice for 1 min. after the pipetting neutralization solution. During this period, tubes were inverted for 6-8 times to mix the ingredients. The cell debris was pelleted by centrifugation (Micomase 230 RF Centrifuge, Thermo IEC, USA) step for 10-15 minutes at 25°C, 13000 rpm. Then, cleaned lysate was transferred into the spin column and centrifuged in a microcentrifuge for only 1 min to bind to DNA to the column. And after discarded the flowthrough, washing steps started with the addition of 750 µl washing solution and centrifuged for 1 min., again. second washing step was done by adding lower solution, 250µl and centrifuged again for 1 min but two times. Finally the plasmid DNA was eluted by 100 µl of nuclease free water and lastly centrifuged for 1 min.. After centrifugation, sample about 15 µl was run on the agarose gel to check the efficiency of purification and the remaining sample was stored at -20°C for further use.

2.3.2 Plasmid DNA Digestion by Using Restriction Enzymes

Purified pQE-31/775 plasmid was digested with one (*PstI*) and combination of two (*PstI* and *SalI*) restriction enzymes. Digestions were performed following the protocol of the manufacturer (Fermentas) and the samples were incubated in a dry block heater (Multi-blok heater, Lab-Line) at 37°C for 1-2 hours.

2.3.3 Agarose Gel Electrophoresis

After isolation and digestion of plasmid DNAs were analysed by agarose gel (0.8% w/v) electrophoresis. We used a submarine agarose gel apparatus (Mini Sub™ DNA Cell, Bio-Rad, Richmond, CA, U.S.A) in these experiments. For gel preparation and electrophoresis 1X TAE buffer was used. DNA samples (of 10-20 µl), mixed with 1/10 vol. of tracking dye, were loaded into wells of gel which was supplemented with ethidium bromide (Et-Br, 0.5 µg/ml). Electrophoresis was carried out at 70 volts and 70 mAmp (Bio-Rad Power Supply, 200/2.0).

After electrophoresis, the bands on the gel were visualized and the image was photographed by a gel imaging and documentation system (Vilber Lourmat TFP-M/WL, Marne La Vallee Cedex 1, France and Vilber Lourmat, CN 3000, EU).

2.4 Purification of the Recombinant TVN0775/sHsp by Ni-NTA Column Chromatography

2.4.1 Preparation of Cell Free Extract

Recombinant pQE31 – 7 (including TVN0775 gene) cells were inoculated into a flask containing 10 ml LB broth and ampicillin, then incubated at 37°C and 207 rpm overnight in a shaker incubator (Heidolph Inkubator 1000, Germany).

To increase the culture volume, 50 ml LB broth supplemented with ampicillin was inoculated using overnight culture of recombinant *E.coli* pQE-31/775 cells. This culture was grown at 37°C by vigorous shaking at 300 rpm. The absorbance at 600 nm was monitored spectrophotometrically at regular intervals. (Shimadzu 1601 UV Visible Spectrophotometer, Shimadzu Analytical Co., Kyoto, Japan). At mid-exponential phase ($OD_{600}=0.5-0.7$), IPTG (1 mM final concentration) was added to induce the expression. Incubation continued about 5 hours. Then, cells were harvested by centrifugation at 4000g (Sigma 3K30 Centrifuge, Germany) for 20 min. at +4°C. After the supernatant was discarded, the cell pellet was dissolved in lysis buffer then lysozyme solution (1mg/ml) was added and the mixture was incubated on ice for 30 min. The cells were disrupted by sonication (Sonicator VC 100, Sonics and Materials, CT, U.S.A). The cell free extract (CFE) was obtained by centrifugation of the lysate (Sigma 3K30 Centrifuge, Germany) at 10000g at 4 °C for 30 min. The supernatant called cell free extract, was stored at -20°C until use.

2.4.2 Purification of TVN0775/sHsp

6XHis tagged proteins were purified using the QIAExpressionist Kit (QIAGEN Inc., Valencia, USA) following the manufacturer's instructions. The kit technology based on the selectivity and affinity of nickel-nitrilotriacetic acid (Ni-NTA) agarose for purification of recombinant proteins tagged with six histidine residues. Ni-NTA column in which has Ni-NTA agarose and it binds polyhistidine residues. The affinity of 6XHis tag facilitates binding to Ni-NTA. Nitrilotriacetic acid (NTA) is a tetra-dentate chelating ligand, in a highly cross-linked 6% agarose matrix. The polyhistidine sequence will bind to nickel, NTA binds Ni^{2+} ions by four coordination sites, leaving two sites free to interact with the 6XHis tag.

Purification of 6xHis-tagged proteins by Ni-NTA affinity chromatography, however, can be performed under native or denaturing conditions (Figure 2.1).

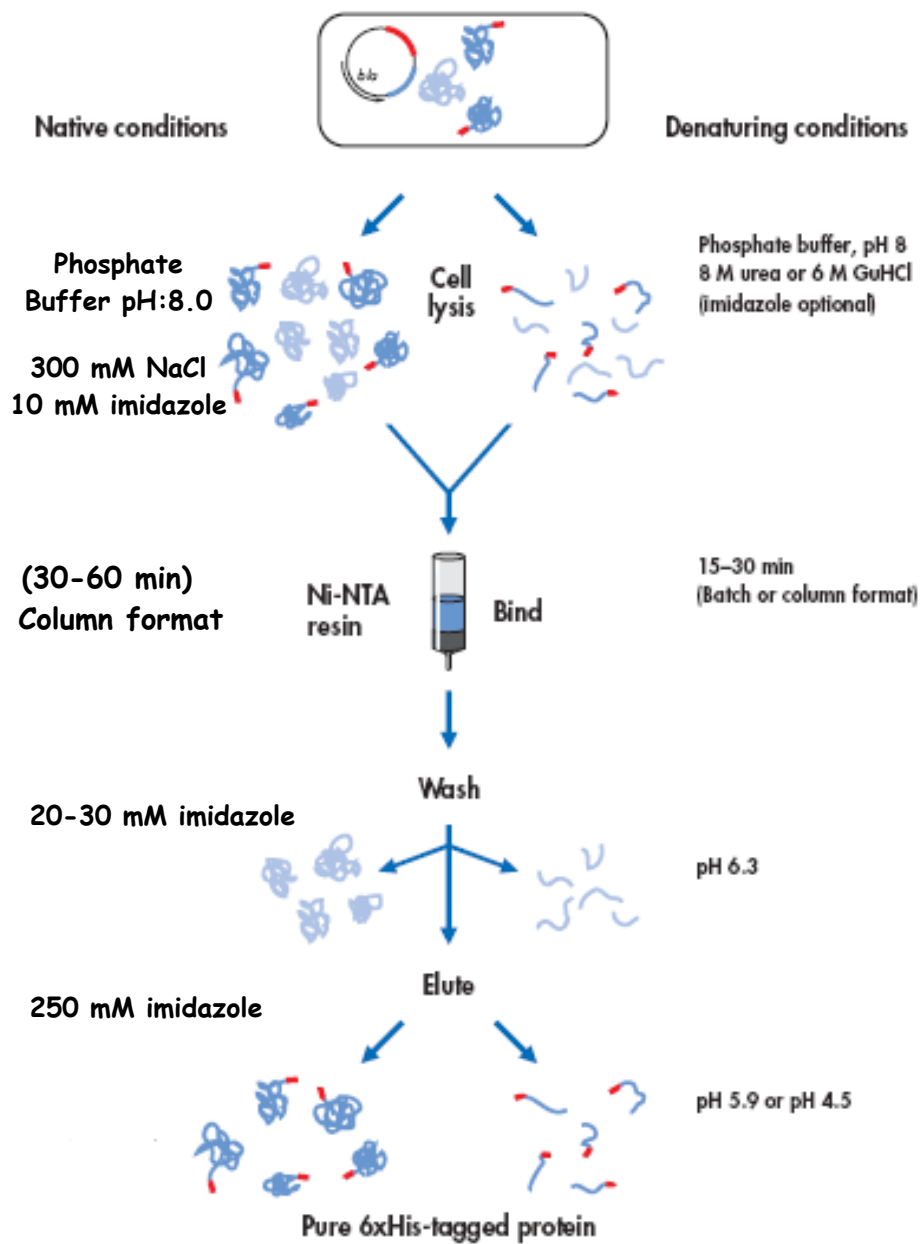


Figure 2.1 Purification of 6X-His tagged proteins using the QIAexpress System (from the handbook, QIAexpressionist Kit, Qiagen).

The cleared lysate was thawed on ice and certain volume (2-4 ml) of the lysate was added to 1 ml of the 50% Ni-NTA slurry, and mixed gently by shaking at 4°C for 60 min. The lysate- Ni-NTA mixture was loaded into a column within the bottom outlet capped. After the agarose has set, bottom cap was removed, and

column flow through was collected in the eppendorph tubes. Then the column was washed twice with 4 ml wash buffer and the wash fractions were collected for SDS-PAGE analysis. The His-tagged sHsp was eluted 4 times with 0.5 ml elution buffer. The eluate and other column fractions were stored at -20°C for further use.

The contents and preparation of the used buffers here, were given in Appendix A.

2.4.3 SDS Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of 0.5 % anionic detergent SDS was performed on the 5 % stacking gel and 12.5 % separating gel according to Laemmli (1970). PageRuler™ Prestained Protein Ladder, (Fermentas UAB, Vilnius, Lithuania) was used as molecular weight standard. Vertical gel electrophoresis was carried out using the Bio-Rad System (Bio-Rad, Richmond, CA, U.S.A). The assembly of the glass plate cassettes and the process of the gel casting were done according to the instruction manual provided with the apparatus.

10 µl aliquots of samples were mixed with 2X sample buffer containing 5% SDS and incubated in boiling water bath for 5 minutes to denaturate the proteins. Afterwards, protein sample and molecular weight standart were loaded onto gel. Running process was carried out at 100 V at +4°C (Bio-Rad Power Supply, 200/2.0). After the electrophoresis, polyacrylamide gel was stained in Page Blue Protein Staining Solution (Fermentas) and then, washed by distilled water by several times. Gel photos were taken by the camera (Vilber Lourmat Gel Imaging and Analysis System, Marne La Vallee Cedex 1, France.Vilber Lourmat) under white light.

2.4.4 Heat treatment of the cell lysates

Frozen cell free extract was thawed on ice and heated at 65°C for 10 minutes in a waterbath (Kottermann Labortechnik, Germany). After heat treatment, cell lysate was kept on ice for 30 minutes and then centrifuged (Sigma 3K30 Centrifuge, Germany) at 12000 rpm at 4°C for 90 minutes to remove denatured proteins of the host cell. The supernatant was kept at -20°C until use.

2.4.5 Ultrafiltration of the Purified sHsp

After protein purification, some of the eluted samples were chosen according to the gel image and they were mixed in a filter (cut off: 5K) (M-141, Sigma Chemical Co., St. Louis, Missouri, USA). Firstly, filter was washed with some lysis buffer by centrifugation using a top clinical centrifuge (Labofuge 200, Heraeus Sepatech, Germany) for 15 min. at 4000xg. Then the sample was applied to filter and buffer exchanged by lysis buffer (without imidazole) and concentration was achieved by repeated centrifugation for 30 minutes at 4000xg. These samples were kept at -20°C until use in chaperone activity assays.

2.5 Determination of Chaperone Activity of sHsps from *Thermoplasma volcanium*

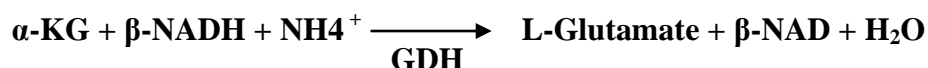
Chaperone activity of purified TVN0775/sHsp was tested in two aspects, first to find out heat protection effect, second to find out refolding activity on chemically denatured substrate protein. The model enzyme used in these experiments was bovine glutamate dehydrogenase (boGDH, EC 1.4.1.3) (Sigma).

2.5.1 Protection of boGDH from Heat Denaturation by the Help of TVN0775/sHsp

Aliquots of enzyme (0.2 U) in the presence (20 μ l) and absence (control) of purified TVN0775/sHsp were heated separately at 45°C, 50°C and 53°C. The residual activities of enzyme samples were measured as described in the manufacture's protocol (Sigma). Standard reaction mixture (1 ml total volume in quartz cuvettes) contained; 90 mM sodium phosphate buffer, 12 mM α -ketoglutarate, 48 mM ammonium acetate, 0.16 mM β -NADH, 0.4 mM EDTA and 0.03-0.06 unit L-glutamic dehydrogenase. (pH: 7.43). The mixture was pre-incubated at 25°C for 3 min.

Absorbance was measured at 340 nm using UV visible double-beam spectrophotometer with a temperature controlled cell holder (Shimadzu 1601 UV/Visible Spectrophotometer, Shimadzu Analytical Co., Kyoto, Japan).

The glutamate dehydrogenase activity is based on this reaction:



The rate of decrease in the absorbency at 340 nm and 25°C, resulting from the oxidation of NADH in the presence of α -ketoglutarate and ammonium ions, is proportional to the catalytic activity of glutamate dehydrogenase.

2.5.2 Refolding of Chemically Denatured boGDH by the Help of TVN0775/sHsp

The aliquots of boGDH (44 U) in the assay buffer were denatured in 1.5 M, 2 M, 2.5 M, 3 M, 4 M and 5 M guanidinium hydrochloride (GdnHCl) for 30 min. and 60 min. at room temperature. For renaturation, denatured enzyme was diluted 100 fold in the assay buffer and incubated at room temperature in the presence (20 μ l)

and absence (control) of the TVN775/sHsp for 2 h. The residual boGDH activity was measured as described above.

2.6 Tolerance of Recombinant *E.coli* cells Expressing sHsps to Heat Shock

To study the effect of sHsps on survivability of the *E.coli* cells at high temperature, overnight cultures of the control *E.coli* cells (transformed with pQE-31 plasmid) and recombinant ones which containing TVN0984 and TVN0775 genes were prepared in LB broth, containing ampicillin. Each of the fresh LB broth (25-30ml) with ampicillin in flasks inoculated with 1 ml overnight culture and cells were grown up to mid-exponential phase by shaking at 37°C (Heidolph Incubator 1000, Germany). Then sHsp expression was induced by adding IPTG to a final concentration of 1mM and incubation was continued under the same conditions. Culture samples were removed by regular time intervals (30 min) and optical densities were measured at 600nm (Schimadzu Corperation, Japan).

After 2.5 hours, the cultures were transferred to new LB broth so OD at 600nm was 0.5-0.7. Then, these cultures were exposed to heat shock at 53°C for 2 hours in a shaking water bath (Kottermann Labortechnik, Germany). During this incubation period, absorbance measurements at 30 min. intervals were made and also series of 10 fold dilutions of these samples were plated onto LB agar plates (with ampicillin) by spreading. The plates were incubated overnight at 37°C. Nextday, colonies were counted.

The numerical results of colony counting and recorded OD values during these experiments were evaluated by plotting graphs using Microsoft Excel program.

2.7 Stress Response of *Thermoplasma volcanium* Cells

Before RNA isolation, *Tp. volcanium* cells were stressed under different factors: heat, pH and hydrogen peroxide (H₂O₂).

2.7.1 Heat Stress

Tp. volcanium cells were grown in 10 ml medium (50 ml of liquid volcanium medium, pH: 2.7) containing glucose and yeast extract in a 250 ml flask. After incubation at until mid-log phase (OD₆₀₀= 0.5-0.7), used for inoculation of three supplemented volcanium media. Following subculturing the cells were grown at 60°C for 4 days. The incubation temperature for two flasks were shifted to 65°C and 70°C, separately for 2 hours. During heat shock, samples were removed at regular time intervals for RNA isolation. Incubation of the third culture flask (control) was proceeded at 60°C and samples for RNA isolation were removed as parallel to test cultures.

2.7.2 pH Stress

In this experiment five different cultures of *Tp. volcanium* was prepared and inoculated as descibed for heat stress. As it is known, the optimum pH range is around 2.0 for *Tp. volcanium* cells. After measuring the pH value of growing cultures, the pH value of test cultures were adjusted by sodium hydroxide (NaOH) up to planned value. These measurements and adjustments were performed by the help of Mettler pHmeter.

Following incubation at 60°C for 4 days the pH of the four cultures were raised to pH: 3.5, pH: 4.0, pH: 4.5 and pH: 5.0, separately. Incubation was continued at 60°C for 3 hours. Control culture (pH: 2.0) incubation was performed at 60°C

without any pH adjustment. Culture samples were removed at 30 minutes time intervals following pH shift both from test cultures and control culture.

2.7.3 Oxidative Stress

Five different cultures of *Tp. volcanium* was prepared and inoculated as described above. After incubation of the cells at 60°C up to mid –log phase, four of the cultures were exposed to oxidative stress by adding H₂O₂ at 0.01 mM, 0.02mM, 0.03mM and 0.05mM final concentrations and incubation continued for 3 hours. Control culture was grown under the same conditions without H₂O₂ addition from both test cultures and control culture.

2.7.4 RNA isolation from *Thermoplasma volcanium* Cells Exposed to Stress

Total RNA isolation was performed by using the protocol of RNeasy Mini Kit (QIAGEN Inc. Valencia, USA).

After the isolation, quality and the quantity of the RNA samples diluted (dilution factor :1/233) in deionized water were determined by OD₂₆₀ and OD₂₈₀ measurements by using UV-visible doublebeam spectrophotometer (Shimadzu 1601 UV/Visible spectrophotometer, Shimadzu Analytical Co., Kyoto, Japan). The samples were deep freeze (-80°C) to use them later for cDNA preparation.

2.7.5 Agarose Gel Electrophoresis of Eluted RNA Samples

Formaldehyde agarose gel (1,2 %) was prepared according to the recipe which was given in appendix A. For observation of the purified RNA bands by gel electrophoresis, an aliquot of defrosted RNA samples (5 µl) mixed with 2X RNA loading dye (1,5 µl) and RNA Ladder (RiboRuler™ Low Range RNA Ladder ,

Fermentas) (2 µl) was mixed with its loading dye (2 µl). Then the samples and the ladder incubated at 65°C in a water bath (Köttermann, GmbH & Co KG, Uetze, Hänigsen, Germany) for 5 min. Then, they were loaded onto the gel and electrophoresis was performed on 70 V and 70 mA using Bio-Rad power supply for about 1 hour.

After electrophoresis, the bands in the gel were visualized and photographed using an imaging system (Vilber Lourmat Gel Imaging and Analysis System, Marne La Vallée Cedex 1, France).

2.8 Two step Reverse Transcription PCR (RT-PCR) Amplification of Gene Fragments of Hsp20 related genes, TVN0775 and TVN0984;

To study gene expression levels of variously stressed cells, we have performed a two-step RT-PCR; RT-PCR on a conventional thermal cycler (Techgene, Techne Inc. NJ, USA) for single strand cDNA synthesis and quantitative RT-PCR on the LightCycler® Carousel-Based System, the LightCycler® 480 System, Roche Diagnostics, Switzerland.

2.8.1 Designed Primers for RT-PCR

For the first step of RT-PCR analysis, we needed single primers for TVN0775 (375 nt) and TVN0984 (549nt). The sequences of the primers were obtained from the cloned TVN0775 and TVN0984 genes' sequences. The length of the primer for TVN0775 was 22 bp and for TVN0984 was 21 bp and their GC contents were 40.9% and 47.6%, respectively. These primers were ordered from the company of TIB MOLBIOL (GmbH, Berlin, Germany) (Table 2.1).

Table 2.1 Sequences of the primers in the reverse transcription .

Primers	Sequence of the Primers
RP Reverse Primer for TVN0775	CTAACTGTAAGTATGCCTTCAC
RP Reverse Primer for TVN0984	GTATAGAGACCTTTCTGCCAC

2.8.2 cDNA Synthesis

The reaction mixture for RT-PCR of each RNA sample was prepared in 0.2 ml PCR tubes that were kept on ice. The reaction mixture in a total volume of 20 μ l contained 1 μ g RNA sample, 0.4 μ M of primer, 400 μ M of the dNTP mix. The reaction tubes were placed in the Thermal Cycler and preheated at 70°C for 10 minutes and then, M-MuLV Reverse Transcriptase enzyme (1 μ l) and 5X M-MuLV buffer (5 μ l) were added. The reverse transcription was carried out at 42°C for 1 hour followed by heating at 94°C for 3 min. to stop reaction. The amplification product was kept at 4°C until use.

M-MuLV Reverse Transcriptase enzyme used in this experiment is an RNA-dependent DNA polymerase which uses single-stranded RNA or DNA as a template in the presence of a primer to synthesize a complementary DNA (cDNA) strand.

In these experiments, Transcriptor First Strand cDNA Synthesis Kit was used following the Manufacturer's protocol (Roche Diagnosis, Switzerland Roche).

2.8.3 Primers for Quantitative Reverse Transcription (RT-PCR)

In qRT-PCR experiments, we used forward primer (Fp) and reverse primer (Rp) set for each sHsp gene. For TVN0775 gene, the length of Fp is 24 bp and the length of Rp is 22 bp. The amplified cDNA fragment of the TVN0775 gene is 77 bp. For TVN0984 gene, the length of Fp is 20 bp and the length of Rp is 21 bp. The amplified cDNA fragment of the TVN0984 gene is 164 bp (Table 2.2).

Table 2.2 Forward and Reverse Primers that are used in qRT-PCR for sHsp genes

Primers	Sequence of the Primers
Fp Forward Primer for TVN0775	ACTGTGAAAGAGGTCTCATCCTTT
Rp Reverse Primer for TVN0775	GCTTCCAATACCAGATCAGAGC
Fp Forward Primer for TVN0984	TTACGCTTAACGTGGACGAA
Rp Reverse Primer for TVN0984	TGTAGTCGAAGTCAATGCTCTTG

2.8.4 Real-Time PCR Analysis

The cDNA samples synthesized in the first step reverse transcription were used as template in the Real-Time PCR. The LightCycler® FastStart DNA Master SYBR Green I and LightCycler® FastStart DNA Master PLUS SYBR Green I (Roche Diagnosis, Switzerland) kits were used in the studies by following the kit protocols cDNA amplifications were monitored Real-Time, using LightCycler® 1.5 instrument. (Roche Dagnostics, Roche Instrument Center AG, Switzerland). The kit protocol based on the detection of amplified PCR products by the

fluorescence signal of Sybr Green I dye at 530 nm. Sybr Green I is a kind of fluorophore which is a functional group in a molecule which will absorb energy of a specific wavelength and re-emit energy at a specific wavelength, causes a molecule to be fluorescent. Intercalating into the double stranded DNA is the specificity of Sybr Green I. This dye is preferred in this reaction because it is stable and also binds non-covalently to the surface of DNA.

Fast Start DNA Master PLUS SYBR Green I kit protocol is achieved by four programs in light cycler: the first one is the pre-incubation stage, for activation of the DNA polymerase and denaturation of the DNA. Amplification of the target DNA is the following step and the fluorescence signal of Sybr Green I increases by binding to newly synthesized DNA at this step. Then, the stage in which melting curve for PCR product identification comes. Melting occurs by gradually increasing the temperature of the reaction. As the temperature is increased, the fluorescence decreases because of the separation of double strands of the DNA. At the end of the Real-Time PCR reaction, rotor and the thermal chamber is left for cooling.

In each Real-Time PCR experiment a control sample reaction was run, which was prepared as the test samples reaction, except instead of cDNA, ddH₂O is included into mixture (negative control). The validation of the specific amplifications will be provided by T_m (melting temperature) curve analysis.

2.8.5 Agarose (MS) Gel Electrophoresis of the Products of RT-PCR

The product of Real-Time PCR, were analyzed by agarose gel electrophoresis which is using agarose MS (Roche Diagnostics Switzerland) is specific for high resolution of small size DNA fragments. Electrophoresis was run as described before with 1X TAE as the running buffer. As size marker, DNA ladder (Gene Ruler™ 50bp DNA ladder Plus) from MBI Fermentas was used.

CHAPTER 3

RESULTS

3.1 Restriction digestion of the recombinant plasmids carrying TVN0775 and TVN0984 genes

The recombinant TVN0775 and TVN0984 genes heterologously expressed in *E.coli* previously in our laboratory. Digestion of pQE 30/7-1 and pQE 31/7 plasmids with *PstI* linearized the recombinant plasmids, yielding 618 bp and 447 bp length fragments respectively. Double digestion with *Sall* / *PstI* excised the cloned TVN0775/sHsp gene (489 bp) and TVN0984/sHsp gene (659 bp) from recombinant pQE 31 and pQE 30 plasmids respectively. The gel electrophoresis profiles of the restriction fragments are shown in the figure 3.1

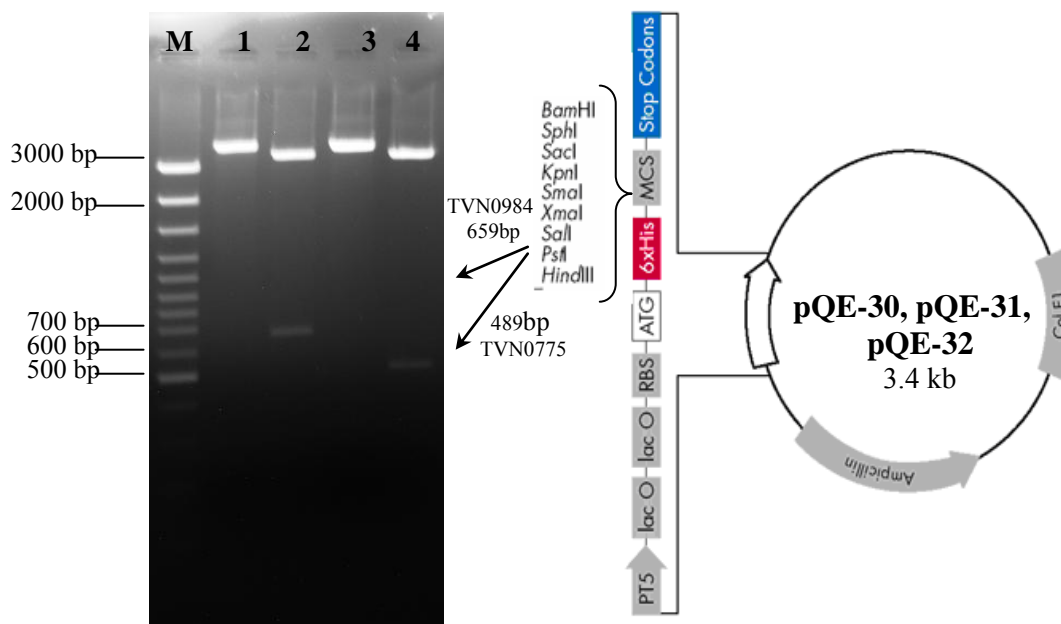


Figure 3.1 Agarose gel electrophoresis of restriction enzyme digested recombinant plasmids. **Lane 1:** pQE-30/984 gene digested with *PstI* enzyme; **Lane 2:** pQE-30/984 gene digested with *PstI* and *Sall* enzymes; **Lane 3:** pQE-31/775 gene digested with *PstI* enzyme; **Lane 4:** pQE-31/775 gene digested with *PstI* and *Sall* enzymes. **M:** GeneRuler™, 100 bp Plus DNA Ladder (Fermentas, UAB, Vilnius, Lithuania).

3.2 Heat Tolerance of Recombinant *E.coli* cells

3.2.1 Heat Tolerance of Recombinant *E.coli* pQE-31/775 cells

The growth curves obtained by OD₆₀₀ measurements for *E.coli* pQE-31/775 and *E.coli* pQE-31 (control) cultures overlap before and after induction and up to heat shock application. After heat shock there has been some retardation in the growth of the control culture, as compared to test culture (Figure 3.2).

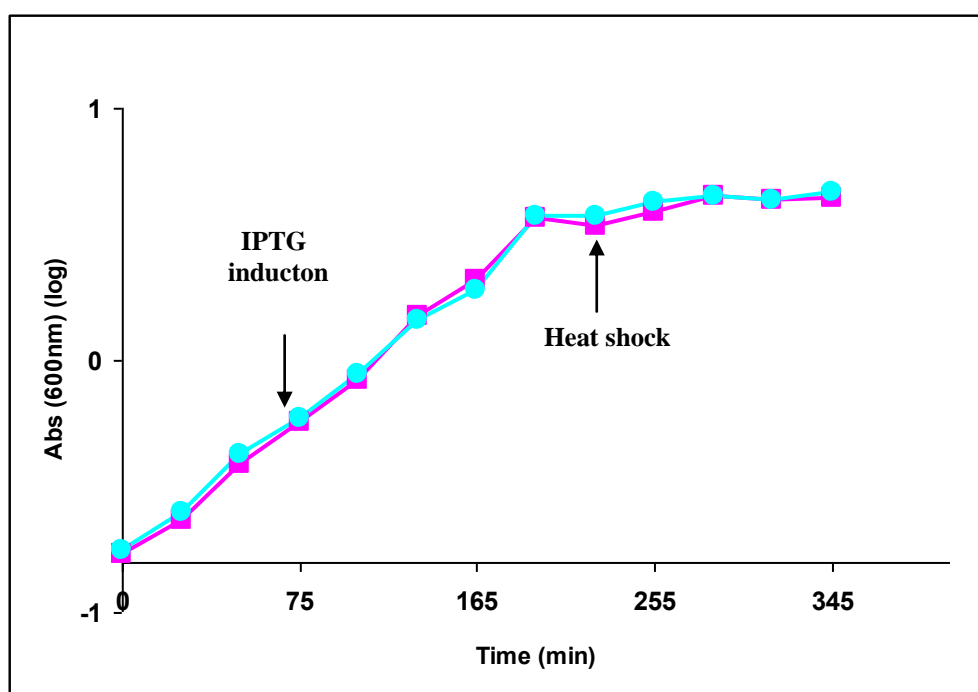


Figure 3.2 Growth curves of the control and recombinant *E.coli* pQE-31/775 culture before and after heat shock exposure. First arrow shows the IPTG induction at 75th min. and the second arrow indicates the heat shock at 150th min. after IPTG induction. —■— *E.coli* pQE-31 (control) —●— *E.coli* pQE-31/775 (test)

Following the sub-culturing and subsequent heat shock application between 30th - 120th min., OD₆₀₀ measurements of the test culture was found higher than that of the control culture (Figure 3.3). These results indicate that the control culture is

more sensitive to heat shock condition than recombinant *E.coli* pQE-31/775 culture and cell density of the test culture is relatively higher.

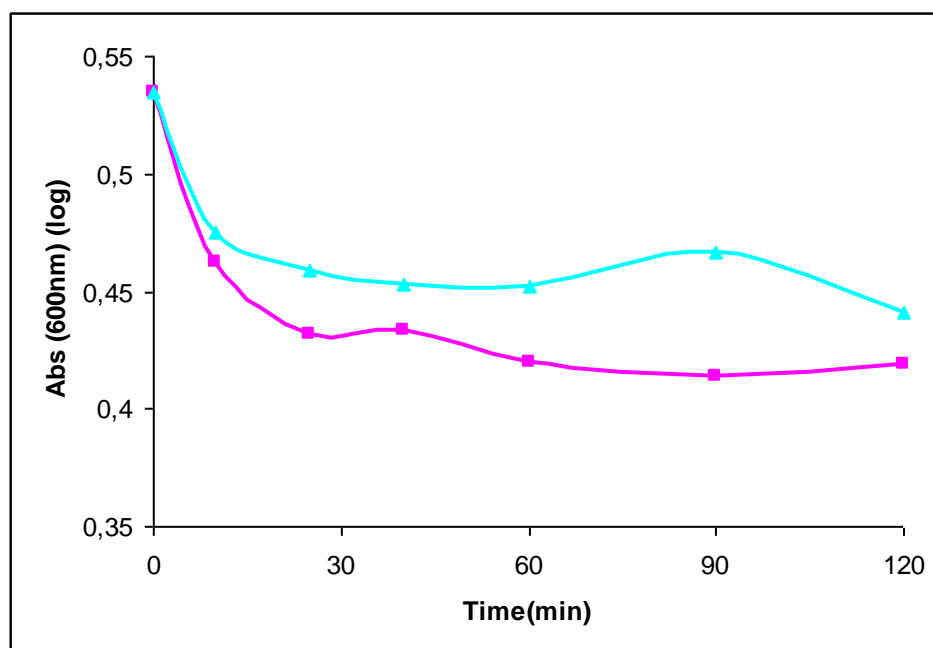


Figure 3.3 Effect of heat shock at 53°C for 2 hours on the growth of control and recombinant *E.coli* pQE-31/775 culture. —■— *E.coli* pQE-31 (control), —▲— *E.coli* pQE-31/775 (test).

The colony count data was in good correlation with the spectrophotometric absorbance (at 600 nm) measurement results (Figure 3.4). There was a sharp decline in the number of viable cells of the control and test cultures in the first 20 min. of heat shock treatment. Afterwards, cell viability was stabilized at higher levels in the cultures of *E.coli* pQE-31/775 cells ($\sim 2 \times 10^3$ /ml) as compared to control culture (after 2 h of heat shock, 4×10^2 /ml).

These results indicated that over expression of TVN0775/sHsp gene increased the thermotolerance (at 53°C) of the mesophilic host cell (*E.coli*).

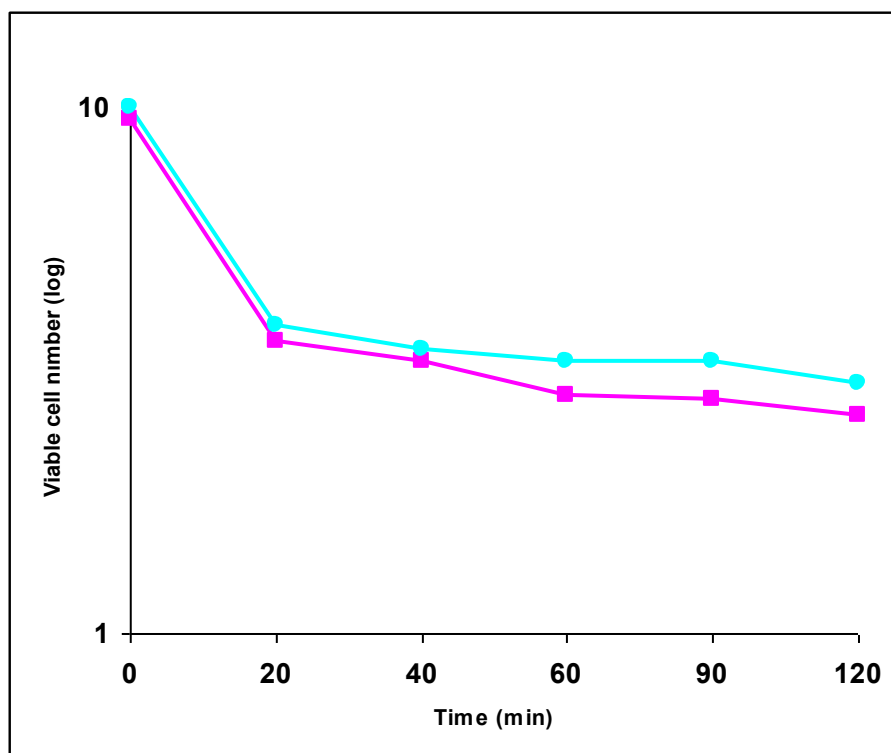


Figure 3.4 Effect of heat shock at 53°C for 2 hours on the growth of viable cells counts of the control and recombinant *E.coli* pQE-31/775 cells by colony counting at logarithmic scale. —■— *E.coli* pQE-31 (control), —●— *E.coli* pQE-31/775 (test).

3.2.2 Heat Tolerance of Recombinant *E.coli* pQE-30/984 cells

As revealed by time dependent OD₆₀₀ measurements, the growth curves of the control (*E.coli* pQE-30) and the test (*E.coli* pQE-30/984) cultures were very similar, before and after IPTG induction (Figure 3.5).

There was a continuous decline in the cell densities of the both cultures up to first 20th min. of heat shock. Then, no detectable change in the cell OD₆₀₀ measurements was observed by the end of the 2 h heat shock application (Figure 3.6).

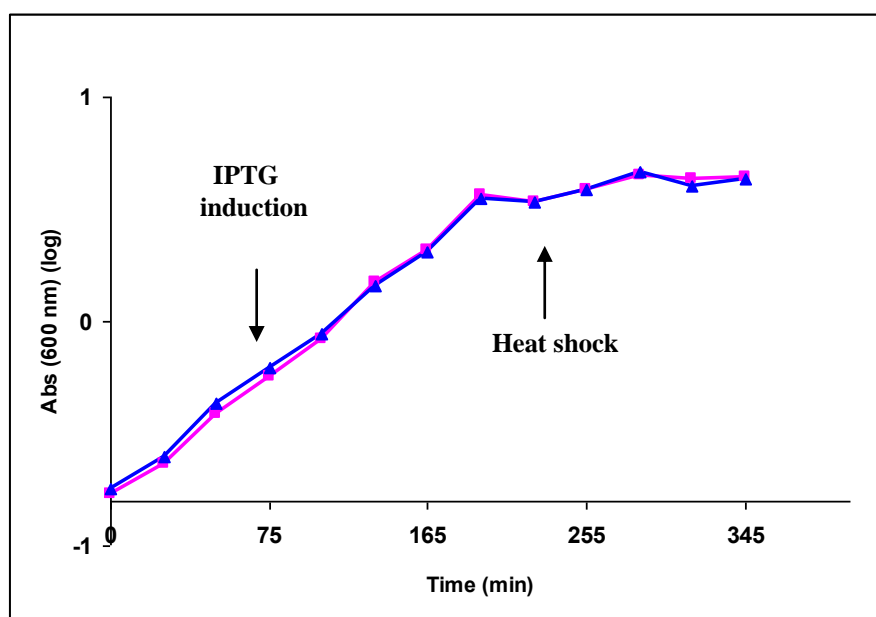


Figure 3.5 Growth curves of the control and recombinant *E.coli* pQE-30/984 culture before and after heat-shock exposure. The arrow shows the IPTG induction at 75th min. of the growth. —■— *E.coli* pQE-30 (control), —▲— *E.coli* pQE-30/984 (test).

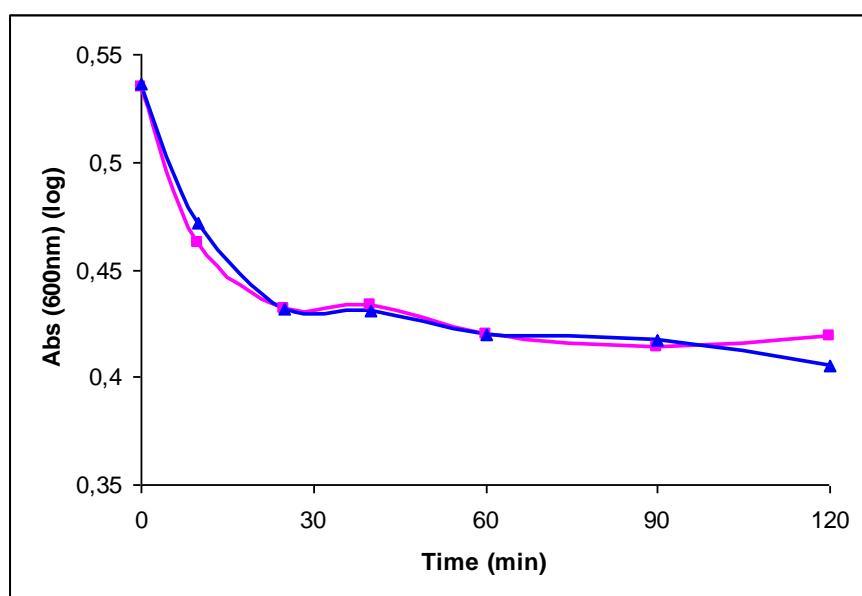


Figure 3.6 Effect of heat shock at 53°C for 2 hours on the growth of control and recombinant *E.coli* pQE-30/984 cells. —■— *E.coli* pQE-30 (control), —▲— *E.coli* pQE-30/984 (test).

The results of viable colony counts were in good correlation with the results of optical density measurements. At the end of 2 h heat shock treatment, the cell retardation in the growth of test culture ($5 \times 10^2/\text{ml}$) was more noticeable than control culture ($4 \times 10^2/\text{ml}$) (Figure 3.7).

Finally, there was no serious finding about the effectiveness of TVN0984 gene contained cultures on the heat resistance of *E.coli* cells.

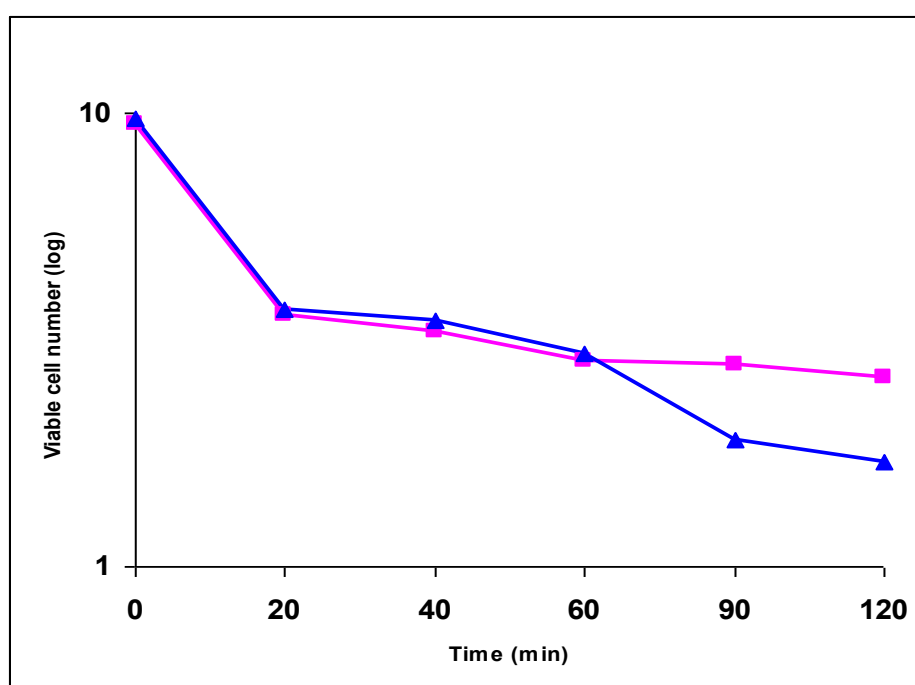


Figure 3.7 Effect of heat shock at 53°C for 2 hours on the growth of viable cell counts of the control and recombinant *E.coli* pQE-30/984 cells compared to control culture by colony counting at logarithmic scale. —■— *E.coli* pQE-30 (control), —▲— *E.coli* pQE-30/984 (test).

3.3 Studies on Stress Response at the Gene Expression Level

3.3.1 RNA Isolations

RNA isolation was performed as described in the section 2.7.4. Experimental conditions were optimized to isolate control and test RNA samples from *Tp. volcanium* cultures under three different stress conditions: heat shock, pH stress and oxidative stress.

Culture samples were taken in a time dependent manner to isolate RNA. Purity of RNA samples were checked by formamide agarose gel electrophoresis (Figures 3.8 - 3.14). RNA concentration and purity were assessed by OD₂₆₀ and/or OD₂₈₀ measurements (Table 3.1). Clear bands of 16S rRNA and 23S rRNA were distinguished without any degradation in the gel photographs of all RNA samples.

Cultures exposed to heat-shock at 65°C and 70°C between 15 min. and 180 min. were used for RNA isolation. Agarose gel electrophoresis results of representative RNA samples are shown in the figure 3.8. The RNA samples have been stored in a deep freeze at -80°C (Thermo Scientific).

The pH stress was induced by increasing the pH of the culture to pH: 3.5, pH: 4.0, pH: 4.5 and pH: 5.0, separately, as described in the Materials and Methods. The pH of control culture under these experimental conditions was 3.0. Representative results of RNA samples are shown in the figures 3.9 - 3.13.

Also, RNA was isolated from *Tp. volcanium* cultures treated with H₂O₂ at 4 different concentrations (0.01 mM, 0.02 mM, 0.03 mM and 0.05 mM). Representative results of RNA samples are shown in the figure 3.14.

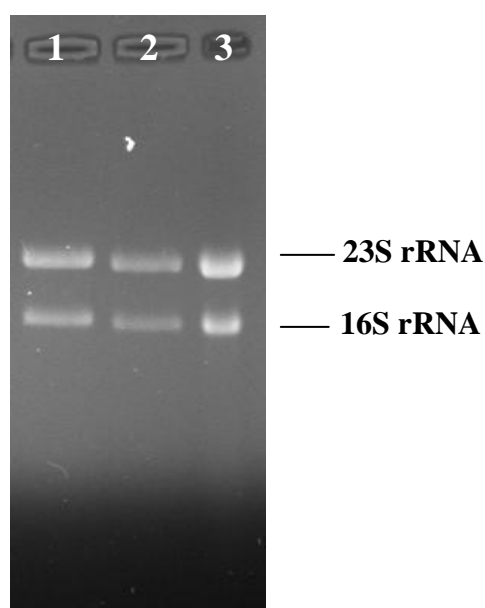


Figure 3.8 Agarose gel electrophoresis of the RNA samples isolated from heat shocked culture at 65°C. **Lane 1, 2 and 3** show the samples at the 15th, 150th and 180th minutes of the heat shock application to *Tp. volcanium* culture, respectively.

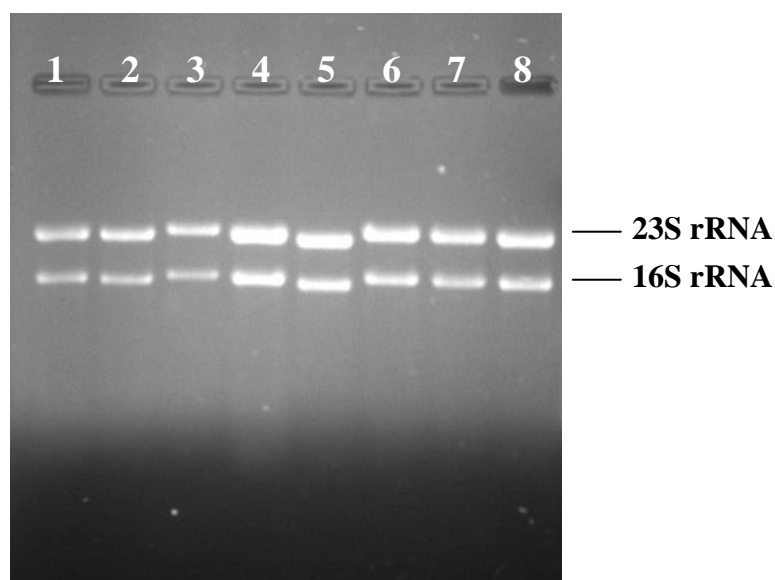


Figure 3.9 Agarose gel electrophoresis of the RNA samples isolated from cultures under the pH stress and not. **Lane 1, 2, 3 and 4** show the treated samples at pH: 3.5 which were isolated at 30th, 60th, 90th and 120th min of the stress application to *Tp. volcanium* cells, respectively. **Lane 5, 6, 7 and 8** show the control RNA samples isolated at pH: 3.0 at 30th, 60th, 90th and 120th min, respectively.

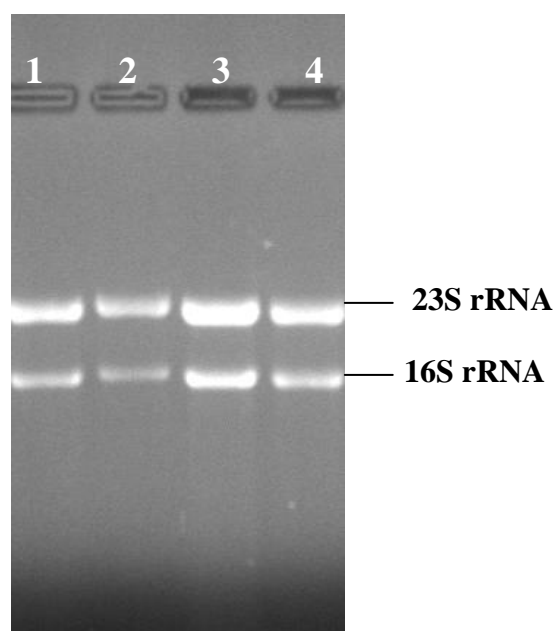


Figure 3.10 Agarose gel electrophoresis of the RNA samples isolated from culture under the pH stress. **Lane 1, 2, 3 and 4** show the treated samples at pH: 4.0 which were isolated at 30th, 60th, 90th and 120th min of the stress application to *Tp. volcanium* cells, respectively.

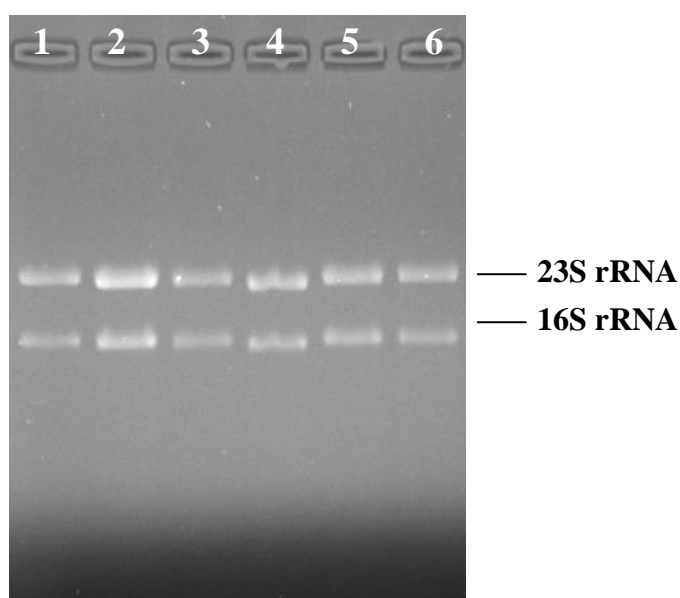


Figure 3.11 Agarose gel electrophoresis of the RNA samples isolated from culture under the pH stress. **Lane 1, 2, 3, 4, 5 and 6** show the treated samples at pH: 5.0 which were isolated at 30th, 60th, 90th, 120th, 150th and 180th min of the stress application to *Tp. volcanium* cells, respectively.

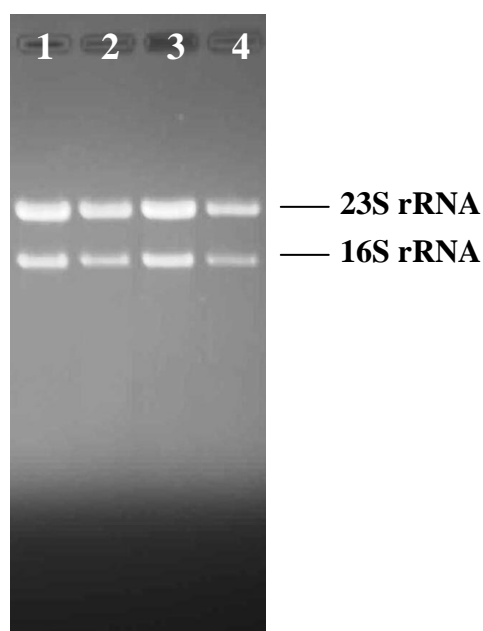


Figure 3.12 Agarose gel electrophoresis of the RNA samples isolated from culture under the pH stress. **Lane 1, 2, 3 and 4** show the treated samples at pH: 4.5 which were isolated at 30th, 60th, 90th and 120th min of the stress application to *Tp. volcanium* cells, respectively.

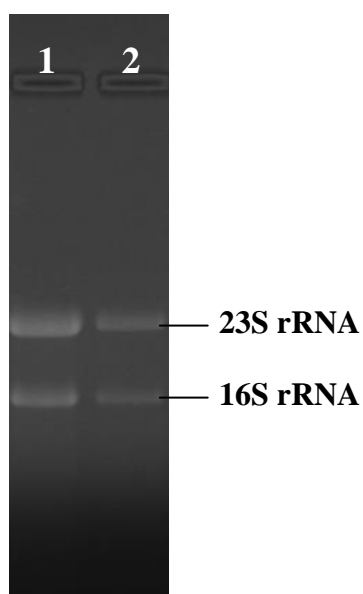


Figure 3.13 Agarose gel electrophoresis of the RNA samples isolated from culture under the pH stress. **Lane 1 and 2** show the treated samples at pH: 4.0 which were isolated at 150th and 180th min of the stress application to *Tp. volcanium* cells, respectively.

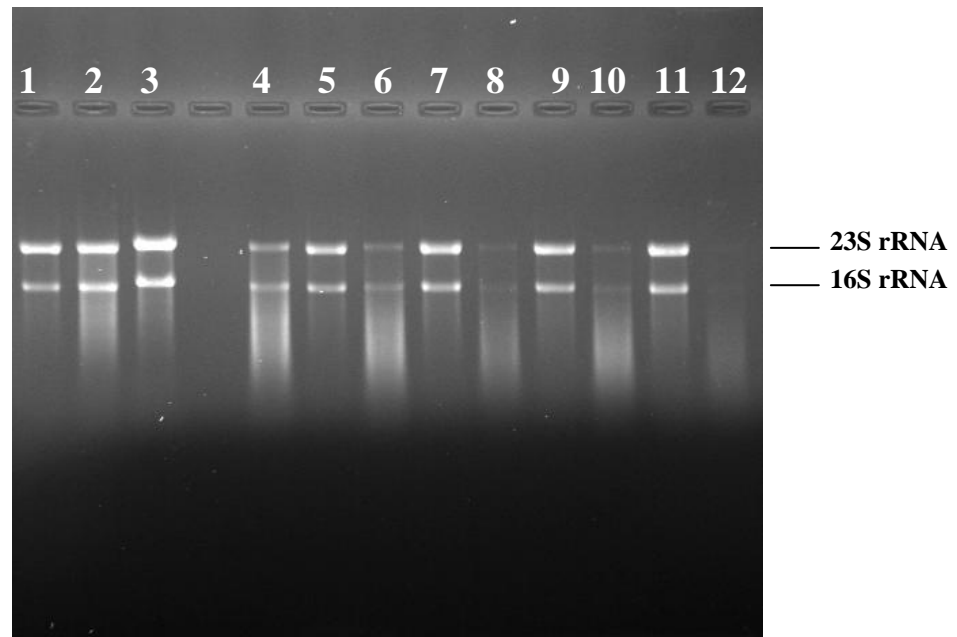


Figure 3.14 Agarose gel electrophoresis of the RNA samples isolated from cultures under the oxidative stress. **Lane 1, 3, 5, 7, 9 and 11:** RNA samples from H_2O_2 (0,03 mM) exposed culture which were isolated at 30th, 60th, 90th, 120th, 150th and 180th min of the oxidative stress, respectively. **Lane 2, 4, 6, 8, 10 and 12:** RNA samples from H_2O_2 (0,05 mM) exposed culture which were isolated at 30th, 60th, 90th, 120th, 150th and 180th min of the oxidative stress application to the *Tp. volcanium* cells, respectively.

3.3.2 Real-Time PCR Experiments

3.3.2.1 Detection of the Real-Time PCR Products

Real-Time PCR experiments were performed using single stranded cDNA as template to analyze the expressions of TVN0775 and TVN0984 genes under stress conditions as described in the section 2.8.4.

The amplified cDNA fragments of TVN0775 and TVN0984 genes were unique 77 bp and 164 bp amplicons, as shown in the figures 3.15 and 3.16, respectively.

Table 3.1 Concentrations and the A_{260}/A_{280} ratios of RNA samples.

Figure No	Stress	Time (min)	OD260/OD280	Conc. $\mu\text{g}/\mu\text{l}$	Figure No	Stress	Time (min)	OD260/OD280	Conc. $\mu\text{g}/\mu\text{l}$
3.8	65°C	15	1,21	0,8	3.9	pH: 3.5	30	1,12	0,8
		150	1,12	0,9			60	1,24	0,8
		180	1,12	0,8			90	1,19	0,8
3.10	pH: 4.0	30	1,26	0,7			120	1,21	0,8
		60	1,18	0,9		Control pH:3.0	30	1,18	0,8
		90	1,27	0,8			60	1,21	0,8
		120	1,21	0,9			90	1,18	0,8
3.12	pH: 4.5	30	1,27	0,8			120	1,24	0,8
		60	1,28	0,8	3.11	pH: 5.0	30	1,18	0,8
		90	1,32	0,7			60	1,19	0,8
		120	1,33	0,7			90	1,15	0,8
3.14	0.03mM H_2O_2	30	1,35	0,7			120	1,33	0,7
	0.05mM H_2O_2	30	1,30	0,7			150	1,15	0,8
	0.03mM H_2O_2	60	1,22	0,8			180	1,11	0,9
	0.05mM H_2O_2	60	1,27	0,7	3.13	pH: 4.0	150	1,17	0,58
	0.03mM H_2O_2	90	1,21	0,8			180	1,20	0,55
	0.05mM H_2O_2	90	1,31	0,7					
	0.03mM H_2O_2	120	1,31	0,7					
	0.05mM H_2O_2	120	1,22	0,8					
	0.03mM H_2O_2	150	1,30	0,7					
	0.05mM H_2O_2	150	1,19	0,8					
	0.03mM H_2O_2	180	1,23	0,7					
	0.05mM H_2O_2	180	1,2	0,8					

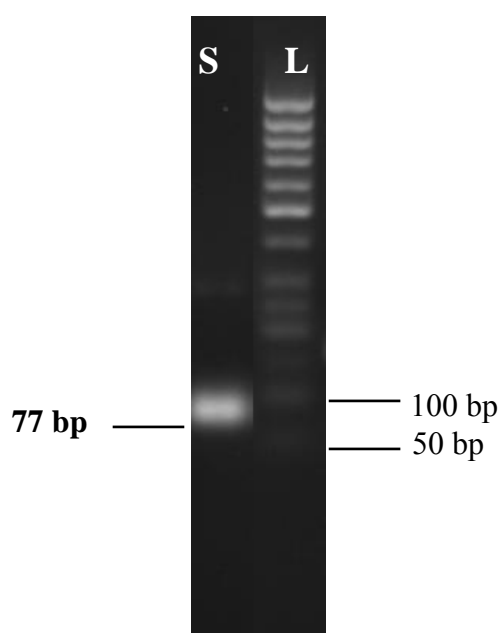


Figure 3.15 Agarose gel electrophoresis of Real-Time PCR product of TVN0775 gene. **S:** Amplified cDNA fragment of TVN0775. **L:** 50 bp ladder (GeneRuler™ and O'GeneRuler™ 50 bp DNA Ladders (50-1000 bp), Fermentas UAB, Vilnius, Lithuania).

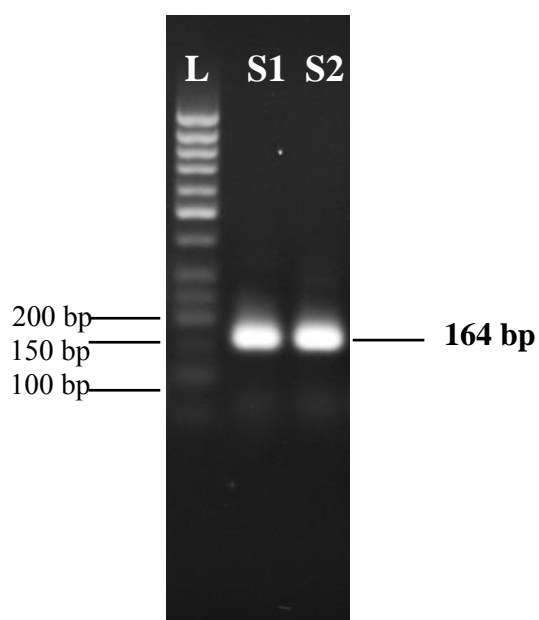


Figure 3.16 Agarose gel electrophoresis of Real-Time PCR products of TVN0984 gene. **L:** 50 bp ladder (GeneRuler™ and O'GeneRuler™ 50 bp DNA Ladders (50-1000 bp), Fermentas UAB, Vilnius, Lithuania) **S1 and S2:** Amplified cDNA fragments of TVN0984.

3.3.2.2 Differential Expression of TVN0775 Gene under Stress Conditions

3.3.2.2.1 Differential Expression of TVN0775 Gene under Heat-Shock

The TVN0775 gene expression was induced during 150 min. heat shock at 65°C as revealed by lower Ct values of the tests relative to that of control (Table 3.2). At 180th min. of heat shock expression level of test sample was lower than that of control.

The lower the Ct value the earlier the amplification of the cDNA sample is expected. Melting curve analysis for each Real-Time experiment verified the specific amplification of the TVN0775 gene's cDNA, average T_m being 82,5°C (± 0.31). Representative amplification curves of TVN0775 cDNA samples of 65°C heat-shock (15th min and 30th min) were given in the figures 3.17 and 3.18.

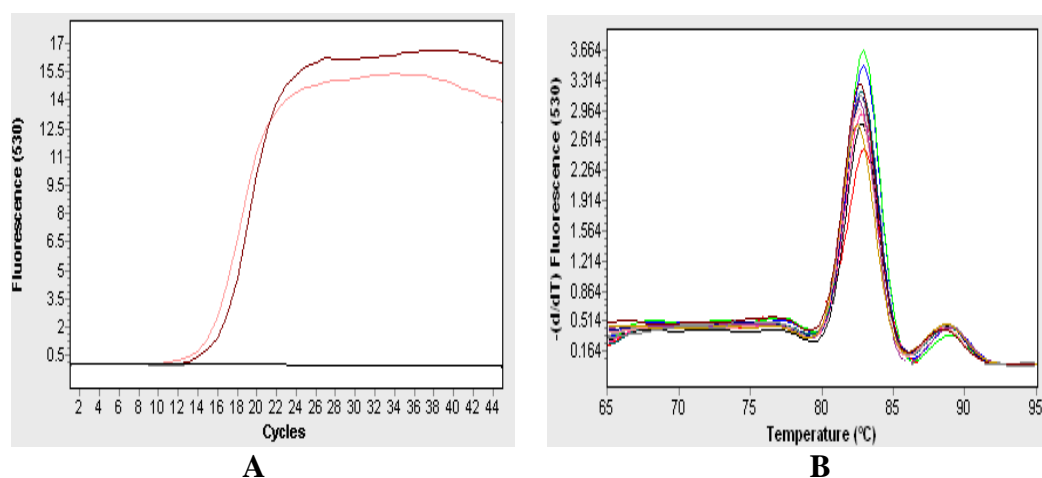


Figure 3.17 Real-Time PCR graphics of heat shocked (test) at 65°C and non treated (control, at about 58°C) samples of TVN0775 gene. — Test (15th min), Ct: 13.94, — Control Ct: 14.97, — Negative control (A). Melting curves for TVN0775 gene under heat shock at 65°C (B).

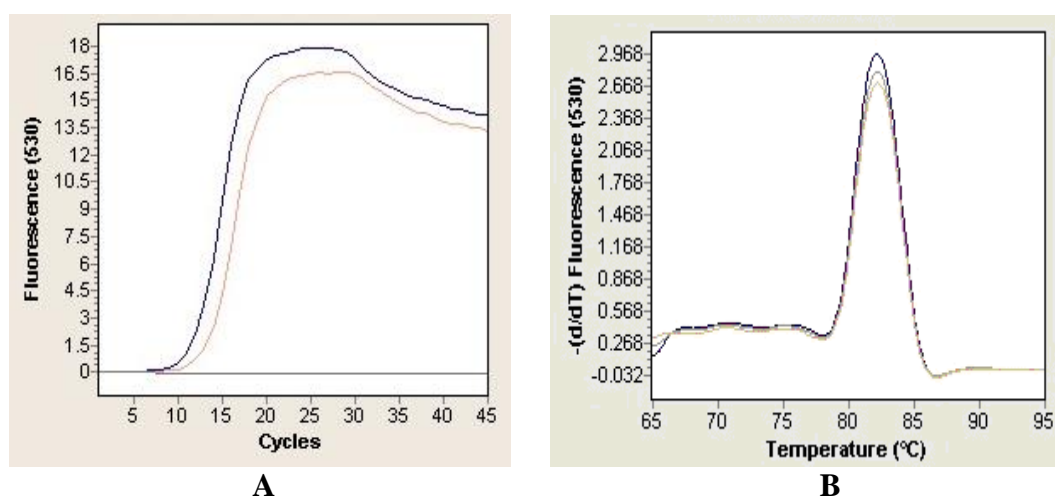


Figure 3.18 Real-Time PCR graphics of heat shocked (test) at 65°C and non treated (control, at about 58°C) samples of TVN0775 gene. — Test (30th min), Ct: 10.62 — Control Ct: 12.17, The line at the bottom shows “negative control” (A). Melting curves for TVN0775 gene under heat shock at 65°C (B).

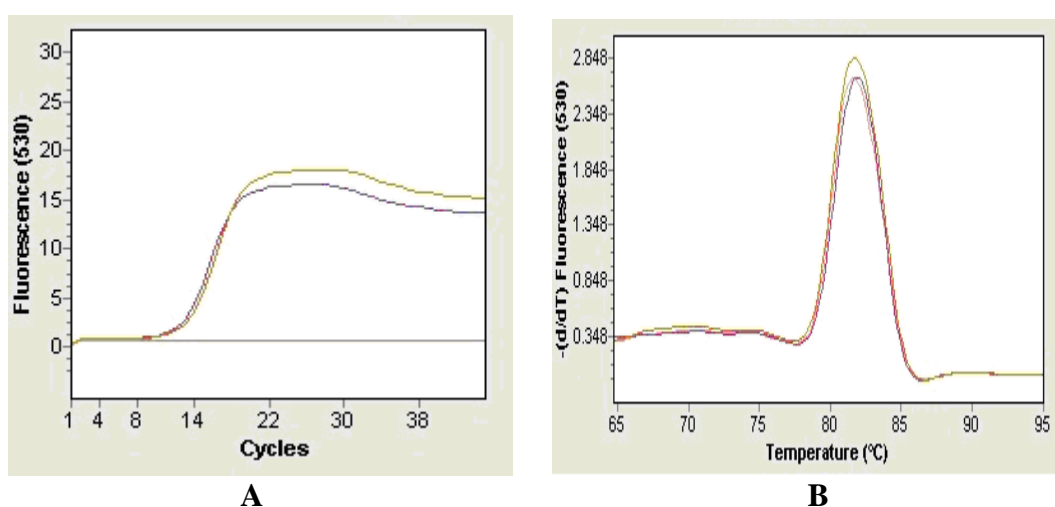


Figure 3.19 Real-Time PCR graphics of heat shocked (test) at 70°C and non treated (control, at about 58°C) samples of TVN0775 gene. — Test (120th min), Ct: 11.56 — Control Ct: 12.07, — Negative control (A). Melting curves for TVN0775 gene under heat shock at 70°C (B).

The TVN0775 gene transcription is also induced by heat shock at 70°C for 2 h (except 90th min), as evidenced by lower Ct values of the tests as compared to that

of controls (Table 3.2). Representative amplification curve of TVN0775 cDNA sample of 70°C heat shock (120th min) is shown in the Figure 3.19.

3.3.2.2.1.1 Fold Analysis of TVN0775 Gene Expression Results under Heat-Shock

There are two methods to analyze the RT-PCR data in literature. One of them is absolute quantification and the other one is relative quantification. Input copy number was determined in the absolute quantification method and this quantification is based on construction of a standard curve (Liu and Saint, 2002). In relative quantification method, as its name suggests, signal of the tested transcript is evaluated relative to that of control. In this method, test transcript represents a treated sample and the control represents an untreated sample.

We have evaluated the RT-PCR results according to the relative quantification method. The fold difference was calculated using the formula: $2^{-\Delta\Delta C_t}$. Here the expected PCR efficiency is 2. “Ct” shows the threshold cycle number, which is defined as the “fractional cycle number at which the amount of amplified target reaches a fixed threshold” (Livak and Schmittgen, 2001).

As well as graphical illustration of the data of RT-PCR results, quantitatively estimations were made using the Ct values and the efficiency of the RT-PCR reaction. A computer programme, Lin_reg_pcr, was used to calculate the efficiency of RT-PCR (Ramakers, et. al., 2003). For relative quantification method, the PCR efficiency values must be around 2.0.

Table 3.2 Cp and Tm values for differential expression of TVN0775 gene under heat shock condition.

Heat Stress	Time (min)	Ct	TM
65°C	15	13.94	82.69
	Control	14.97	82.74
	30	10.62	82.39
	Control	12.17	82.41
	60	13.46	82.86
	Control	14.97	82.74
	90	13.76	82.85
	Control	14.97	82.74
	120	11.64	82.67
	Control	11.75	82.31
	150	14.62	82.66
	Control	14.97	82.74
	180	15.08	82.57
	Control	14.97	82.74
70°C	30	14.29	83.0
	Control	14.97	82.74
	60	14.49	82.98
	Control	14.97	82.74
	90	12.5	81.98
	Control	12.07	82.04
	120	11.56	82.11
	Control	12.07	82.04

Average fold-difference values of at least from 3 different repeated heat shock experiments were used to plot column type charts using Microsoft Excel Program (Figure 3.20).

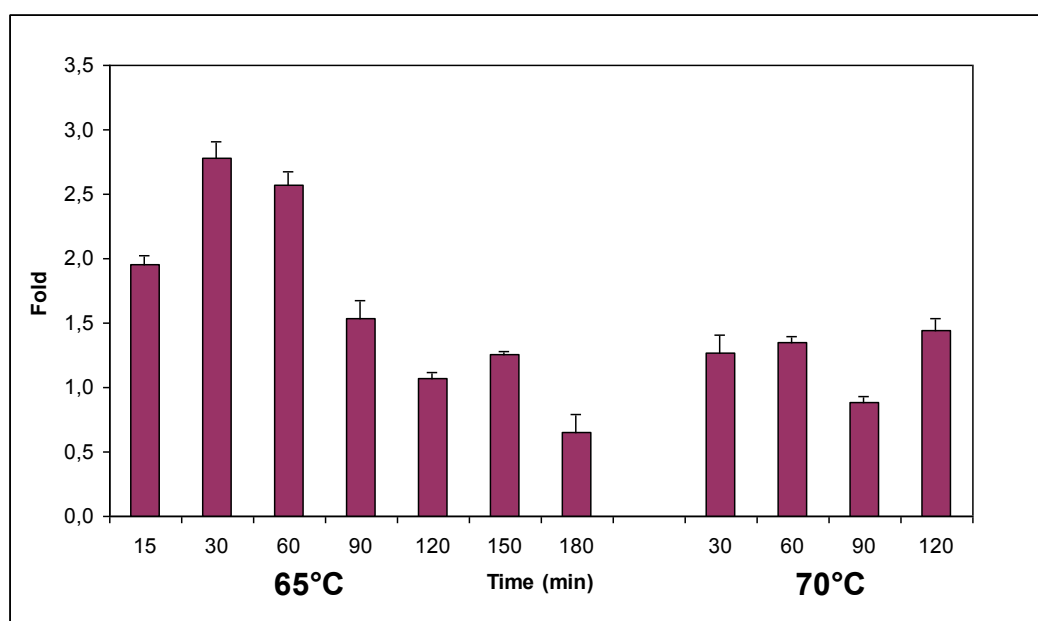


Figure 3.20 Fold analysis of TVN0775 gene expression under heat shock at 65°C and 70°C, time-dependent. Each column indicates the fold of the induced gene expression compared to control under the stated stress factor at the specified times.

There was a time-dependent increase in the expression of TVN0775 gene under heat shock at 65°C up to 90 min. There was >2.5 fold more transcription of the TVN0775 gene after 30-60 min. exposure to 65°C, as compared to control. After 2 h heat shock, transcription was same as the control, while a detectable decrease was observed in the transcription at 180th min of the exposure than the control.

At 70°C, there was also a time dependent increase in the level of TVN0775 gene expression and after 2 h, the expression was about 1.5 fold higher in the test cultures as compared to control cultures.

3.3.2.2.2 Differential Expression of TVN0775 Gene under pH Stress

The optimal pH is about 2.0 for *Tp. volcanium* cells. To exert stress on these cells, the pH of the mid-log cultures were raised to pH: 3.5, pH: 4.0, pH: 4.5 and pH:

5.0 as described in the Materials and Methods. The samples were removed by regular time intervals during 2-3 h pH stress.

The lower the Ct value the earlier the amplification of the cDNA sample is expected. Melting curve analysis for each Real-Time experiment verified the specific amplification of the TVN0775 gene's cDNA average T_m being 81.44°C (± 1.67). Representative amplification curves of TVN0775 cDNA samples of pH stress (pH: 3.5-120th min, pH: 4.0-120th min, pH: 4.5-120th min and pH: 5.0-120th min) were given in the figures 3.21-3.24.

When Ct values are considered at pH: 3.5, it is expected that TVN0775 expression was initially retarded (30th min), but then (from 60th min) slightly increased up to the level of control cultures. At pH 4.0, Ct values of the tests at 30th min, 60th min, 90th min and 120th min was lower than that of control. At pH: 4.5, Ct values of the tests were higher than that of the control and at pH: 5.0, Ct values of the tests were lower than that of the control up to 150th min of pH stress (Table 3.3).

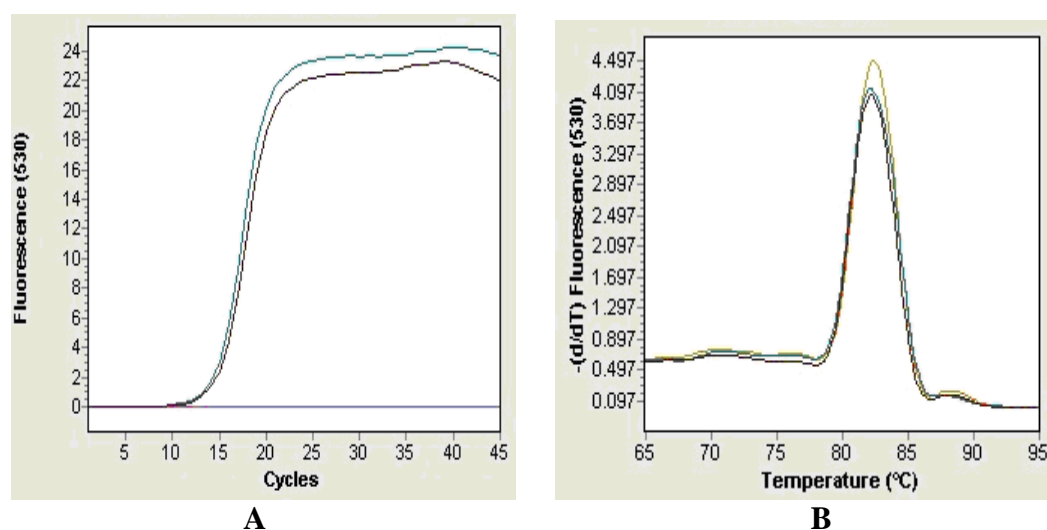


Figure 3.21 Real-Time PCR graphics of stressed at pH: 3.5 (test) and non treated (control, at pH: 3.0) samples of TVN0775 gene. — Test (120th min), Ct: 13.42 — Control Ct: 13.72 (A). Melting curves for TVN0775 gene under stress at pH: 3.5 (B).

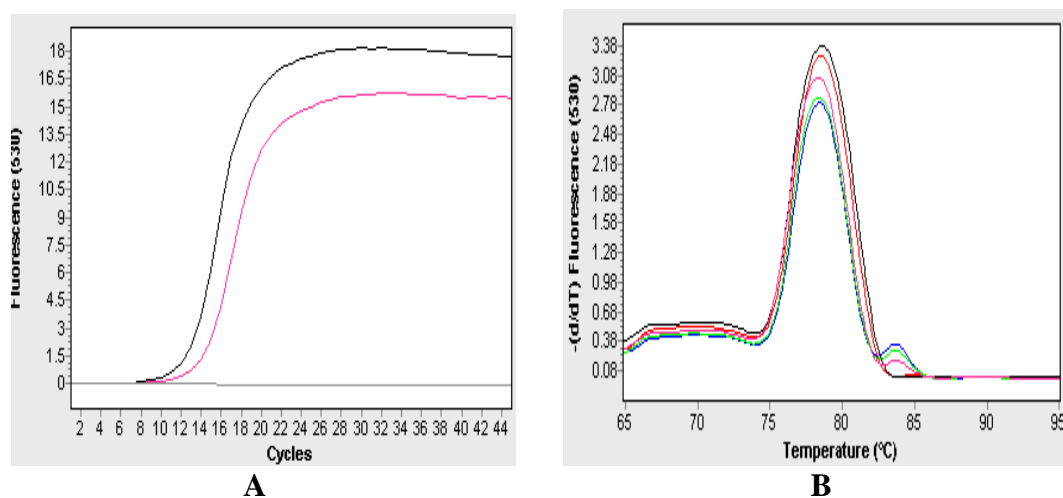


Figure 3.22 Real-Time PCR graphics of stressed at pH: 4.0 (test) and non treated (control, at pH: 3.0) samples of TVN0775 gene. — Test (120th min), Ct: 11.55 — Control Ct: 13.01 (A). Melting curves for TVN0775 gene under stress at pH: 4.0 (B).

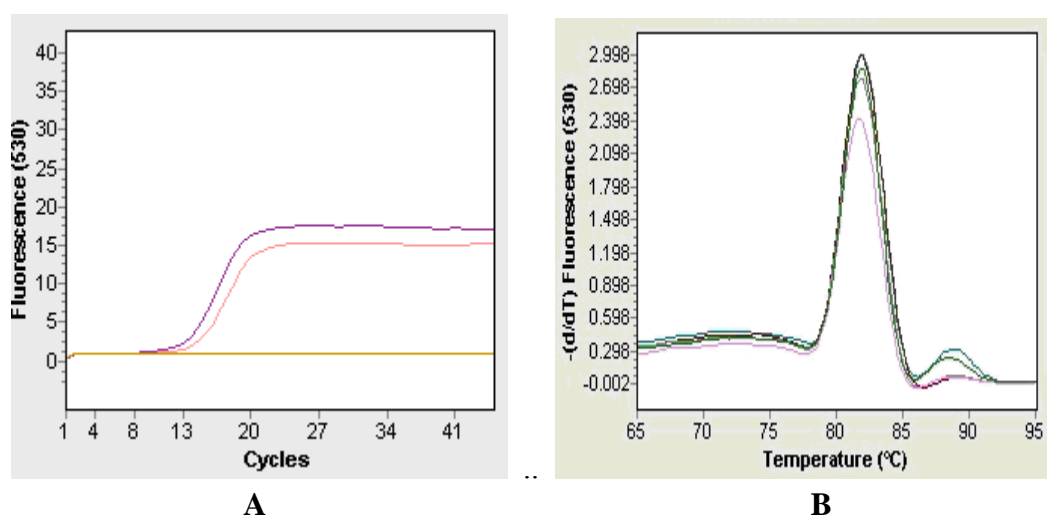


Figure 3.23 Real-Time PCR graphics of stressed at pH: 4.5 (test) and non treated (control, at pH: 3.0) samples of TVN0775 gene. — Test (120th min), Ct: 13.26 — Control Ct: 12.42 (A). Melting curves for TVN0775 gene under stress at pH: 4.5 (B).

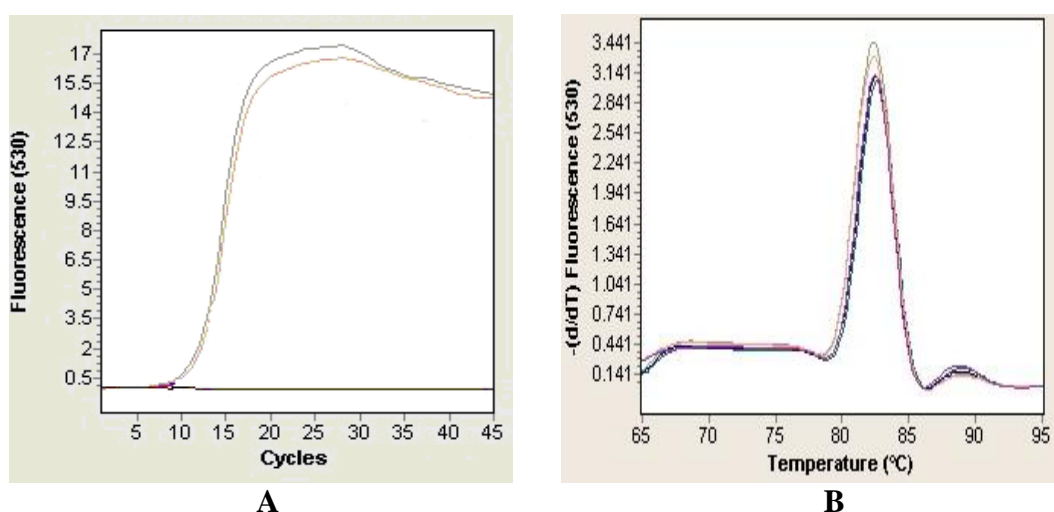


Figure 3.24 Real-Time PCR graphics of stressed at pH: 5.0 (test) and non treated (control, at pH: 3.0) samples of TVN0775 gene. — Test (120th min), Ct: 10.73 — Control Ct: 11.74 (A). Melting curves for TVN0775 gene under stress at pH: 5.0 (B).

3.3.2.2.2.1 Fold Analysis of TVN0775 Gene Expression Results under pH Stress

The fold difference analysis of the RT-PCR experiments results were in agreement with the estimations based on Ct values of tests relative to that of controls.

TVN0775 gene expression exhibited the highest increase (2.5 fold) relative to control cell under pH: 4.0. The expression level was lower than that of control cells at pH: 4.5 and 150th and 180th min of pH: 5.0

Column charts were plotted using average fold-difference values from at least two different RT-PCR experiments versus pH value specified (Figure 3.25).

Table 3.3 Cp and Tm values for differential expression of TVN0775 gene under pH stress condition.

pH Stress	Time	Ct	TM
pH: 3.5	30	14.43	82.44
	Control	13.04	82.05
	60	13.3	82.2
	Control	13.04	82.05
	90	13.77	82.64
	Control	13.72	82.46
	120	13.42	82.55
	Control	13.72	82.46
pH: 4.0	30	12.86	78.63
	Control	13.01	78.51
	60	12.67	78.36
	Control	13.01	78.51
	90	11.68	78.79
	Control	13.01	78.51
	120	11.55	78.84
	Control	13.01	78.51
pH: 4.5	30	13.56	82.64
	Control	12.42	81.63
	60	14.28	82.54
	Control	12.42	81.63
	90	13.47	82.31
	Control	12.42	81.63
	120	13.26	82.06
	Control	12.42	81.63
pH: 5.0	30	11.17	82.73
	Control	11.74	82.54
	60	11.0	82.62
	Control	11.74	82.54
	90	10.49	82.49
	Control	11.74	82.54
	120	10.73	82.47
	Control	11.74	82.54
	150	12.71	78.55
	Control	12.66	81.84
	180	12.79	78.91
	Control	12.66	81.84

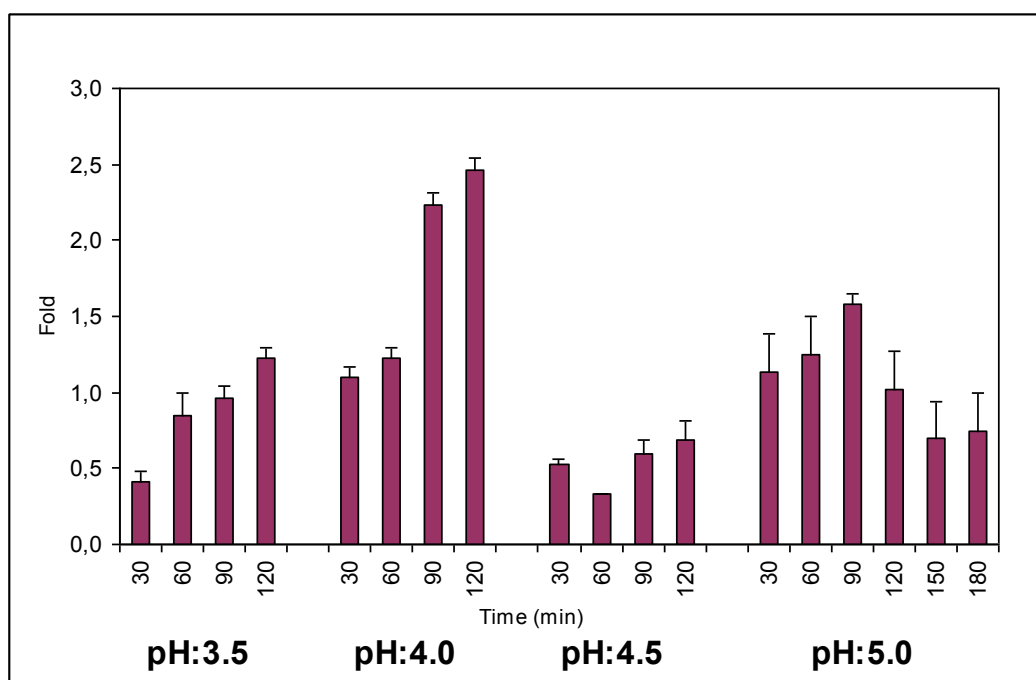


Figure 3.25 Fold analysis of TVN0775 gene expression under pH stress at pH: 3.5, pH: 4.0, pH: 4.5 and pH: 5.0 for 2-3 hours. Each column indicates the fold of the induced gene expression compared to control under the stated stress factor at the specified time.

3.3.2.2.3 Effect of Oxidative Stress on Differential Expression of TVN0775 Gene

Different concentrations of hydrogen peroxide (H_2O_2) (between 0.01 mM to 0.05 mM) were used to maintain oxidative stress for 2 h as explained in the Materials and Methods.

Oxidative stress imposed by exposure of the cultures to 0.01 mM and 0.02 mM H_2O_2 were expected to enhance expression of the TVN0775 gene since Ct values of the tests were lower than that of control (Table 3.4). Highest Ct values were achieved at 0.01 mM H_2O_2 concentration after 90 min and at 0.02 mM after 60 min. Test Ct values were higher than that of control at 0.03 mM and 0.05 mM H_2O_2 during 3 h oxidative stress.

The lower the Ct value the earlier the amplification of the cDNA sample is expected. Melting curve analysis for each Real-Time experiment verified the specific amplification of the TVN0775 gene's cDNA average T_m being 82.07°C (±1.52). Representative amplification curves of TVN0775 cDNA samples of oxidative stress (0.01 mM H₂O₂ - 90th min and 0.05 mM H₂O₂ - 60th min) were given in the figures 3.26-3.27.

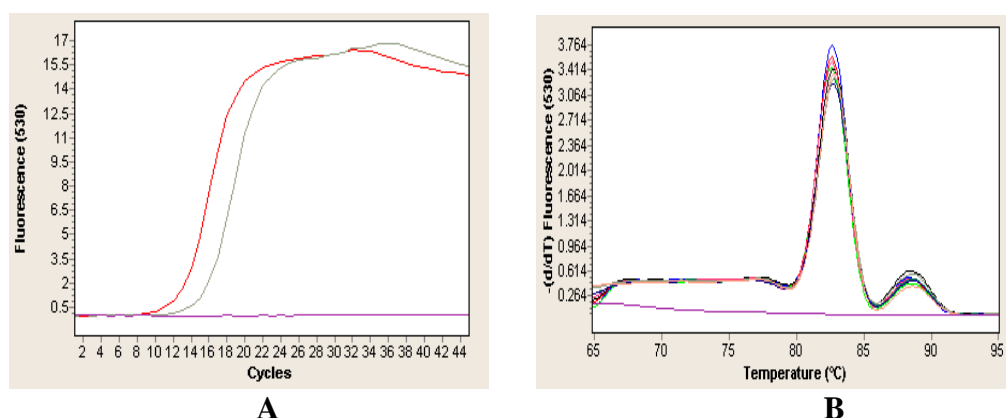


Figure 3.26 Real-Time PCR graphics of stressed at 0.01 mM H₂O₂ (test) and non treated (control) samples of TVN0775 gene. — Test (90th min), Ct: 11.83. — Control Ct: 14.47 (A). Melting curves for TVN0775 gene under oxidative stress at 0.01 mM H₂O₂ (B).

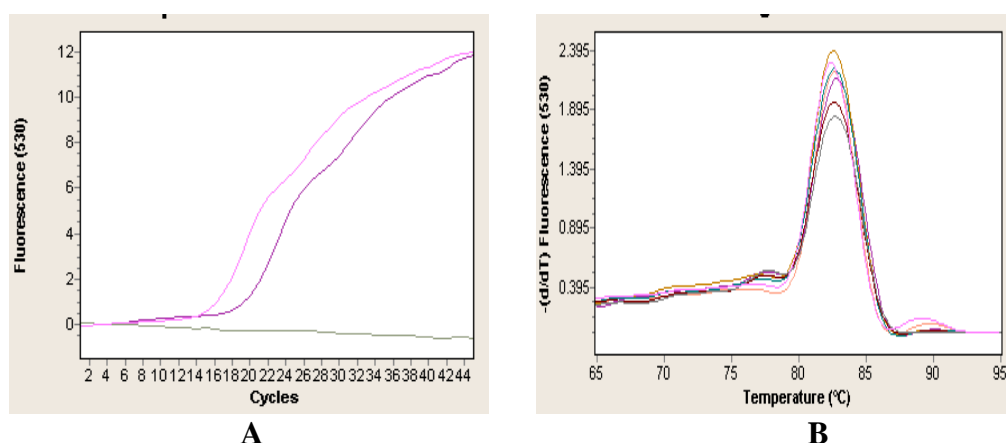


Figure 3.27 Real-Time PCR graphics of stressed at 0.05 mM H₂O₂ (test) and non treated (control) samples of TVN0775 gene. — Test (60th min), Ct: 18.56 — Control Ct: 14.97 (A). Melting curves for TVN0775 gene under oxidative stress at 0.05 mM H₂O₂ (B).

Table 3.4 Cp and Tm values for differential expression of TVN0775 gene under oxidative stress condition.

H₂O₂ concentration	Time (min)	Ct	TM
0.01mM	30	14.19	82.74
	60	13.22	82.67
	90	11.83	82.72
	120	13.83	82.82
	Control	14.47	82.91
0.02mM	30	14.04	78.15
	Control	15.43	79.68
	60	12.83	82.75
	Control	14.47	82.91
	90	13.74	82.88
	Control	14.47	82.91
	120	13.67	82.82
	Control	14.47	82.91
0.03mM	30	12.24	82.2
	Control	11.75	82.01
	60	12.49	82.5
	Control	11.75	82.01
	90	14.25	77.7
	Control	13.11	77.59
	120	12.46	82.14
	Control	11.75	82.01
	150	11.92	82.22
	Control	11.75	82.01
	180	11.92	82.21
	Control	11.75	82.01
0.05mM	30	17.76	82.79
	60	18.56	83.04
	90	17.79	82.85
	120	19.12	82.87
	150	19.27	82.89
	180	19.01	83.0
	Control	14.97	82.6

3.3.2.2.3.1 Fold Analysis of TVN0775 Gene Expression Results under Oxidative Stress

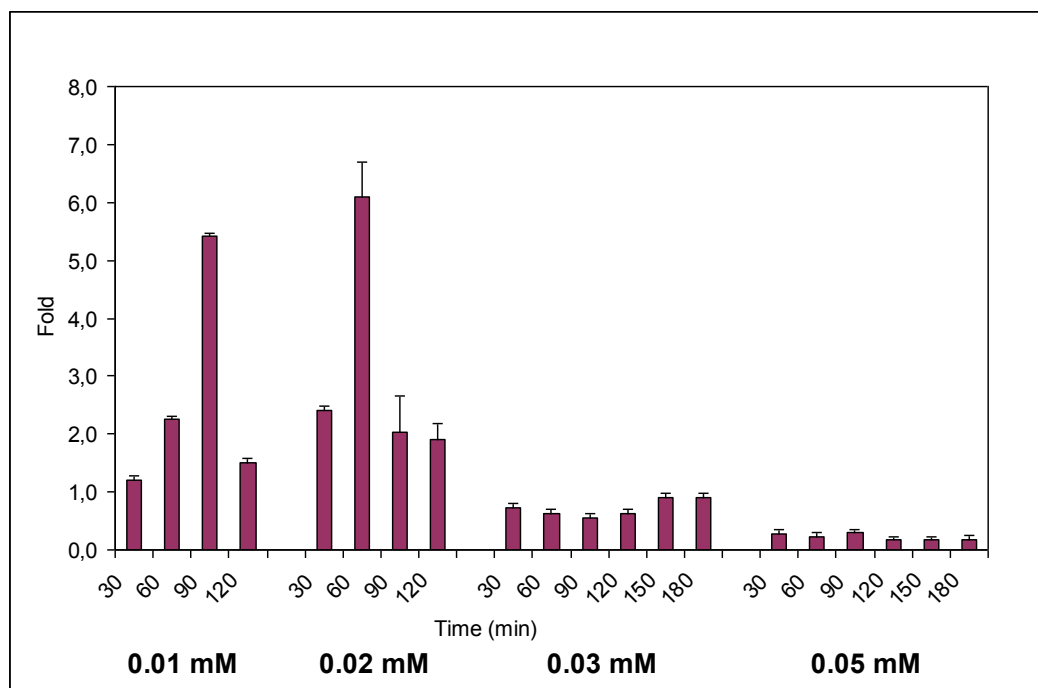


Figure 3.28 Fold analysis of TVN0775 gene expression under oxidative stress at 0.01 mM, 0.02 mM, 0.03 mM and 0.05 mM, for 2-3 hours. Each column indicates the fold of the induced gene expression compared to control under the stated stress factor at the specified time.

Fold analysis results showed that oxidative stress exposed by 0.01 mM H_2O_2 induced the highest expression relative to control was achieved at 90th min (5.4 fold). Oxidative stress imposed by 0.02 mM H_2O_2 induced TVN0775 gene transcription >2 fold relative to control. The highest expression was observed at 60th min of stress exposure. When H_2O_2 concentration was ≥ 0.03 mM, TVN0775 gene expression was less than that of the control. Down regulation of the TVN0775 gene transcription was clearly soon at 0.05 mM H_2O_2 for 3 h.

The column charts were plotted using the average fold values from two different RT-PCR experiments for each oxidative stress conditions.

3.3.2.3 Differential Expression of TVN0984 Gene under Stress Conditions

3.3.2.3.1. Differential Expression of TVN0984 Gene under Heat-Shock

TVN0984 gene expression was also studied under heat shock conditions at 65°C and 70°C.

The Ct values of tests at 65°C and 70°C were higher than that of controls, indicating heat shock induction of the TVN0984 gene expression. The highest Ct value was at 60th min of heat shock at 65°C and at 120th min of heat shock at 70°C.

The lower the Ct value the earlier the amplification of the cDNA sample is expected. Melting curve analysis for each Real-Time experiment verified the specific amplification of the TVN0984 gene's cDNA, average T_m being 84.27°C (±0.37). Representative amplification curves of TVN0984 cDNA samples of 65°C (60th min and 120th min) and 70°C heat-shock (60th min and 120th min) were given in the figures 3.29-3.32.

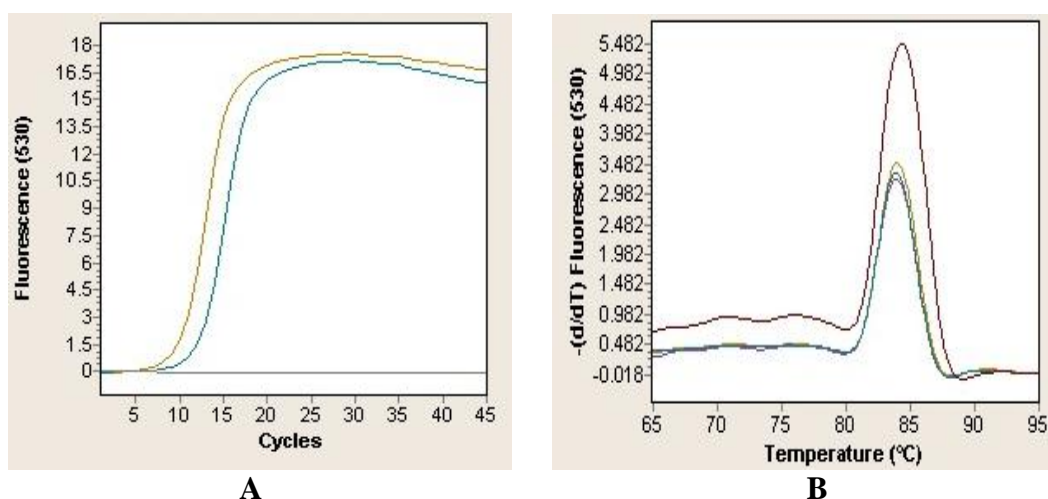


Figure 3.29 Real-Time PCR graphics of heat shock at 65°C (test) and non treated (control, at about 58°C) samples of TVN0984 gene. — Test (60th min), Ct: 8.78 — Control Ct: 10.96 (A). Melting curves for TVN0984 gene under heat stress at 65°C (B).

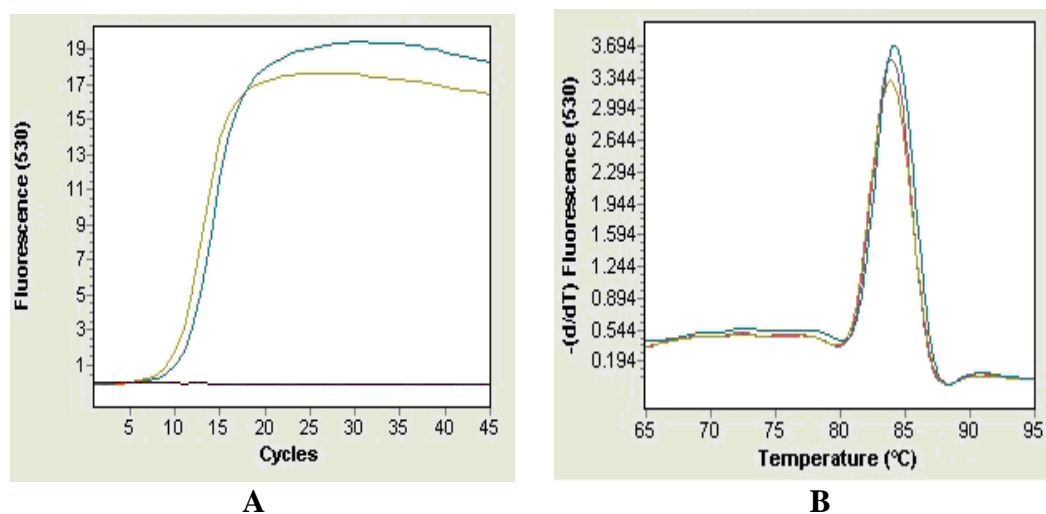


Figure 3.30 Real-Time PCR graphics of heat shock at 65°C (test) and non treated (control, at about 58°C) samples of TVN0984 gene. — Test (120th min), Ct: 8.88 — Control Ct: 9.92 (A). Melting curves for TVN0984 gene under heat stress at 65°C (B).

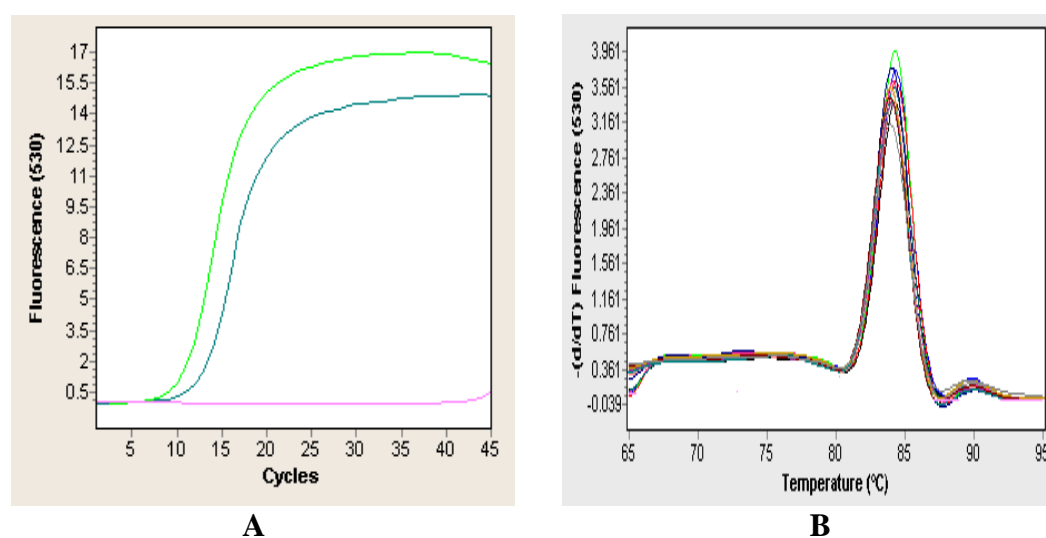


Figure 3.31 Real-Time PCR graphics of heat shock at 70°C (test) and non treated (control, at about 58°C) samples of TVN0984 gene. — Test (60th min), Ct: 9.74 — Control, Ct: 11.72 (A). Melting curves for TVN0984 gene under heat stress at 70°C (B).

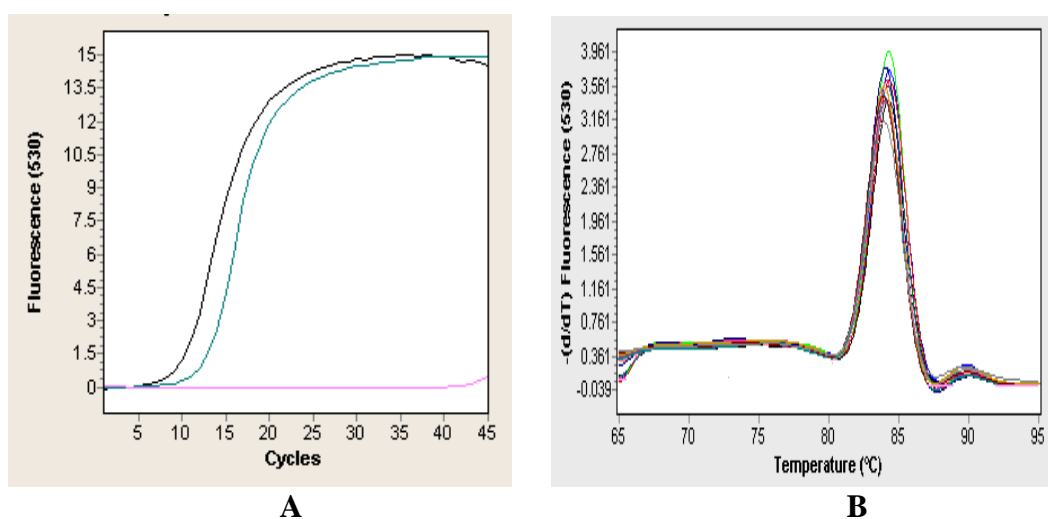


Figure 3.32 Real-Time PCR graphics of heat shock at 70°C (test) and non treated (controls, at about 58°C) samples of TVN0984 gene. — Test (120th min), Ct: 9.13. — Control Ct: 11.72 (A). Melting curves for TVN0984 gene under heat stress at 70°C (B).

3.3.2.3.1.1 Fold Analysis of TVN0984 Gene Expression Results under Heat Shock

Fold analysis revealed that TVN0984 gene transcription increased in a time dependent manner at 65°C for 2 h, then gradual decrease was observed up to 3rd hour. The highest expression level was between 60th min of heat shock exposure (2.6 fold increase). However heat shock at 70°C, continuously enhanced the TVN0984 gene expression and by the end of 2 h heat shock exposure a 4,5 fold increase in the transcription level was obtained (Figure 3.33).

The column charts were plotted using the average fold values from at least two independent RT-PCR experiments under specified temperature and time.

Table 3.5 Cp and Tm values of differential expression of TVN0984 gene under heat shock condition.

Heat shock	Time (min)	Ct	TM
65°C	15	10.76	84.48
	Control	12.23	84.98
	30	9.48	84.09
	Control	10.96	84.07
	60	8.78	84.16
	Control	10.96	84.07
	90	9.83	84.15
	Control	11.72	84.04
	120	8.88	84.02
	Control	9.92	84.36
	150	10.43	84.14
	Control	11.72	84.04
	180	10.64	84.13
	Control	11.72	84.04
70°C	30	10.75	84.39
	Control	11.72	84.04
	60	9.74	84.36
	Control	11.72	84.04
	90	9.26	84.69
	Control	11.15	84.29
	120	9.13	84.36
	Control	11.72	84.04

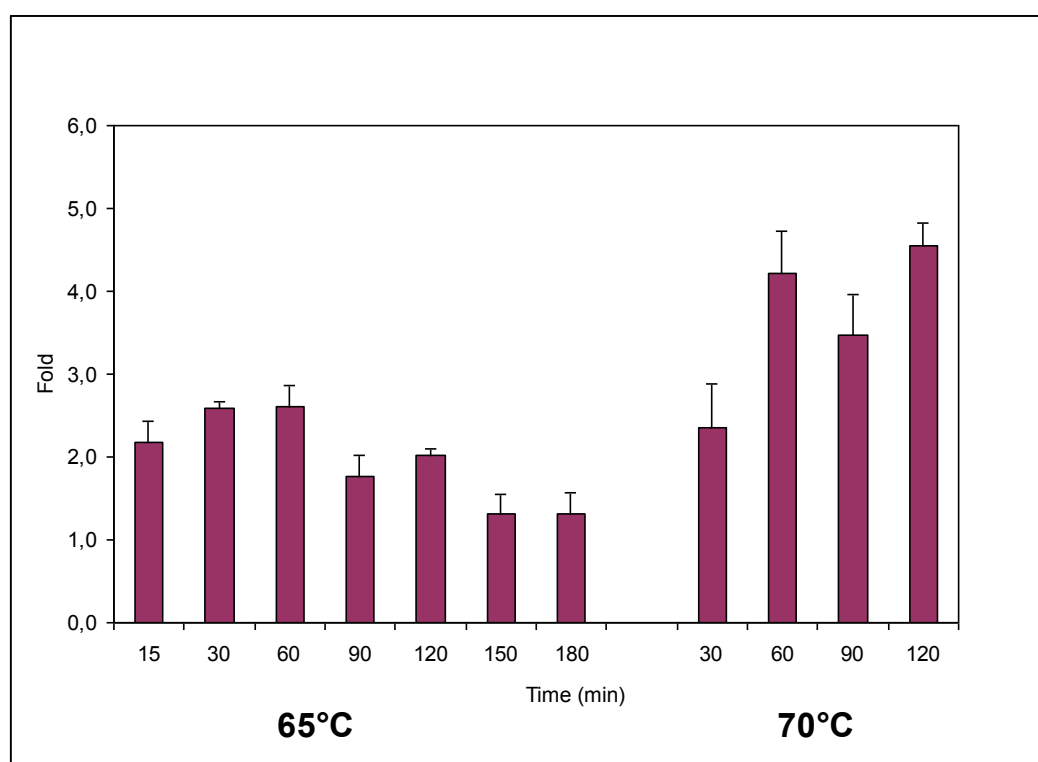


Figure 3.33 Fold analysis of TVN0984 gene expression under heat shock at 65°C and 70°C, time-dependent. Each column indicates the fold of the induced gene expression compared to control under the stated stress factor at the specified time.

3.3.2.3.2 Differential Expression of TVN0984 Gene under pH Stress

The Real-Time PCR experiments for which cDNA templates prepared from RNA samples of the pH-stressed culture yielded Ct values for control and test samples given in the table 3.6.

When Ct values of the tests as compared to that of controls considered it is concluded that increased pH within the range of pH: 3.5 to pH: 5.0 did not induce TVN0984 gene expression. The Ct values of the test at pH: 4.5 were significantly higher than that of the controls.

The lower the Ct value the earlier the amplification of the cDNA sample is expected. Melting curve analysis for each Real-Time experiment verified the specific amplification of the TVN0984 gene's cDNA, average T_m being 82.71°C (±2.08). Representative amplification curves of TVN0984 cDNA samples of pH stress (pH: 3.5 - 120th min, pH: 4.0 - 120th min, pH: 4.5 - 120th min and pH: 5.0 - 90th min and 120th min) were given in the figures 3.34-3.37.

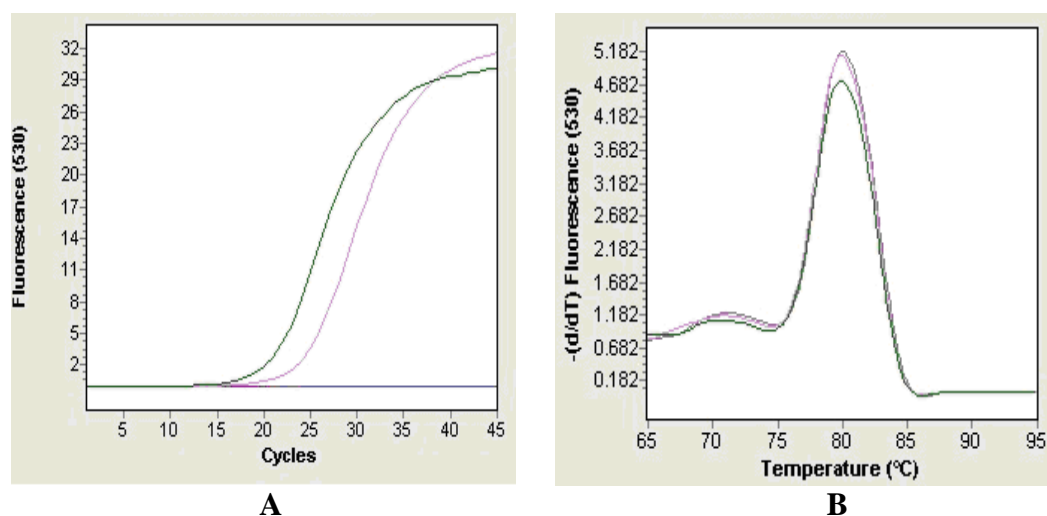


Figure 3.34 Real-Time PCR graphics of stressed at pH: 3.5 (test) and non treated (control, at pH: 3.0) samples of TVN0984 gene. — Test (120th min), Ct: 24.11 — Control Ct: 20.56 (A). Melting curves for TVN0984 gene under stress at pH: 3.5 (B).

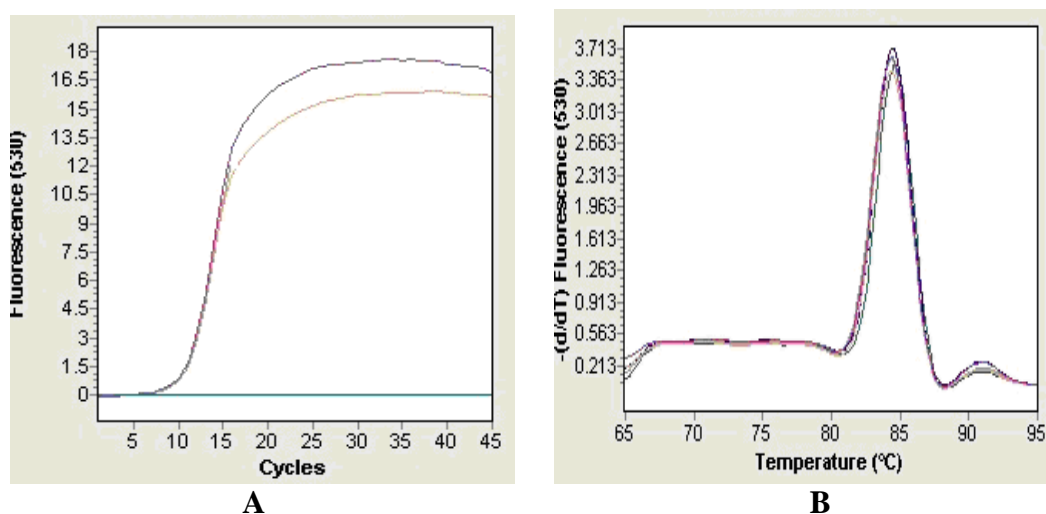


Figure 3.35 Real-Time PCR graphics of pH stress at pH: 4.0 (test) and non treated (control, at pH: 3.0) samples of TVN0984 gene — Test (120th min), Ct: 9.64 — Control, Ct: 9.83 (A). Melting curves for TVN0984 gene under stress at pH: 4.0 (B).

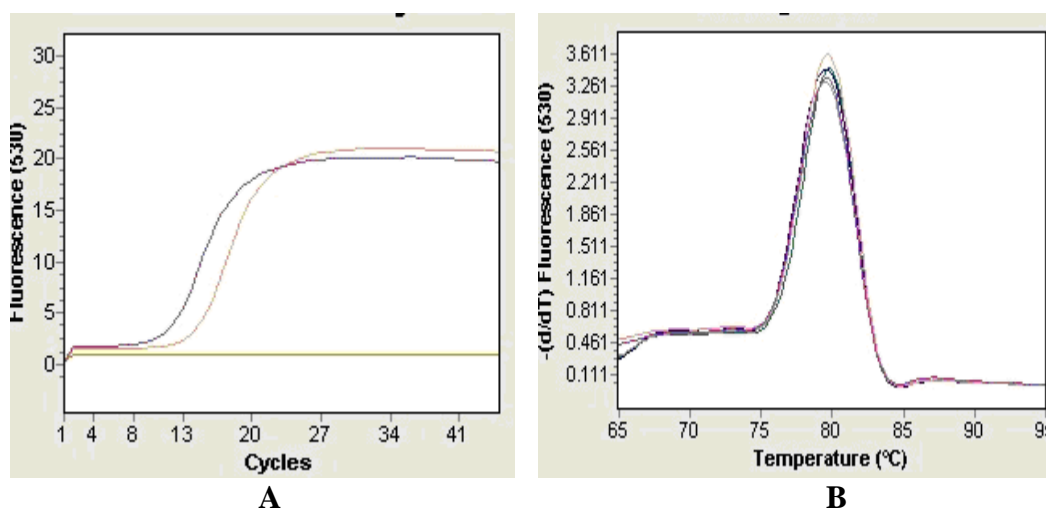


Figure 3.36 Real-Time PCR graphics of stressed at pH: 4.5 (test) and non treated (control, at pH: 3.0) samples of TVN0984 gene. — Test (120th min), Ct: 13.13 — Control Ct: 10.58 (A). Melting curves for TVN0984 gene under stress at pH: 4.5 (B).

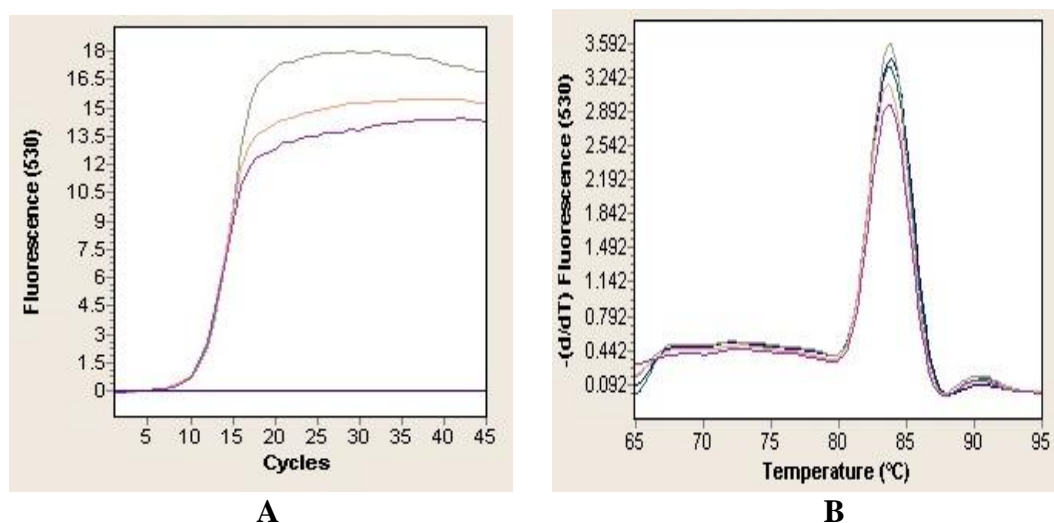


Figure 3.37 Real-Time PCR graphics of stressed at pH: 5.0 (test) and non treated (control, at pH: 3.0) samples of TVN0984 gene. — Test (90th min), Ct: 10.58 — Test (120th min), Ct: 9.94 — Control Ct: 9.82 (A). Melting curves for TVN0984 gene under stress at pH: 5.0 (B).

3.3.2.3.2.1 Fold Analysis of TVN0984 Gene Expression Results under pH Stress

The TVN0984 gene expression was not induced under pH stress. At pH values slightly higher than optimum pH for *Tp. volcanium*, the gene expression reduced slightly as compared to control. But, at higher pH values (pH: ≥ 4.5) TVN0984 gene transcription declined about 5-fold (Figure3.38).

The column charts were plotted using the average fold values from at least three independent RT-PCR experiments at specified pH value and time.

Table 3.6 Cp and Tm values of differential expression of TVN0984 gene under pH stress condition.

pH Stress	Time (min)	Ct	TM
pH: 3.5	30	12.26	84.2
	Control	10.25	84.1
	60	11.83	80.02
	Control	10.68	80.1
	90	12.03	79.12
	Control	10.96	78.97
	120	24.11	80.47
	Control	20.56	80.45
pH: 4.0	30	10.28	84.74
	Control	9.83	84.49
	60	10.58	84.57
	Control	9.83	84.49
	90	9.92	84.46
	Control	9.83	84.49
	120	9.64	84.51
	Control	9.83	84.49
	150	11.66	79.67
	Control	10.72	79.79
	180	12.07	83.72
	Control	10.23	83.56
pH: 4.5	30	13.1	79.9
	60	13.9	79.76
	90	13.01	79.75
	120	13.13	79.85
	Control	10.58	79.73
pH: 5.0	30	10.86	83.95
	Control	9.82	83.87
	60	11.12	84.03
	Control	9.82	83.87
	90	10.58	83.9
	Control	9.82	83.87
	120	9.94	83.82
	Control	9.82	83.87
	150	11.68	84.38
	Control	9.22	84.1
	180	11.45	84.38
	Control	9.22	84.1

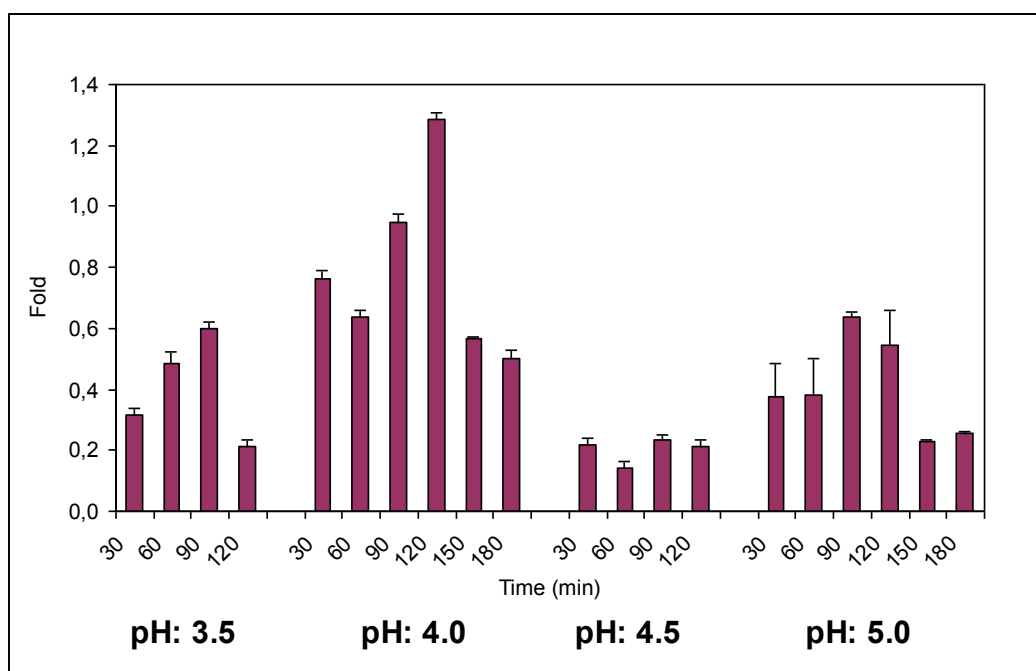


Figure 3.38 Fold analysis of TVN0984 gene expression under pH stress at pH: 3.5, pH: 4.0, pH: 4.5 and pH: 5.0 for 2-3 hours. Each column indicates the fold of the induced gene expression compared to control under the stated stress factor at the specified time.

3.3.2.3.3 Effect of Oxidative Stress on Expression of TVN0984 Gene

Different concentrations of hydrogen peroxide (H_2O_2) (0.01 mM, 0.02 mM, 0.03 mM and 0.05 mM) were tested to see the effect of oxidative stress on the TVN0984 gene expression.

The expression of TVN0984 is expected to be induced by oxidative stress generated by H_2O_2 at 0.01 mM and 0.02 mM concentrations for 2 h. Under these conditions, Ct values of the test were lower than that of the controls (Table 3.7). However at higher concentrations of the H_2O_2 (*i.e.*, 0.03 mM and 0.05 mM) similar or higher Ct values for the test relative to controls were obtained, indicating no change or decline in the expression level.

The lower the Ct value the earlier the amplification of the cDNA sample is expected. Melting curve analysis for each Real-Time experiment verified the specific amplification of the TVN0984 gene's cDNA, average T_m being 81.96°C (±2.30). Representative amplification curves of TVN0984 cDNA samples of oxidative stress (0.01 mM H₂O₂ - 90th min., 0.02 mM H₂O₂ - 90th min., 0.03 mM H₂O₂ - 90th min. and 0.05 mM H₂O₂ - 90th min) were given in the figures 3.39-3.42.

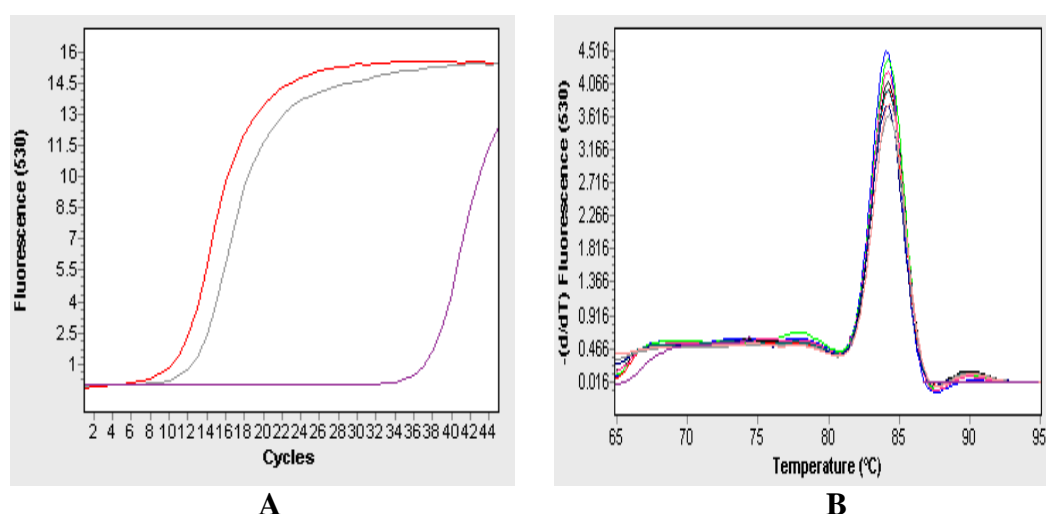


Figure 3.39 Real-Time PCR graphics of stressed at 0.01 mM H₂O₂ (test) and non treated (control) samples of TVN0984 gene. — Test (90th min), Ct: 10.14, — Control Ct: 11.87 (A). Melting curves for TVN0775 gene under oxidative stress at 0.01 mM H₂O₂ (B).

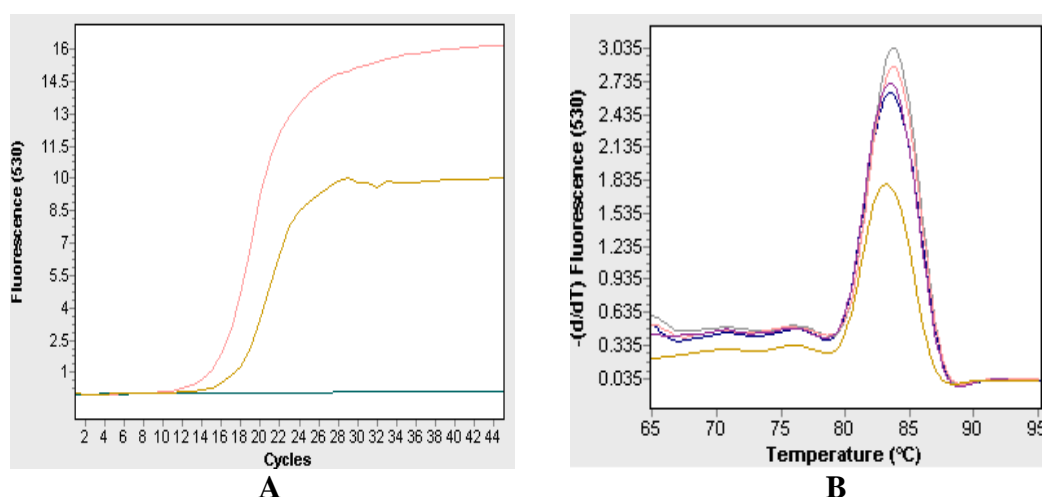


Figure 3.40 Real-Time PCR graphics of stressed at 0.02 mM H₂O₂ (test) and non treated (control) samples of TVN0984 gene. — Test (90th min), Ct: 14.76, — Control Ct: 16.68 (A). Melting curves for TVN0775 gene under oxidative stress at 0.02 mM H₂O₂ (B).

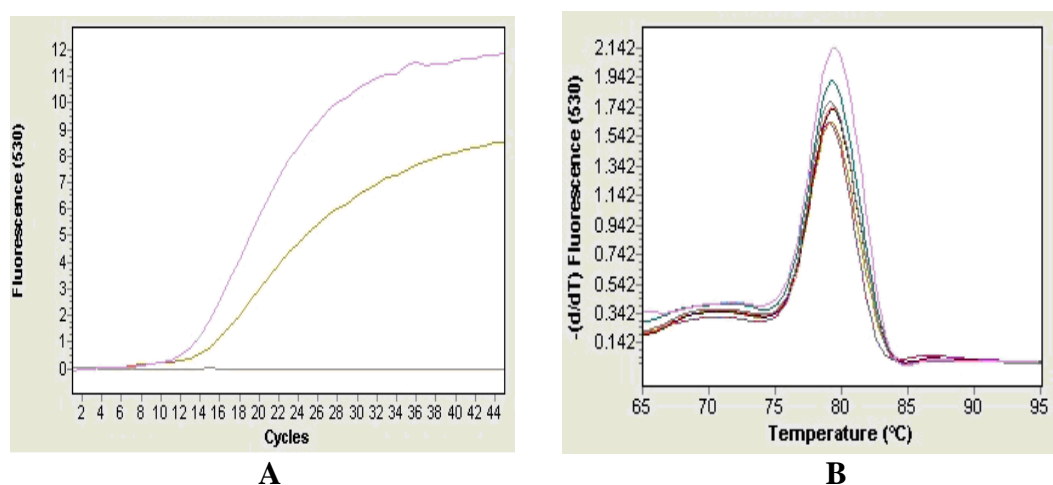


Figure 3.41 Real-Time PCR graphics of stressed at 0.03 mM H₂O₂ (test) and non treated (control) samples of TVN0984 gene. — Test (90th min), Ct: 13.56, — Control Ct: 12.52 (A). Melting curves for TVN0775 gene under oxidative stress at 0.03 mM H₂O₂ (B).

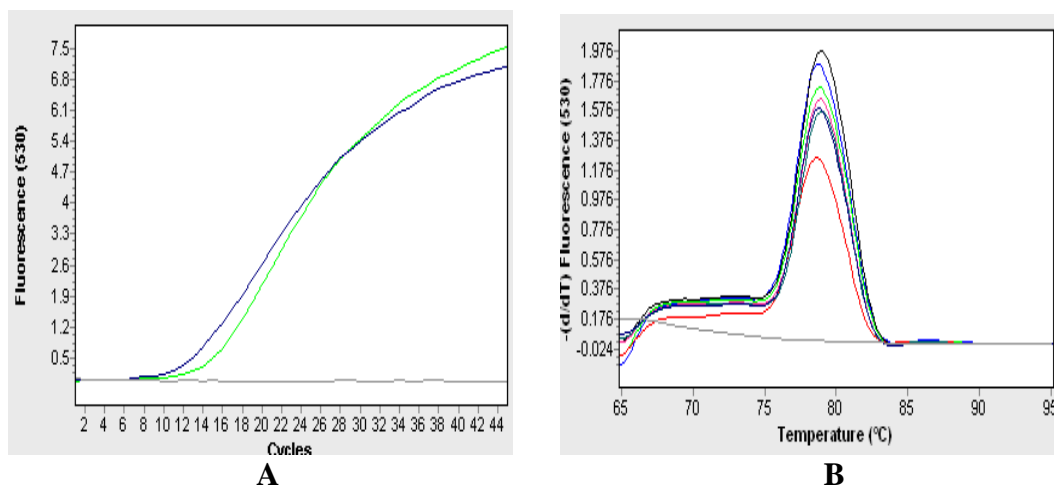


Figure 3.42 Real-Time PCR graphics of stressed at 0.05 mM H₂O₂ (test) and non treated (control) samples of TVN0984 gene. — Test (90th min), Ct: 13.44, — Control Ct: 11.63 (A). Melting curves for TVN0775 gene under oxidative stress at 0.05 mM H₂O₂ (B).

3.3.2.3.3.1 Fold Analysis of TVN0984 Gene Expression Results under Oxidative Stress

Fold analysis indicated that H₂O₂ at 0.01 mM and 0.02 mM concentration increased TVN0984 gene expression, at most 2.5-fold (90 min) and 4.7-fold (30 min), respectively. At 0.03 mM H₂O₂ concentration, gene expression profile was not different than that of the control, but at 0.05 mM H₂O₂, expression level was about one half of the control (Figure3.43).

The column charts were plotted using the average fold values from two independent RT-PCR experiments of specified oxidative stress conditions.

Table 3.7 Cp and Tm values of differential expression of TVN0984 gene under oxidative stress condition.

H₂O₂ concentration	Time (min)	CP	TM
0.01mM	30	12.04	84.17
	60	11.53	84.29
	90	10.14	84.29
	120	11.2	84.29
	Control	11.87	84.33
0.02mM	30	13.92	83.84
	Control	16.68	83.5
	60	11.08	84.26
	Control	11.87	84.33
	90	14.76	84.01
	Control	16.68	83.5
	120	11.5	84.22
	Control	11.87	84.33
0.03mM	30	12.83	79.72
	60	12.4	79.34
	90	13.56	79.58
	120	12.68	79.69
	150	12.74	79.54
	180	13.18	79.62
	Control	12.52	79.79
0.05mM	30	13.08	79.18
	60	13.99	79.26
	90	13.44	78.97
	120	15.19	79.32
	150	15.14	79.25
	180	16.17	79.29
	Control	11.63	79.19

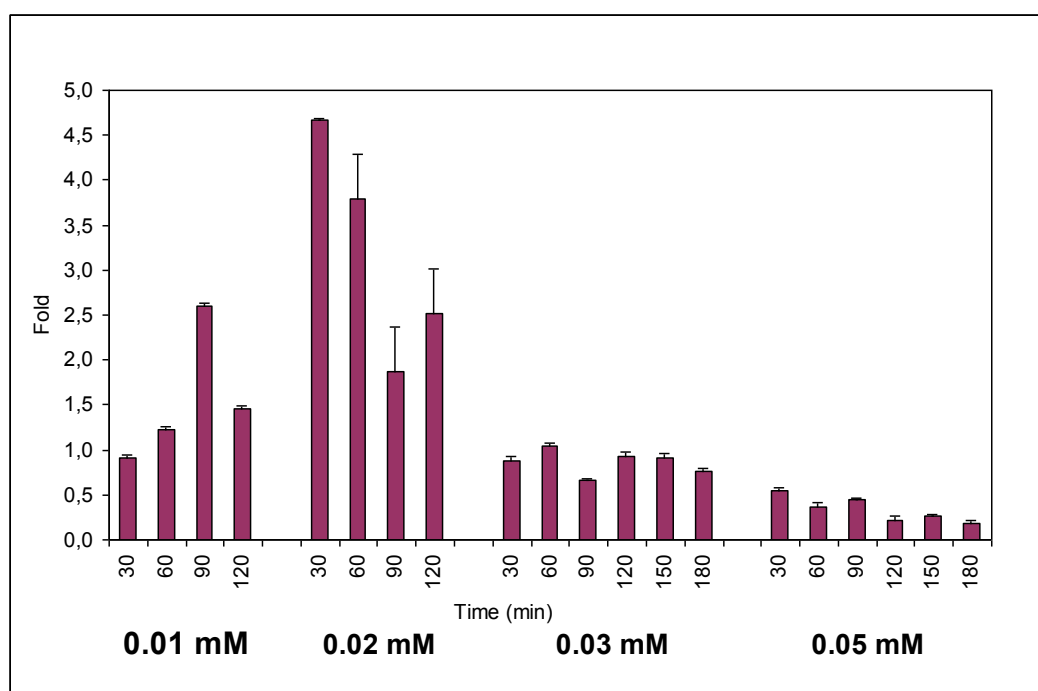


Figure 3.43 Fold analysis of TVN0984 gene expression under oxidative stress at 0.01 mM, 0.02 mM, 0.03 mM and 0.05 mM, for 2-3 hours. Each column indicates the fold of the induced gene expression compared to control under the stated stress factor at the specified time.

3.4 Purification of Recombinant TVN0775/sHsp by Ni-NTA Affinity Chromatography

The TVN0775/sHsp, that has been expressed as an N-terminal 6X-His tag fusion was purified using Ni-NTA affinity chromatography. For purification of the recombinant sHsp optimization was made considering a number of parameters such as Ni-NTA agarose/cell extract ratio, imidazole concentration of the wash and elution buffers. Also, heat treatment of the cell extract to remove heat sensitive *E.coli* proteins was carried out to obtain better purified protein samples.

SDS-gel electrophoresis of the purification result, obtained by application of the cell extracts before heat treatment is shown in the figure 3.44.

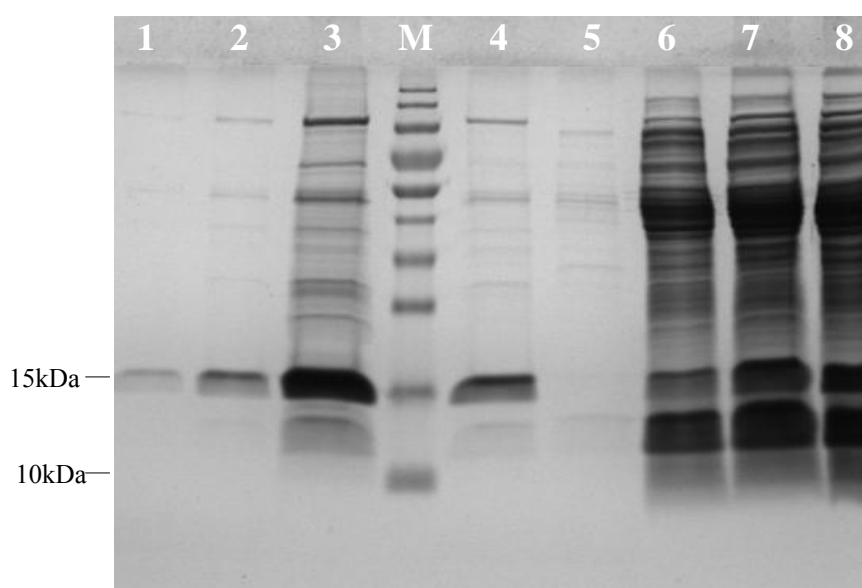


Figure 3.44 SDS-PAGE gel electrophoresis of TVN0775 gene product purification (no heat treatment). **Lane 1-4:** Eluted samples, respectively. **Lane 5, 6:** The proteins coming from the washing step. **Lane 7:** Flow through. **Lane 8:** All proteins before purification, called crude extract. **M** is the PageRuler™ Prestained Protein Ladder (See Appendix B).

The elution fractions were found to be contaminated with other proteins, but still the major band was that of the TVN0775/sHsp. The contaminating proteins were considerably reduced by heat treatment of the cell extract and by including 30 mM and 250 mM imidazole into wash buffer and elution buffer, respectively (Figure 3.45).

The elution buffer was exchanged by the lysis buffer, by ultrafiltration of the purified sHsp sample to be used in the chaperone activity assays (Figure 3.46). This purification has been repeated several times to collect sufficient amount sHsp sample for chaperone activity assays.

The purification protocol applied in this study yielded highly purified recombinant sHsp which produced a single band on the SDS-polyacrylamide gel. The estimated molecular mass of the His-tagged TVN0775/sHsp (about 15 kDa) was

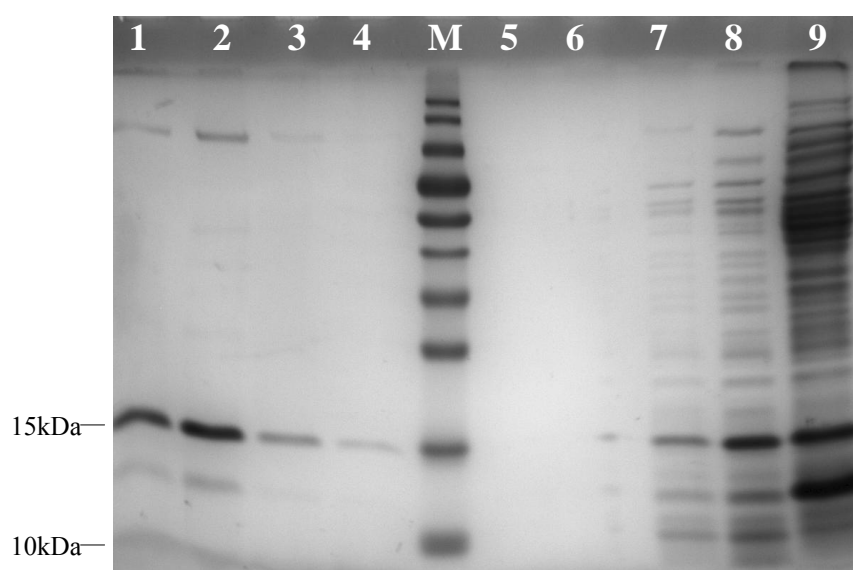


Figure 3.45 SDS-PAGE gel electrophoresis of TVN0775 gene product purification (with heat treatment). **Lane 1-4:** Eluted samples. **Lane 5, 6:** Samples after washing. **Lane 7:** The proteins coming after flow through-4. **Lane 8, 9:** Cell free lysates – after heat and before heat, respectively. **M** is the PageRuler™ Prestained Protein Ladder (See Appendix B).

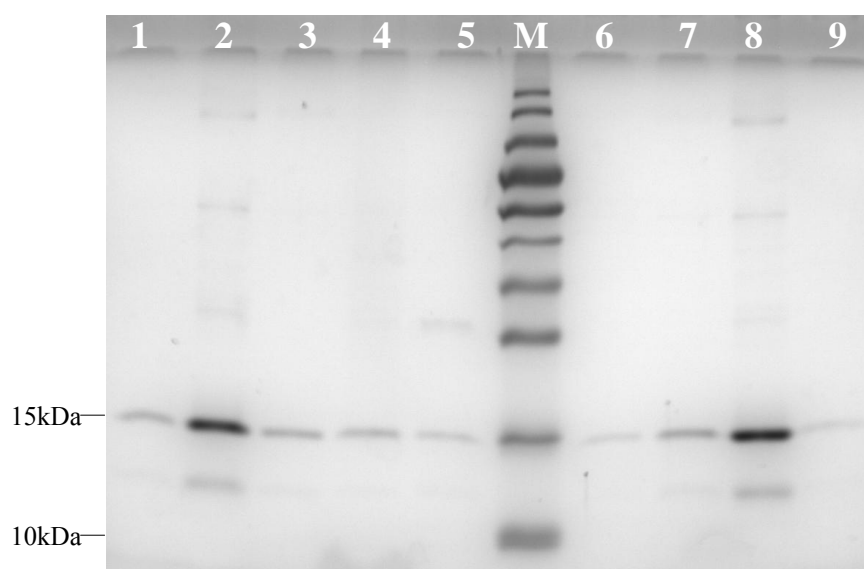


Figure 3.46 SDS-PAGE gel electrophoresis of TVN0775 gene product purification. **Lane 1-3 and 6-9:** Eluted samples. **Lane 4, 5:** Concentrated elution samples. **M** is the PageRuler™ Prestained Protein Ladder (See appendix B).

similar to the calculated molecular weight of the untagged TVN0775 gene product (NCBI).

3.5 Chaperone Activity of the Recombinant TVN0775/sHsp

As described in the Materials and Methods section, bovine glutamate dehydrogenase (boGDH) was used as the model enzyme to study chaperone activity of purified TVN0775/sHsp of *Tp. volcanium*. For this purpose boGDH enzyme activity was evaluated in the presence or absence of interested sHsp after two different treatments; in the first experiment series, the substrate enzyme was subjected to heat treatment and in the second experiment series, the boGDH was denatured by a chemical treatment using guanidinium hydrochloride (GdnHCl).

The enzyme samples were heated at 45°C, 50°C and 53°C in the presence (test) and absence (negative control) of sHsp for 10 minutes. Then the remaining enzyme activity was measured spectrophotometrically as described in the Materials and Methods.

The activity assays with boGDH samples which were not heated, also included into each experiment as the positive control.

The boGDH activity declined after heat treatment at 45°C for 10 min. (negative control, $\Delta\text{mAb}_{340}/\text{min}$: -84), as compared to untreated boGDH enzyme activity (positive control, $\Delta\text{mAb}_{340}/\text{min}$: -140) (Figure 3.47). However, when heat treatment was performed in the presence of sHsp the boGDH activity was higher ($\Delta\text{mAb}_{340}/\text{min}$: -96) than that of the negative control.

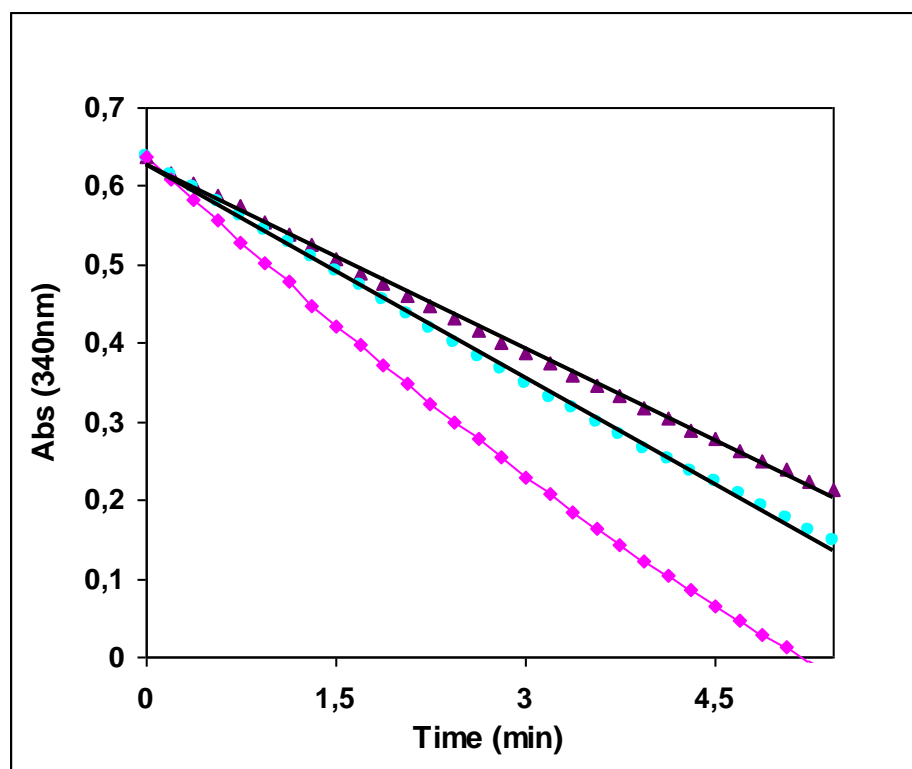


Figure 3.47 The boGDH enzyme activity after heating at 45°C for 10 min.

- The curve of enzyme activity (no heat treatment, no sHsp), (Positive control)
- ▲— The curve of heat treated enzyme activity, in the absence of sHsp (Negative control)
- ◆— The curve of heat treated enzyme activity after heat treatment (test), in the presence of sHsp.

The remaining boGDH activity, before and after heat treatment at 50°C, in the presence and absence of sHsp was shown in the figure 3.48. The boGDH activity of the negative control and test were - 37.4 mAb₃₄₀/min. and - 55.1 mAb₃₄₀/min. respectively.

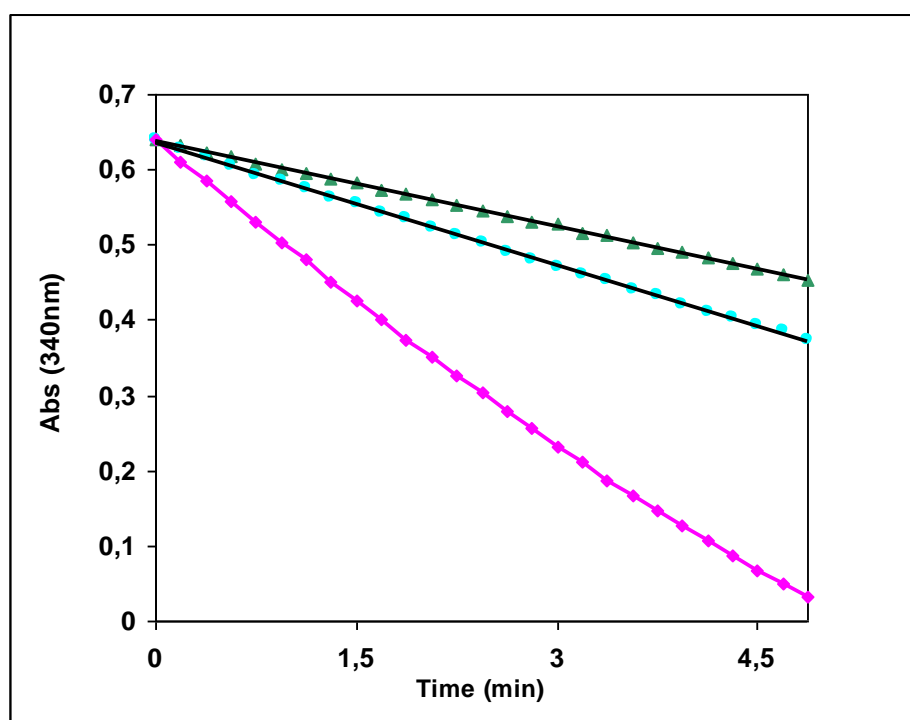


Figure 3.48 The boGDH enzyme after heating at 50°C for 10 min.

- The curve of enzyme activity (no heat treatment, no sHsp) (Positive control).
- ▲— The curve of heat treated enzyme activity, in the absence of sHsp (Negative control).
- The curve of heat treated enzyme activity, in the presence of sHsp.

The remaining boGDH activity, before and after heat treatment at 53°C and in the presence and absence of sHsp was shown in the figure 3.49. Under this condition, boGDH activities of the negative control and test -11.6 mAb₃₄₀/min. and -25.3 mAb₃₄₀/min. respectively.

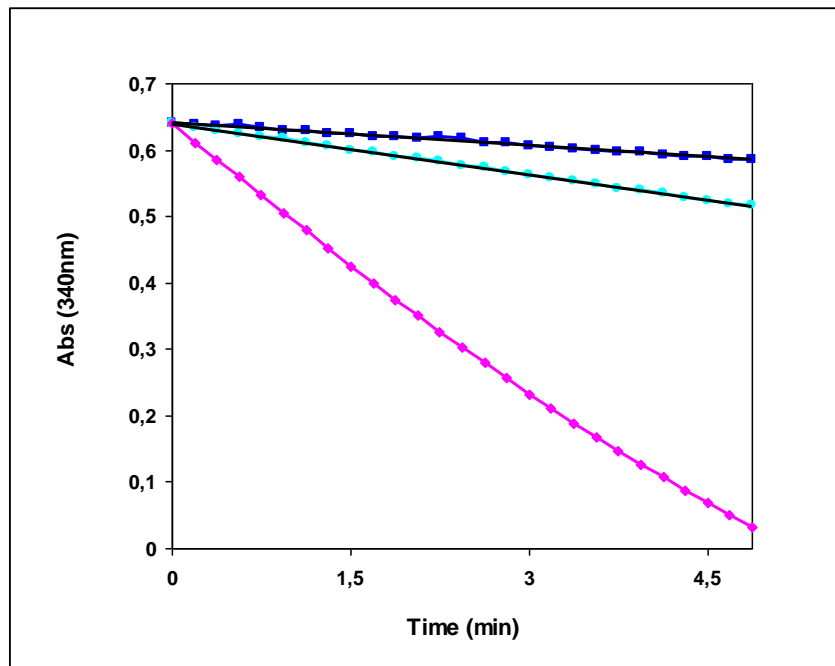


Figure 3.49 The activity graphic of boGDH enzyme after denaturation at 53°C for 10 min.

- The curve of enzyme activity (no heat treatment, no sHsp) (Positive control).
- ▲— Enzyme activity (heat treatment), in the absence of sHsp (Negative control).
- Enzyme activity, in the presence of sHsp.

This result indicated that heat treatment by itself decreased enzyme activity 12 fold. When heat treatment was performed in the presence of sHsp about 2 fold increase was observed in the remaining activity. Thus, although sHsp at 53°C could not exert a full protection from heat denaturation, still provided some activity regain at this temperature.

3.5.1 Self Renaturation of the boGDH Denatured at Different Concentrations of GdnHCl

When boGDH enzyme was denatured with GdnHCl at different concentrations (1.5 M, 2 M, 2.5 M, 3 M and 4 M) for 30 min or 60 min self renaturation was not achieved at 3 and 4 M denaturation conditions. The lower GdnHCl concentrations

denaturation was incomplete; at 1.5 M denaturation condition, almost full activity was regained (-106 mAb₃₄₀/min) and at 2M and 2.5 M denaturation condition, only some activity was regained (-29 mAb₃₄₀/min and -15 mAb₃₄₀/min). Therefore 2.5 M GdnHCl was used to test the chaperone activity of sHsp in renaturation of chemically denatured boGDH. There was no detectable effect of denaturation time (30 min and 60 min) on the renaturation profile of the boGDH activity at the selected concentrations (Figure 3.50 and 3.51).

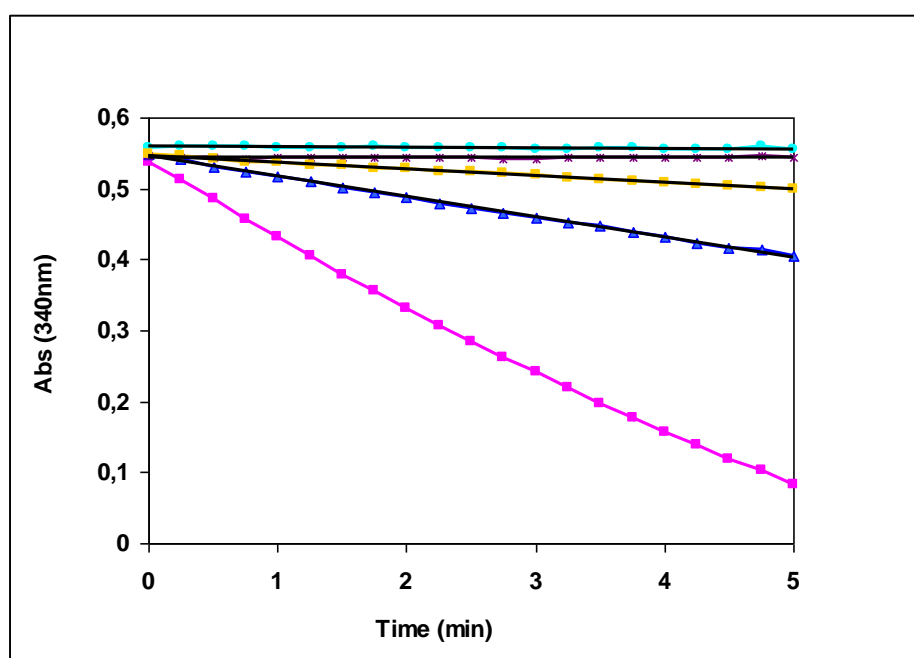


Figure 3.50 Self renaturation of the boGDH enzyme after denaturation at different GdnHCl concentrations for 30 min, in the absence of sHsp

- Regained activity of boGDHenzyme denatured with 1.5 M GdnHCl
- ▲— Regained activity of boGDHenzyme denatured with 2 M GdnHCl.
- Regained activity of boGDHenzyme denatured with 3 M GdnHCl.
- *— Regained activity of boGDHenzyme denatured with 4 M GdnHCl.
- Self renaturation of the enzyme (in the absence of sHsp)

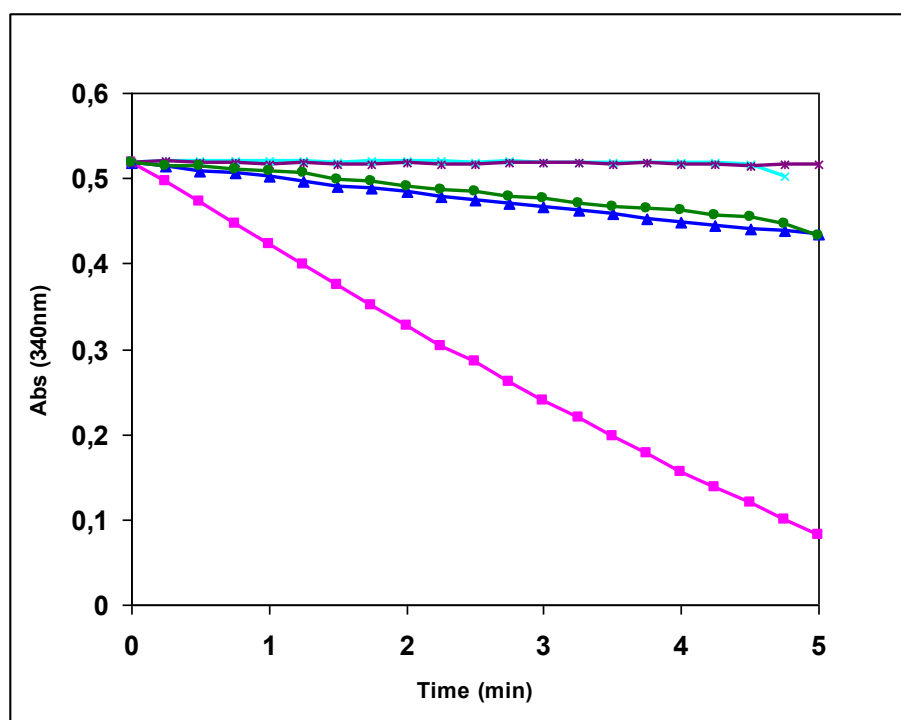


Figure 3.51 Self renaturation of the boGDH enzyme after denaturation at different GdnHCl concentrations for 60 min, in the absence of sHsp

- Regained activity of boGDHenzyme denatured with 1.5 M GdnHCl
- ▲— Regained activity of boGDHenzyme denatured with 2 M GdnHCl.
- ×— Regained activity of boGDHenzyme denatured with 3 M GdnHCl.
- *— Regained activity of boGDHenzyme denatured with 4 M GdnHCl.
- Self renaturation of the enzyme (in the absence of sHsp).

3.5.2 Renaturation of Chemically Denatured boGDH Enzyme by the Help of TVN0775/sHsp

The boGDH enzyme after denaturation at 2.5 M GdnHCl (for 30 min) was incubated in the renaturation buffer for 2h in the presence and absence of the sHsp, as described in the Materials and Methods. The activity regained in the presence of sHsp (-20 mAb₃₄₀/min) was about 2-fold higher as compared to activity in the absence of sHsp (-12 mAb₃₄₀/min) (Figure 3.52).

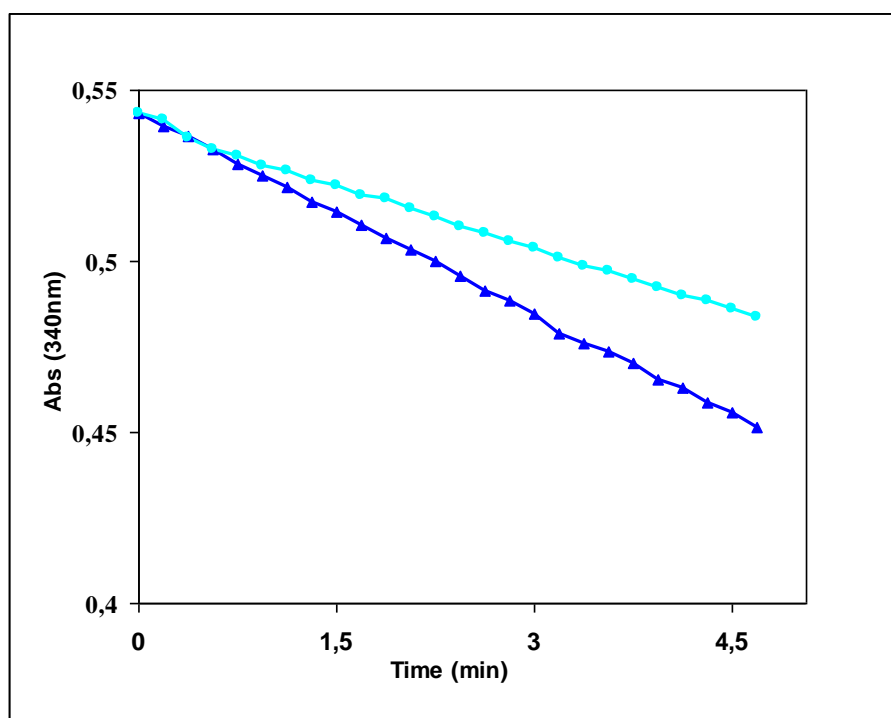


Figure 3.52 Renaturation of the boGDH enzyme denatured at 2.5 M GdnHCl for 30 min in the presence and absence of sHsp

- ▲— Renaturation of the enzyme in the presence of sHsp.
- Self renaturation of the enzyme (in the absence of sHsp)

This result showed that the chaperone activity of sHSP/775 enhanced activity regain of the chemical denatured boGDH under renaturation conditions.

CHAPTER 4

DISCUSSION

Molecular chaperones are one of the important members of cell protection mechanisms under stress conditions. They assist in the folding of newly synthesized proteins, catalyze the refolding of denatured proteins and prevent the denaturation and aggregation of proteins (Rutherford 2003). The molecular chaperone system of thermophilic archaea is very simple compared with that of other organisms. The Hsp104/ClpB and Hsp90 genes are absent in their genomes. Moreover, genes for the Hsp70/DnaK system were not found in the total genomic sequences of many thermophilic and hyperthermophilic archaea (Large et. al, 2009). Therefore, it is believed that the sHsps and type II chaperonins (thermosomes) should constitute the main role in the molecular chaperoning systems of archaea.

There are several lines of evidence that sHsps are critical factors in the cellular chaperone network, thus playing a particularly important role ubiquitously under the conditions of severe stress. They are also potentially important in many diseases related to aging and protein misfolding (Clark and Muchowski, 2000; Sun and MacRae, 2005). On the other hand, sHsps might have several uses in various biotechnological applications for improvement of enzymatic performance (Hant et. al. , 2008).

In this study we investigated the role of two sHsps (*i.e.*, TVN0775 and TVN0984 gene products) of *Thermoplasma volcanium* in stress response. Also, molecular chaperone activity of purified TVN0775/sHsp was analyzed using a eukaryotic mesophilic enzyme (boGDH) as the model protein.

So far, sHsps from various hyperthermophilic archaea (*Methanococcus jannaschii*, *Thermococcus* sp. Strain KS-1, *Sulfolobus tokodaii* strain 7, *Pyrococcus furiosus*, *Archaeoglobus fulgidus*, *Methanococcoides burtonii* and *Sulfolobus solfataricus*) were studied *in vivo* and *in vitro* (Kocabiyik, 2009; Laksanalamai et. al., 2009; Wang et. al., 2010). This is the first report for the investigation of the role of an archaeal sHsp in stress response at transcription level. The novelty of this research is also due to purification of a recombinant sHsp from a moderately thermophilic archaea (*i.e.*, *Thermoplasma*) and studying its chaperoning function.

Our results showed that heterologous, high level expression of TVN0775/sHsp gene in *E.coli* increased its thermotolerance at 53°C. The second sHsp protein of the *Tp. volcanium* (TVN0984/sHsp) was not effective in improvement of the thermal resistance of the mesophilic bacterium (*i.e.*, *E.coli*). Previously, influences of sHsps from some hyperthermophilic archaea, on enhancement of the heat tolerance of mesophilic cells were reported. For example, *Pyrococcus furiosus* is an hyperthermophilic archaea, with an optimum growth temperature of 100°C. Pfu-sHsp in *E.coli* cells protected the host proteins from aggregation at 105°C and so increased the resistance of *E.coli* cells. The effect of this protection lasted for 40 minutes (Laksanalami et. al., 2001). A similar study was performed using a sHsp from a cold adapted microorganism, *Methanococcoides burtonii*. The sHsp from this organism (Mb-sHsp) when overexpressed in *E.coli* prevented the aggregation of host cell proteins at 60°C for 4 hours (Laksanalamai et. al., 2009). The sHsp gene of *S. solfataricus* (SsHsp 14.1) was cloned in *E.coli* cells. The SsHsp 14.1 enhanced thermotolerance of *E. coli* cells at 50°C for 4 hours by preventing aggregation of the cell proteins (Wang et al., 2010).

On the other hand, not only archaea-originated sHsps but also sHsps from other organisms were also used in thermotolerance studies. For example, the sHsp from rice, OsHsp 16.9 provided thermoprotection to *E.coli* cells at 47°C for 1 hour (Yeh et. al., 1997).

In the second part of our research, the expression profiles of interested genes, TVN0775/sHsp and TVN0984/sHsp, under different stress conditions were studied. Firstly, *Tp. volcanium* cells were exposed to various stress conditions; high temperature, high pH and hydrogen peroxide. Secondly, RNAs of the treated cells were isolated. Thirdly, interested genes' mRNAs were reverse transcribed and then, Real-Time PCR was performed. The Ct values obtained and the PCR efficiency values calculated by an on-line computer program were used to determine the fold difference. The expression level of the TVN0775/sHsp gene increased about 3 fold after heat-shock at 65°C for 30 - 60 min. But then it was same as the control at 120th min and then declined up to the end of 3rd h. The expression of the TVN0775/sHsp at 70°C showed a time dependent increase and the expression was about 1.5-fold higher in the test samples for 60-120 min, as compared to control samples. These results showed that expression of TVN0775/sHsp was more induced under heat shock at 65°C than that at 70°C. The highest level of transcription was attained by heat-shock at 65°C for 30-60 min.

The TVN0984/sHsp gene expression was better induced at 70°C (at most 5 fold increase) for 120 min where as at 65°C at most 3 fold increase in the expression level was observed.

There are reports on induction of gene expression level of heat shock proteins under heat shock in other archaea detected by different methods. For example Shockley et al., studied heat shock response of *Pyrococcus furiosus* at 105°C for 60 min. In that study, not only sHsp genes but also thermosome (Hsp60-like chaperonin), proteasome and two other molecular chaperone genes were strongly expressed as shown by northern hybridization and microarray assays. In another gene induction study by using *A. fulgidus*, whole genome microarray gene expression under heat shock was investigated (Rohlin et. al., 2005), as well as expression levels of sHsp genes and thermosome genes but also a few more heat shock genes of *A. fulgidus* were found to be highly induced under heat shock

at 89°C for 1 hour. Before the heat treatment, the *A. fulgidus* cells had grown at 78°C up to mid-exponential phase. As well as microarray analysis, Real-Time PCR was also performed to verify the results.

Thermoplasma volcanium cells were exposed to high pH (pH: 3.5, pH: 4.0, pH: 4.5, pH: 5.0), and the change in the sHsp gene expression profile was analyzed by Real-Time PCR technology. This is the first study about pH stress on archaeal sHsps. The results showed that TVN0775/sHsp gene expression was more sensitive to increased pH than that of TVN0984/sHsp gene expression. The TVN0775/sHsp gene transcription increased at most 2.5-fold at pH 4.0 for 90-120 min. The gene expression did not change or reduced at other pH values. On the other hand, TVN0984/sHsp gene expression did not change at pH 4.0 but significantly reduced at higher pH values (*i.e.*, pH 4.5 and 5.0). On the other hand, extreme pH concentrations (pH: 4.7 and pH: 8.0) was tested on sHsps from human, α A-crystallin and α B-crystallin (Takeuchi et. al., 2003). After the pH stress exposure, survival of the host cells (α A-crystallin cells, *E. coli* cells transformed with pET-11a harbouring human α A-crystallin gene; α B-crystallin cells, *E. coli* cells transformed with pET-11a harbouring human, α B-crystallin gene; GST (glutathione S-transferase) cells, *E. coli* cells transformed with pGEX-2T vector) and the solubility of the expressed target proteins in the cytosol were examined. As a result of this study, the cells expressing α B-crystallin protein under the pH stress demonstrated significantly improved survival when compared with the other cells, and the expressed protein in the cytosol was almost soluble, in contrast with the α A-crystallin protein. Accumulation of heat shock proteins depending on changes in temperature and pH was studied in the acidophilic green alga, *Chlamydomonas acidophila* (Elias et. al., 2005). Results regarding expression of the sHsps under pH stress demonstrated that the increased accumulations of sHsps at very low pH (pH: 1.5) and at the higher pH values of 6 and 7.

The effect of oxidative stress on the expression of TVN0775 and TVN0984 genes was investigated by treatment of *Tp. volcanium* cells with 0.01 mM, 0.02 mM, 0.03 mM and 0.05 mM H₂O₂. For both sHsp genes transcription was induced at lower concentrations of H₂O₂ (0.01 mM and 0.02 mM). The highest expression of TVN0775 gene was observed at 0.01 mM H₂O₂ for 90 min, and at 0.02 mM H₂O₂ for 60 min. The TVN0984 gene transcription was induced 2.5 fold at 0.01 mM H₂O₂ for 90 min and about 5 fold at 0.02 mM H₂O₂ for 60 min. At higher concentrations of H₂O₂ expression of both genes either did not change or down regulated. A similar oxidative stress experiment had previously been done in our laboratory for other heat shock protein genes, *i.e.*, group II chaperonin genes, and it was found that these genes were induced by H₂O₂ stress (Doldur, MS thesis, 2008). Oxidative stress induced expression of heat shock genes was also reported in *Drosophila* by microarray analysis (Landis et. al., 2004).

Lastly, in this study we have purified the TVN0775/sHsp gene which has previously been cloned in *E.coli*, in our laboratory. The TVN0775/sHsp gene as an N-terminal 6X His-tag fusion was purified to homogeneity on Ni-NTA matrices as revealed by SDS-PAGE. The heat treatment of cell extract before loading to column has removed majority of the host cell proteins. Purified protein samples after ultrafiltration for buffer exchange were used in the chaperone activity assays. Glutamate dehydrogenase enzyme from bovine liver (boGDH) was used as substrate in chaperone activity assays. BoGDH is a mesophilic enzyme with an optimal assay temperature of 25°C. It has six subunits and the activity of it was measured by spectrophotometrically by the reduction in absorbance measurements at 340 nm at 25°C because of NADH utilization. Aggregation prevention and refolding activity related to the role of sHsps was followed after the enzyme was denatured by heat or guanidine hydrochloride. We have found out that the recovery of glutamate dehydrogenase activity at 45°C, 50°C and 53°C in the presence of the *Tp. volcanium* sHsp was higher than that of spontaneous refolding. On the other hand, activity measurements after renaturation of the enzyme that was denatured at 2.5 M GdnHCl concentrations

for 30 min revealed that presence of sHsp increased the recovery of the enzyme activity.

In the previous studies, boGDH was used to study the chaperone activity of sHsps or other chaperones. For instance, after the boGDH enzyme was exposed to 56°C, it was treated with Pfu-sHsp, (a sHsp of *Pyrococcus furiosus*) and the enzyme activity did not decline (Laksanalamai et al., 2001). So it means that the Pfu-sHsp protected the boGDH enzyme from aggregation by heat. In another study, bovine glutamate dehydrogenase enzyme was denatured by heat to determine the chaperone activity of Mb-sHsp from *Methanococcoides burtonii*. This study showed that, Mb-sHsp could stabilize the denatured enzyme at 50°C. About 70-80% of the activity was retained for 3 h (Laksanalamai et. al., 2009).

There are other reports on chaperone activity of archaeal sHsps with different substrates, such as, citrate synthase (CS), green fluorescent protein (GFP), 3-isopropylmalate dehydrogenase (IPMDH) and single chain monellin (SCM). The results from these studies are in agreement with the hypothesis that sHsps have role in preventing the protein aggregation and in helping them refolding during abnormal conditions. For example, MjHsp16.5 suppressed the precipitation of porcine heart CS at 40°C (Kim et. al., 1998). The CS is a thermosensitive enzyme and so it normally can not tolerate this temperature. As well as CS, SCM protein was also worked in the same study for aggregation prevention activity of sHsp. In this study it was also shown that MjHsp16.5 protected SCM from denaturation at 80°C for 20 min. In another study, T. sHsp, a chaperone from *Thermococcus sp. KS-1* could prevent CS activity at 45°C (Usui et. al., 2001). Two more sHsps; Hsp21 (from *Arabidopsis thaliana*) and $\alpha\beta$ -crystallin (from human) were also kept the CS activity stable at 47°C for 20 min (Ahrman et. al., 2007). Another study was performed with mitochondrial citrate synthase from pig heart and Hsp20.2 from *A. fulgidus*. The aggregation inhibition of CS denatured in 6 M guanidinium chloride increased dramatically when Hsp20.2 was thermally activated (Haslbeck et. al., 2008). In accordance with the suppression of thermal aggregation related

studies, IPMDH enzyme was also measured at high temperatures with St sHsp14.1 and this enzyme could show activity at 87°C (Saji et. al. , 2007). In an another study, it was found that, although acid denatured GFP had no fluorescence in the absence of T.sHsp, the activity was followed by the fluorescence increase in the presence of T.sHsp (Usui et. al., 2001). This result was an indication of the refolding activity of sHsp.

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APPENDIX A

BUFFERS AND SOLUTIONS

TP. volcanium Medium (1000 ml) PH: 2.3 (Adjust with 98% H₂SO₄)

Potassium – di hydrogen Phosphate (KH ₂ PO ₄).....	3g
Magnesium sulphate (MgSO ₄).....	1g
Calcium chloride with dihydrous (CaCl ₂ .2H ₂ O).....	0.25 g
DiAmmonium sulphate ((NH ₄) ₂ SO ₄).....	0.2 g

SDS Running Buffer (500ml) pH: 8.7 (no adjustment)

Tris	1.51 g
Glycin.....	7.2 g
SDS.....	0.5 g

Agarose Gel (8 %)

Running buffer

50x TAE Buffer.....	6 ml
distilled water.....	300 ml

Gel

Agarose.....	0.32 g
Running buffer.....	40 ml
Etidium Bromide.....	5 µl

50x TAE Buffer (1000 ml)

Tris base.....242 g
Glacial acetic acid.....57.1 ml
EDTA(0.5M- pH: 8.0).....100 ml

10x Formaldehyde (FA) Gel Buffer (250 ml) Ph: 7.0

MOPS (100 mM) (Mw: 209.27)..... 20.93 g
NaOAc (25 mM) (Mw: 82.03)2.05 g
EDTA (5 mM) (Mw: 372.24).....1.8612 g

TE Buffer (100 ml) Ph: 8.0

Tris (10 mM) (Mw: 121.14).....0.12 g
EDTA (1 mM) (Mw: 372.24).....0.037 g

5x RNA Loading Buffer

Saturated aqueous bromophenol blue solution.....16 µl
EDTA (500 mM) (pH 8.0).....80 µl
Formaldehyde (37%) (12.3 M)720 µl
Glycerol (100%).....2 ml
Formamide.....3.084 ml
10 x FA gel buffer.....4 ml
RNase-free water.....completed to 10 ml

Formaldehyde Agarose Gel (1.2 %)

Agarose.....0.48 g,
10x FA gel buffer.....4 ml
RNase free water.....completed to 40 ml

Before pouring the gel;

Formaldehyde..... 720 μ l

Etidium Bromide.....5 μ l

1x FA gel running buffer

10x FA gel buffer.....30 ml

37% (12.3 M)

Formaldehyde.....6 ml

RNase free water.....completed to 300 ml

Buffers for Protein Extraction-Purification

Stock Buffer (750ml)

50mM NaH₂PO₄..... 5.85g

300mM NaCl 13.14g

pH: 7.99 (adjusted with NaOH)

Lysis Buffer(100ml) (+10mM imidazole)

Add 1ml imidazole (from 1M imidazole solution) into 100 ml stock buffer

No pH adjustment

Wash Buffer (100ml) (+20mM imidazole)

Add 0.136g imidazole into 100 ml stock buffer

pH: 8.04 (adjusted with NaOH and HCl)

Elution Buffer (100ml) (+250mM imidazole)

Add 1.7g imidazole into 100 ml stock buffer

pH: 8.00 (adjusted with NaOH and HCl)

SDS Gel Preparation (12.5 %)

	Seperating (Running) Gel	Stacking gel
Acr/Bis	2.5 ml	330 µl
Double Distilled Water	1.1 ml	870 µl
1.88 Tris (pH: 8.9)	1.2 ml	400 µl
SDS (0.5 %)	1.2 ml	400 µl
AmoniumPerSulfate (APS) (10 %)	30 µl	10 µl
TEMED	5 µl	2 - 3 µl

APPENDIX B

MARKER AND LADDERS

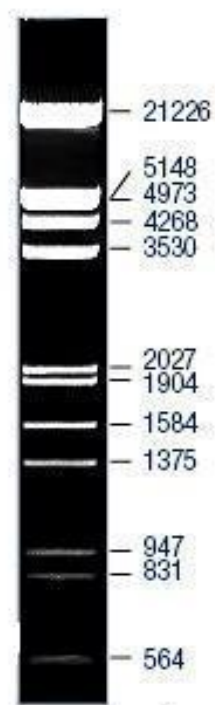


Figure B.1 EcoRI+HindIII cut Lambda DNA

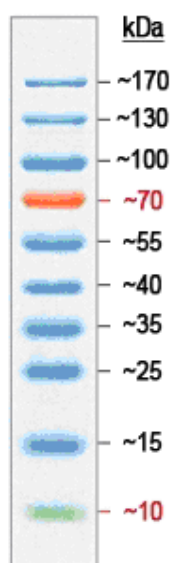


Figure B.2 PageRuler™ Prestained Protein Ladder
(Fermentas UAB, Vilnius, Lithuania)

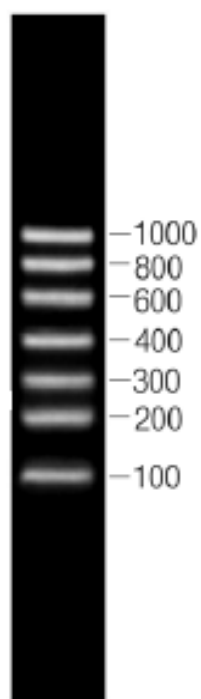


Figure B.3 RiboRuler™ Low Range RNA Ladder
(Fermentas UAB, Vilnius, Lithuania)

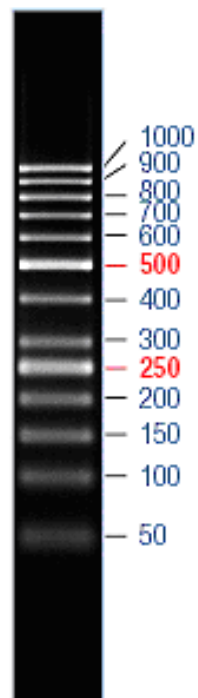


Figure B.4 GeneRuler™ and O'GeneRuler™ 50 bp DNA Ladder (50-1000 bp) (Fermentas UAB, Vilnius, Lithuania)

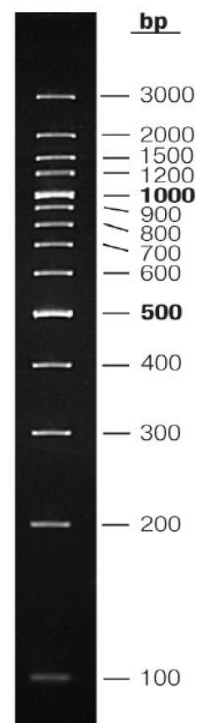


Figure B.5 GeneRuler™, 100 bp Plus DNA Ladder (Fermentas UAB, Vilnius, Lithuania)