Thermo and pH stable ATP-independent chaperone activity of heat-inducible Hsp70 from *Pennisetum glaucum*

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Heat shock proteins (Hsps) are a class of molecular chaperones that play an essential role in preserving cellular functions under stressful conditions. The over production of recombinant proteins often causes cellular stress that results in aggregation/misfolding of proteins, which sometimes leads to the formation of inclusion bodies. Here we report the cloning and characterization of heat-inducible PgHsp70 from *Pennisetum glaucum,* a heat and drought tolerant plant that showed stability and chaperone activity at elevated temperatures. The predicted amino acid sequence of PgHsp70 revealed a high homology with Hsp70 from other plants, and the overall 3D structure homology modeling is similar to that of the constitutively expressed bovine cytosolic Heat Shock Cognate (HSC)-70. The purified recombinant protein had an apparent molecular mass of 70 kDa and displayed optimal chaperone activity at 50°C, and pH 8.0. Under these conditions, the $T_{1/2}$ of PgHsp70 increased from 10 to 15 h in the presence of glycerol. The PgHsp70 exhibited a higher chaperone activity towards glutamate dehydrogenase than alcohol dehydrogenase. The expression of recombinant carbonic anhydrase (CA) in *E. coli* in a catalytically active soluble form rather than in inclusion bodies was made feasible by co-expression of PgHsp70. Circular dichroism (CD) studies of the recombinant PgHsp70 did not reveal any discernible changes in the α-helix content, with increase in temperature from 35 to 85°C, thus suggesting a critical role of α-helix content in maintaining the chaperone activity.

Introduction

All living organisms respond to sudden change in temperature, which causes the induction of a set of highly conserved proteins called heat shock proteins (Hsps).[1,2] These Hsps function as molecular chaperones and are essential for the cellular metabolic homeostasis.[3,4] As molecular chaperones, they ensure the stability of other cellular proteins and protect them from various physiological and environmental insults.[5,7] They have a broad range of functions ranging from the prevention of protein aggregation, refolding of misfolded proteins, degradation of unstable proteins and dissolution of protein complexes, besides some act as transcription factors.[4,8]

Hsps are found in different cellular compartments and have been broadly classified according to their molecular weights such as, Hsp-100, 90, 70, 60 and small Hsps. Hsp70 (DnaK-DnaJ-GrpE) and Hsp100 (ClpA and ClpB) chaperones have been shown to play a role in refolding and increased thermal stability of bacterial luciferases in *Escherichia coli*.[9] The Hsp70 belongs to a highly conserved family of heat shock proteins, is present in various sub-cellular compartments and helps in combating diverse forms of cellular stresses. It is known to protect various substrate proteins such as, citrate synthase, malate dehydrogenase, α-lactalbumin and insulin from thermal or chemical aggregation.[8,10] Eukaryotic Hsp70s conventionally have three functional domains: an N-terminal ATPase domain (~45 kDa), a substrate binding domain (~15 kDa), and a C-terminal domain (~25 kDa).[8] The C-terminal domain has a terminal sequence motif EEVD, which has been implicated in binding proteins such as Hop (Hsp70 Hsp90 organizing protein) through their tetratricopeptide motifs.[11]

Chaperones of some of the Hsp70, 60, 40 and small heat shock proteins are unable to refold their substrates without ATP.[12,13] In their ATP bound forms, Hsp70s bind and release substrates quickly, while, in the ADP bound state, substrate peptides are bound in a hydrophobic pocket covered by an α-helical lid, which must open to allow substrate binding or release.[14,15] The importance of α-helical lid in Hsp70 was explained clearly by Slepenkov et al.[16] The genes for various HSPs have been cloned and characterized and overexpression of some of these has resulted in developing transgenic plants showing tolerance to various stresses.[17,18] However, detailed biochemical analysis and the role of plant HSPs as chaperones have been investigated only in a few cases.[17,19-22] The overexpression of tobacco NtHSP70-1 confers

drought-stress tolerance in plants.[23] The expression of *AtHSP70* and *AtHSP101* genes was shown to be upregulated in calmodulin-binding protein phosphatase AtPP7 overexpression lines after heat shock, suggesting a possible role for AtPP7 in regulating the expression of (HSP) genes.[24] Recently a transcriptional profiling of Arabidopsis heat shock proteins and transcription factors revealed an extensive overlap between heat and non-heat stress response pathways.[25] In another study, Su and Li[26] have recently reported that Arabidopsis stromal Hsp70s [cpHsc70-1 (At4g24280) and cpHsc70-2 (At5g49910)] are essential for plant development and important for thermotolerance of germinating seeds.

Protein folding has been recognized as one of the central problems in biology.[8,27] Many proteins in a living cell will not fold properly without the assistance of molecular chaperones. The improper folding of linear amino acid sequences into non-native protein conformation leads to illegitimate interaction with other cellular components and subsequent protein aggregations.[8,27] The overexpression of recombinant proteins under artificially induced conditions puts an additional demand for protein folding and protein quality control system assisted by molecular chaperones.[8]

The present investigation deals with the in-vitro chaperone activity of a thermostable inducible Hsp70 (PgHsp70) from a heat tolerant *Pennisetum glaucum* (Pearl millet). The purified recombinant PgHsp70 was characterized for elucidating its structural and biochemical features. We have shown that the maintenance of alpha helix conformation is essential for the ATP-independent chaperone activity of PgHsp70. When the carbonic anhydrase (CA) gene from *Pennisetum glaucum* was overexpressed in *E. coli* using an isopropyl-β-D-thiogalactopyranoside (IPTG) inducible protein expression system, the recombinant protein aggregated into inclusion bodies. The simultaneous overexpression of PgHsp70 in the same cells, however, resulted in the production of catalytically active soluble CA.

Results and Discussion

Cloning and in silico analysis of PgHsp70. *Pennisetum glaucum*, a Poaceae family member, is a heat and drought-tolerant crop that grows at high temperatures (>45°C). During differential screening of stress induced subtractive cDNA libraries among many other genes, a set of HSPs was observed.[28] The C-terminus of Hsp70s from various species show highly conserved motifs like the cytosolic group is EEVD, for ER it is HDEL, for mitochondria it is PEAEYEEAKK and for plastids it is PEGDVIDADFTDSK.[35] HDEL motif in BiP is an ER retention signal. The EEVD motif in cytosolic Hsp70 is essential for interaction with Hsp70 interacting proteins such as Hip/p48 and p60/Hop.[36] Deletion of EEVD from a rat Hsc70 resulted in a dramatic increase in ATPase activity suggesting a role for EEVD in modulating ATPase activity of Hsc70.[37] The PgHsp70 has a signal peptide and lacks the EEVD motif, and this might be the property of the chloroplast homologues. The full length cDNA of PgHsp70 is 2,377 bp in length and contains an open reading frame of 2,039 bp and 128 bp 5' and 210 bp 3' untranslated regions (UTR) [Acc.No. **EF495353**]. The ORF encodes for a 649 amino acids long protein with an apparent molecular weight of 70 kDa and an estimated isoelectric point of 5.0. The deduced amino acid sequence of PgHsp70 [Pg] possessed an overall 88–90% amino acid sequence identity with chloroplast Hsps picked up from data base such as *Oryza sativa* [Os] (Acc.No. **ABA97211**, showing 88% similarity), *Pisum sativum* [Ps] (Acc.No. **CAA49147**, showing 90% similarity), *Cucumix sativus* [Cs] (Acc.No. **ABM92419**, showing 89% similarity) and *Arabidopsis thaliana* [At] (Acc.No. **NP194159** are showing 88% similarity) (**Fig. 1**). The phylogenetic tree shows protein sequence variation among various plant Hsps.

The PgHsp70 and bovine template sequence (1YUW) share a similarity of about 52%. The target PgHsp70 was modeled from sequence 48 to 589, based on sequence alignment shown in **Figure 2**. A close inspection of the comparative model (**Fig. 3**) and the pair wise alignment helps in identifying important residues involved in interdomain interactions and residues important for the protein function. As expected, the overall predicted 3D structure is similar to the bovine HSP 70,[32] and an exposed linker connects the two domains, Substrate Binding Domain (SBD) and Nucleotide Binding Domain (NBD) (**Figs. 2 and 3A–C**). The PgHsp70 SBD is formed by sequence 431–589 and NBD is formed by sequence 48–424 and the linker is formed by a relatively shorter sequence 424–430 (red colored C_α trace). Substrate binding site is formed by 574–579 (Green C_α trace). The interaction between the domains is stabilized by a number of interacting residues. There is a salt bridge between R559 and D191. A salt bridge corresponding to 1YUW (bovine), K325:E530 is absent in PgHSP70, as the residue corresponding to K530 is substituted in PgHSP70 by K565. The interaction is also stabilized by long range ionic interactions like among E255 and R452 as well as hydrophobic interactions between the residues: V253, F254, V256, V550, V554 and A557. The overall comparison of the template with PgHSP70 displays conservation of important residues in space.

Expression profile of PgHsp70 gene under different stress conditions. To establish detailed expression data of *PgHsp70*, a comprehensive RT-PCR (reverse transcriptase polymerase chain reaction) analysis was carried out from RNA samples isolated from different stress (temperature, cold, drought, ABA [abscisic acid], salicylic acid [SA] and salt) treated *Pennisetum glaucum* seedlings at various time points. The control seedlings were not subject to any stress. The relative transcript changes and those which showed at least more than two-fold changes as compared to the control condition were taken as significant. **Table 1** represents the summarized data of biochemical and physical stress, which revealed that in all other stresses, except heat stress and SA treatment, the level of transcript was decreased. However, in the case of heat stress, the transcript level of *PgHsp70* was upregulated upto 10- to 15-fold (**Table 1**).

Production of recombinant PgHsp70 protein. The PgHsp70 was overexpressed in *E. coli* BL21 (DE3) after addition of IPTG in the late exponential and early stationary phases of growth. The protein profile of the *E. coli* BL21 (DE3) carrying the pET-PgHsp70 was analyzed by 10% SDS-PAGE gels. Analysis of crude bacterial extracts revealed high levels of expression of an approximately 70 kDa recombinant protein in *E. coli* BL21 (DE3) carrying the pET-PgHsp70 construct. Further analysis

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Cs     1 MGASTAQIHGLGAPSFAAASMRK-SNNVSS-----RSVFFGQKLG-NSSAFPAAAFLNLRSNTSRRNSSVRPLRIVNEKVVGIDLGTTNSAVAAMEGGKPTIVTNAEGQR 103
Ps     1 MASSAQIHGLGTASFSSLKKPS-SISGNS-----KTLFFGQRLNSNHSPFTRAAFPKLSSKTFKKG---FTLRVVSEKVVGIDLGTTNSAVAAMEGGKPTIITNAEGQR 100
At     1 MASSAAQIHVLGGIGFASSSSSKRNLNGKGGTFMPRSAFFGTRTGPFSTPTSAFLRMGTRNGGGASRYAVGPVRVVNEKVVGIDLGTTNSAVAAMEGGKPTIVTNAEGQR 110
Os     1 ASFTSQLGAMACGAAPSTSPLAARSG--------QLFVGRKPA------AASVQMRVPRAGRARG---VAMRVACEKVVGIDLGTTNSAVAAMEGGKPTVITNAEGQR  92
Pg     1         MATTTIPT-----------STPFFTAHHG-----PRRS-RPSVSAAVYSRSRRWRPLRVACEKVVGIDLGTTNSAVAAMEGGKPTIVTNAEGAR  77
                   .              *   .           .*.  *******************.  .***** *

Cs   104 TTPSVVAYTKNGDRLVGQIAKRQAVVNPENTFFSVKRFIGRKMSEVDEESKQVSYRVVRDENGNVKLECPAIGKQFAAEEISAQVLRKLVDDASKFLNDKVTKAVTVPA 213
Ps   101 TTPSVVAYTKNGDRLVGQIAKRQAVVNPENTFFSVKRFIGRKMSEVDEESKQVSYRVIRDDNGNVKLDCPAIGKSFAAEEISAQVLRKLVDDASKFLNDKVTKAVVTPA 210
At   111 TTPSVVAYTKSGDRLVGQIAKRQAVVNPENTFFSVKRFIGRKMNEVDEESKQVSYRVVRDENNNVKLDCPAINKQFAAEEISAQVLRKLVDDASRFLNDKVTKAVITVPA 220
Os    93 TTPSVVAYTKGGERLVGQIAKRQAVVNPENTFFSVKRFIGRKMAEVDDEAKQVSYHVVRDDNGNVKLDCPAIGKQFAAEEISAQVLRKLVDDASKFLNDKITKAVVTVPA 202
Pg    78 TTPSVVAYTKTGDRLVGQIAKRQAVVNPENTFFSVKRFIGRKMNEVDEESKQVSYRVVRDDNGNVKLDCPAIGKQFAAEEISAQVLRKLVDDASKFLNDKVTKAVTVPA 187
         *********** *.********************************.***.*.****.*.**.*.****.****.* *.************.*****.****.****

Cs   214 YFNDSQRTATKDAGRIAGLEVLRIINEPTAASLAYGFEKKSNETILVFDLGGGTFDVSVLEVGDGVFEVLSTSGDTHLGGDDFDKRIVDWLAANFKRDEGIDLLKDKQAL 323
Ps   211 YFNDSQRTATKDAGRIAGLEVLRIINEPTAASLAYGFERKNNETILVFDLGGGTFDVSVLEVGDGVFEVLSTSGDTHLGGDDFDKRVVDWLAGDFKRDEGIDLLKDKQAL 320
At   221 YFNDSQRTATKDAGRIAGLEVLRIINEPTAASLAYGFDRKANETILVFDLGGGTFDVSVLEVGDGVFEVLSTSGDTHLGGDDFDKRVVDWLAAEFKKDEGIDLLKDKQAL 330
Os   203 YFNDSQRTATKDAGRIAGLEVLRIINEPTAASLAYGFEKKNNETILVFDLGGGTFDVSVLEVGDGVFEVLSTSGDTHLGGDDFDKKVVDWLASNFKKDEGIDLLKDKQAL 312
Pg   188 YFNDSQRTATKDAGRIAALEVLRIINEPTAASLAYGFEKKNNETILVFDLGGGTFDVSVLEVGDGVFEVLSTSGDTHLGGDDFDKRIVDWLAGTFKNDEGIDLLKDKQAL 297
         ****************** ********************..* *****************************************.***** ** **************

Cs   324 QRLTETAEKAKMELSSLTQANISLPFITATADGPKHIETTITRAKFEELCSDLLDRLKTPVENSLRDAKLSFKDIDEVILVGGSTRIPAVQELVKKMTGKEPNVTVNPDE 433
Ps   321 QRLTETAEKAKMELSSLSQTNISLPFITATADGPKHIETTLTRAKFEELCSDLLDRLRTPVENSLRDAKLSIKDIDEVILVGGSTRIPAVQELVKKLIGKDPNVTVNPDE 430
At   331 QRLTEAAEKAKIELSSLTQTNMSLPFITATADGPKHIETTLTRAKFEELCSDLLDRVRTPVENSLRDAKLSFKDIDEVILVGGSTRIPAVQELVRKVTGKEPNVTVNPDE 440
Os   313 QRLTEAAEKAKMELSTLSQTNISLPFITATADGPKHIETTLSRAKFEELCSDLIDRLKTPVTNALRDAKLSVDNLDEVILVGGSTRIPSVQELVKKITGKDPNVTVNPDE 422
Pg   298 QRLTEAAEKAKMELSSLTQTNISLPFITATADGPKHIETTLTRAKFEELCSDLLDRLKTPVDNALRDAKLQFKDIDEVILVGGSTRIPAVQELVKKITGKDPNVTVNPDE 407
         *****.*****.***.*.*.*.****************..*********** ***.**.*** *.******  .***********.*****.*. **.*********

Cs   434 VVALGAAVQAGVLAGDVSDIVLLDVSPLSLGLETLGGVMTKIIPRNTTLPTSKSEVFSTAADGQTSVEINVLQGEREFVRDNKSLGSFRLDGIPPAPRGVPQIEVKFDID 543
Ps   431 VVALGAAVQAGVLAGDVSDIVLLDVSPLSLGLETLGGVMTKIIPRNTTLPTSKSEVFSTAADGQTSVEINVLQGEREFVRDNKSLGSFRLDGIPPAPRGVPQIEVKFDID 540
At   441 VVALGAAVQAGVLAGDVSDIVLLDVTPLSIGLETLGGVMTKIIPRNTTLPTSKSEVFSTAADGQTSVEINVLQGEREFVRDNKSLGSFRLDGIPPAPRGVPQIEVKFDID 550
Os   423 VVSLGAAVQGGVLAGDVKDVVLLDVTPLSLGLETLGGVMTKIIPRNTTLPTSKSEVFSTAADGQTSVEINVLQGEREFVRDNKSLGSFRLDGIPPAPRGVPQIEVKFDID 532
Pg   408 VVALGAAVQAGVLSGDVSDIVLLDVTPLSLGLETLGGVMTKITPRNTTLPTSKSEVFSTAADGQTSVEINVLQGEREFVRDNKSLGSFRLDGIPPAPRGVPQIEVKFDID 517
         **.****** ***.***.*.*****.*** ************** *********************************************************

Cs   544 ANGILSVTAIDKGTGKKQDITITGASTLPSDEVERMVSEADKFAKEDKEKRDAIDTKNQADSVVYQTEKQLKELGDKVPGPVKEKVESKLGELKEAISGGSTEAIKEAMA 653
Ps   541 ANGILSVAAIDKGTGKKQDITITGASTLPGDEVERMVSEAERFSKEDKEKREAIDTKNQADSVVYQTEKQLKELGEKVPAPVKEKVEAKLGELKEAITGGSTQTIKDALA 650
At   551 ANGILSVSAVDKGTGKKQDITITGASTLPKDEVDQMVQEAERFAKDDKEKRDAIDTKNQADSVVYQTEKQLKELGEKIPGEVKEKVEAKLQELKDKIGSGSTQEIKDAMA 660
Os   533 ANGILSVAAIDKGTGKKQDITITGASTLPKDEVERMVEADKFAQEDKEKRDAIDTKNQADSVVYQTEKQLKELGDKVPAPVKDVKAKLNELKEAIAGGSTQSMKDAMA 642
Pg   518 ANGILSVTAVDKGTGKKQDITITGASTLPKDEVERMVDEAERFAKEDKEKRDAIDTKNQAESVIYQTEKQLKELGDKVPSDVKGKVKSKLQELKDAVAGGSTQMMKDALS 627
         *******.*.****************** ***..**.**..*.***.********.**.************.*.* **.** ** ***.. ***..*.*..

Cs   654 ALNQEVMQLGQSLYNQP---GAGAAPGPG-ASSESGPSESTGK-GPEGDVIDADFSDSK   707
Ps   651 ALNQEVMQLGQSLYNQP---GAAGQAGPTPPGSESGPSESSGKEGPEGDVIDADFTDSK   706
At   661 ALNQEVMQIGQSLYNQPGAGGPGAGPSPGGEGASSGDSSSSKG-GDGDDVIDADFTDSQ   718
Os   643 ALNEEVMQIGQAMYNQQ-PNAGAAGPTPG---ADAGPTSSGGKGPNDGDVIDADFTDSN   697
Pg   628 ALNQEVMQLGQSLYSQQ--GAPGASPTPG--ADATGSPEKPGG---EGDVIDADFTDSK*  680
         ***.****.**..* *          *      .*            *******.**
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Figure 1. Alignment of HSP70 proteins from selected crop species: Protein sequences were analyzed using DNA analysis software DNASTAR. For protein alignment, Clustal V of MegAlign was used in which clusters were aligned as pairs, then collectively as sequence groups to produce the overall alignment. Comparison of PgHsp70 with deduced amino acid sequences of chloroplast of *Oryza sativa* (accession no. ABA97211), *Pisum sativum* (accession no. CAA49147), *Cucumix sativus* (accession no. ABM92419) and *Arabidopsis thaliana* (accession no. Np194159).

revealed that the majority of the recombinant protein was in the soluble fraction (**Fig. 4A**). The recombinant hexa-histidine-tagged PgHsp70 protein was purified by affinity chromatography on a Ni-NTA column in a single step, which yielded ≥95% purity. The HPLC analysis showed one dominant peak (retention time: 30 min) (**Fig. 4B**).

Chaperone activity of PgHsp70. The recombinant PgHsp70 was assayed for chaperone activity by studying its ability to prevent thermal unfolding of glutamate dehydrogenase (GDH) and alcohol dehydrogenase (ADH). In thermal denaturation experiments, the protein substrates, GDH and ADH were protected efficiently by recombinant protein PgHsp70 but not efficiently by BSA (**Fig. 5A and B**) as assayed by light scatter measurements. On a comparative basis for the largest binding capacity for heat-denatured proteins and its early refolding property, ADH proved to be a better affinity substrate for chaperone activity of PgHsp70 as compared to GDH. These data also suggest that if the PgHsp70 protein is expressed with transit peptide and without an Hsp70 escort protein, it can also be active in contrast to the previous reports.[38-40]

PgHsp70 chaperone activity (in-vitro) was not affected by the addition of ATP in the reaction mixture thus suggesting that recombinant PgHsp70 is independent of ATP for its chaperone activity (**Fig. 5C**). Here it is to note that there were no nucleotides present in the buffer in which the PgHsp70 was dissolved. Similar findings have been reported in *Borrelia burgdorferi* for Hsp60 and 70.[41] Earlier, Cho and Bae[21] have also reported ATP-independent thermo-protective activity of tobacco Hsp70. The tobacco Hsc18, however, showed inhibition in the chaperone activity in the presence of ATP.[10] PgHSP70 was much better than BSA in terms of chaperone activity (**Fig. 5**).

Although thermostability is an intrinsic property dictated by the primary structure of a protein, many external factors including ions influence the thermostability and chaperone activity. Majority of heat sock proteins (e.g., Hsp70) are active and stable at slightly acidic or neutral pH.[37] However, PgHsp70 was found to be active over a broad pH range (**Fig. 6A**), which is nearly similar to that reported from Hsp70 of *Plasmodium falciparum*.[38] The recombinant PgHsp70 was stable at pH 8.0 for 10 h at 50°C. Approximately 98% of the residual activity was recorded when

Nucleotide binding domain

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TARGET  48          VG IDLGTTNSAV AAMEGGKPTI VTNAEGARTT PSVVAYTKTG
1yuwA    1  MSKGPA--VG IDLGTTYSCV GVFQHGKVEI IANDQGNRTT PSYVAFTDT-
                 ** ****** * *   .   .  ** *  ..* .* *** ** **.* *

TARGET  90  DRLVGQIAKR QAVVNPENTF FSVKRFIGRK MNEV--DEES KQVSYRVVRD
1yuwA   48  ERLIGDAAKN QVAMNPTNTV FDAKRLIGRR FDDAVVQSDM KHWPFMVVND
            .**.*. **    *  .** **  *  **.***.  ..   .. *. .. ** *

TARGET 138  -DNGNVKLDC PAIGKQFAAE EISAQVLRKL VDDASKFLND KVTKAVITVP
1yuwA   98  AGRPKVQVEY KGETKSFYPE EVSSMVLTKM KEIAEAYLGK TVTNAVVTVP
               .* ..   .   * * .* *.*. ** *.  . * .*   **.**.***

TARGET 187  AYFNDSQRTA TKDAGRIAAL EVLRIINEPT AASLAYGFEK K--NNETILV
1yuwA  148  AYFNDSQRQA TKDAGTIAGL NVLRIINEPT AAAIAYGLDK KVGAERNVLI
            ******** * ***** **.* .********* **..***..* *   . .*.

TARGET 235  FDLGGGTFDV SVLEVGDGVF EVLSTSGDTH LGGDDFDKRI VDWLAGTFKN
1yuwA  198  FDLGGGTFDV SILTIAAGIF EVKSTAGDTH LGGEDFDNRM VNHFIAEFKR
            ********** *.* .. *.* ** **.**** ***.***.*. *. .. . **

TARGET 285  DEGIDLLKDK QALQRLTEAA EKAKMELSSL TQTNISLPFI TATADGPKHI
1yuwA  248  KHKKDISENK RAVRRLRTAC ERAKRTLSSS TQASIEIDSL YEGID----F
             *.   .* .*..** *  *.** *** **..* .     *  .

TARGET 335  ETTLTRAKFE ELCSDLLDRL RTPVDNALRD AKLQFKDIDE VILVGGSTRI
1yuwA  294  YTSITRARFE ELNADLFRGT LDPVEKALRD AKLDKSQIHD IVLVGGSTRI
             *.***.** ** .**.     **..**** ***. .*  . ..********
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Linker domain

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TARGET 385  PAVQELVK-K ITGKDPNVTV NPDEVVALGA AVQAGVLSGD VSDIV----L
1yuwA  344  PKIQKLLQDF FNGKELNKSI NPDEAVAYGA AVQAAILSGD KSENVQDLLL
            * .* *.    . **. * .. **** ** ** ****..**** *. *      *

TARGET 430  LDVTPLSLGL ETLGGVMTKI TPRNTTLPTS KSEVFSTAAD GQTSVEINVL
1yuwA  394  LDVTPLSLGI ETAGGVMTVL IKRNTTIPTK QTQTFTTYSD NQPGVLIQVY
            *********. ** ***** .   ****.**   .. *.* .* * .* * *

TARGET 480  QGEREFVRDN KSLGSFRLDG IPPAPRGVPQ IEVKFDIDAN GILSVTAVDK
1yuwA  444  EGERAMTKDN NLLGKFELTG IPPAPRGVPQ IEVTFDIDAN GILNVSAVDK
            .***   .** .  ** * ** ********** *** ****** ***.*.****

TARGET 530  GTGKKQDITI TGAS-TLPKD EVERMVDEAE RFAKEDKEKR DAIDTKNQAE
1yuwA  494  STGKENKITI TNDKGRLSKE DIERMVQEAE KYKAEDEKQR DKVSSKNSLE
             .***   *** ***   *.*. ..****.*** ..  **   * * . .** *
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Substrate binding domain

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TARGET 579  SVIYQTEKQL K
1yuwA  544  SYAFNMKATV E
            *  .    .
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Figure 2. Sequence alignment of PgHsp70 and protein sequence corresponding to the template (1YUW). The domain regions such as nucleotide binding, linker and substrate binding are boxed and marked. Stars and dots in the sequences indicate the positions of identical amino acids and semi conserved substitutions, respectively.

the recombinant PgHsp70 was exposed to pH 4.0 and pH 10.0 for 6 and 7 h, respectively (**Fig. 6B**).

PgHsp70 chaperone activity enhanced consistently with an increase in temperature from 35 to 45°C, and thereafter remained consistent (90%) up to 85°C, before showing a slight decline at 95°C (**Fig. 6C**). Thus PgHsp70 exhibits very high thermostability. Half-life values of the PgHsp70 at 50° and 60°C were 10 and 8 h, respectively (**Fig. 6D**), which are similar to those reported for thermophilic catalysts of some bacteria and fungi.[42]

Secondary structure and thermostability. The contribution of aromatic residues in maintaining the secondary structure of proteins is well documented.[43] In the context of PgHsp70, it is of particular interest to study the role of the aromatic residues at N-terminal e.g., Tyrosines [(Y) at 31, 35, 132, 188 and 222] and Tryptophans [(W) at 37 and 277] for their possible role in stability and chaperone activity.

Modifications of crucial amino acids such as Y and W in the substrate binding site are believed to bring about important changes in their chaperone activity as reported earlier by Bernd Bukau (www.zmbh.uni-heidelberg.de/Bukau/images/Bukau.pdf). With a view to probe into the nature of molecular forces that stabilize PgHsp70, the structural investigation of PgHsp70 was carried out in the presence of perturbants of different kinds: acrylamide (neutral), I⁻ (anionic), Cs⁺ (cationic) and denaturants Gdn.HCl, SDS and urea. The spectrum of native PgHsp70 indicated the presence of 80% {α}-helix, 5.1% {β}-turn, and 14.4% remainder (analyzed by Dichroweb software see in supplementary data). The secondary structure of PgHsp70 appeared entirely different from that reported for Bovine Hsp70, which had 15% {alpha}-helix, 24% {β}-sheet, 24% {beta}-turn, and 38% remainder. High percent of α-helices of PgHsp70 makes it very different from other reported Hsps. It, however, showed some similarity to the Salmonella Hsp70, which consists of 85% α-helices.[44] The coiled α-helices structure in proteins confers a variety of functional capabilities;[45] this property thus might be responsible for the thermostability of PgHsp70 (>45°C).

In order to understand the charge, stability effect of PgHsp70 (in the presence of 0.2 mM KI), a 15% decrease in α-helix content was recorded. The KI treatment resulted in the slight loss of both chaperone activity (**Fig. 7A**) and secondary structure (**Fig. 7B**). Charged quenchers are known to probe only the surface-exposed amino acid residues, while the polar and uncharged acrylamide can diffuse into the protein core and change the conformation.[46] A decrease (10%) in the α-helix of PgHsp70 was observed at 0.1 mM acrylamide, and further addition resulted in only minimal changes. Surprisingly, there was no quantitative change in the chaperone activity of the PgHsp70 even at high concentration of acrylamide (0.5 mM) (**Fig. 7C**). As suggested by Eftink and Ghiron,[46] we have concluded that, the acrylamide interacts by means of simple collision process and does not apparently interfere with the protein substrate-binding site of PgHsp70. The addition of different concentrations of CsCl (0.1–0.5 mM) to the reaction mixture also had only slight effect on both secondary structure (10% decreases in α-helix) and chaperone activity of PgHsp70 (results not shown).

Gdn.HCl is known to interact reversibly with the carboxyl groups of amino acids.[47] At low concentrations, Gdn.HCl (0.1 mM) is believed to shield the carboxyl groups in the
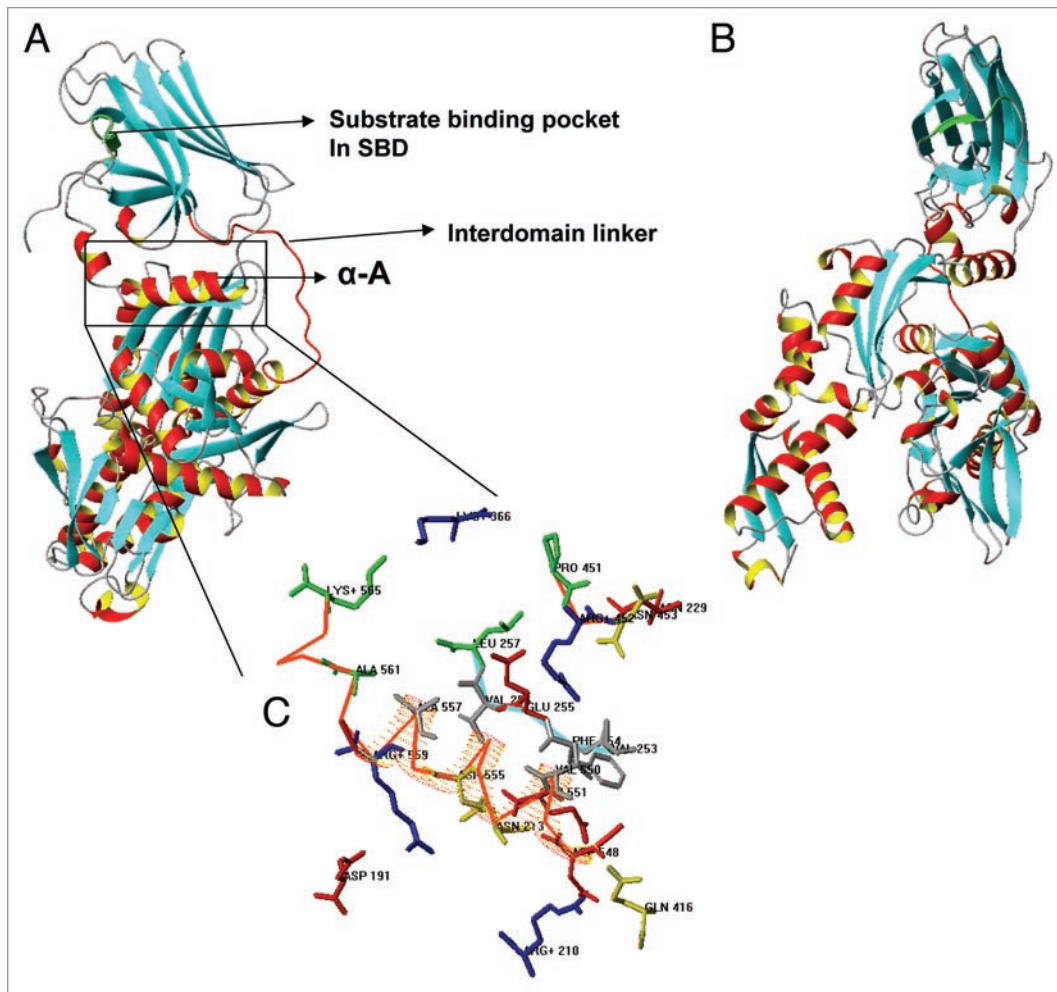
Figure 3. (A and B) Ribbon representation of PgHsp70 comparative model, Helix A highlighted in a box. The region above Helix A corresponds to the substrate binding domain (SBD) and the region below, to the nucleotide binding domain (NBD). Interdomain linker represented as red C_α trace. (C) Detailed view of interactions between SBD (C_α trace in red orange) and NBD (C_α trace in Cyan) and few discontinuous residues without C_α trace, side chains of residues identical or conserved to the template in blue, red, yellow or dark grey for +, -, polar, or nonpolar. PgHsp70 residues not conserved with respect to the template residues are shown in green.

Table 1. The relative fold change of Pg*Hsp70* transcript under different abiotic stress conditions

Gene	Control (water, rt)	Heat (48°C)	Drought (withholding water, 34 h)	Salt (250 mM NaCl)	Cold (4°C)	ABA (100 μM)	SA (1 mM)
PgHsp70	NS	+++	-	NS	--	-	+

The fold change between 2 to 5 are given a single (+) or (-) sign for upregulation and downregulation respectively; Similarly, (--) denotes 5 to 10 fold decrease, (+++) denotes 10- to 15-fold increase and NS denotes for not significant change and in the expression values; rt, room temperature.

vicinity of amino acid residues in the chaperone active site. As the Gdn.HCl concentration was increased, significant loss of conformational occurred (40% decrease in α-helix content and increase in random coil) (**Fig. 8A**), and also the chaperone activity decreased at 0.1 to 0.5 mM Gdn.HCl (**Fig. 8B**). Stabilization of secondary structure of PgHsp70 was carried out by incorporating glycerol (0.001% v/v) to a fixed concentration (0.1 mg ml⁻¹) of PgHsp70 and recording the conformational changes and chaperone activity of PgHsp70 (**Table 2**); this confirmed the protective nature of glycerol against denaturation

[Gdn.HCl (0.5 mM)]. Glycerol is thought to interact non-covalently with proteins and bring about protection against denaturation with Gdn.HCl. In a similar manner, Hsp70 chaperon activity was found to be stabilized by addition of ADP.[38] The CD spectra in the presence of urea clearly revealed the unfolding of PgHsp70, which led to a 40% decrease in α-helix content (**Fig. 9A**). These conformational changes occurring in PgHsp70 could be responsible for a decline in the chaperone activity (**Fig. 9B**). A 38% inactivation of PgHsp70 chaperone activity was observed at 0.5 mM urea. These experimental results suggest that
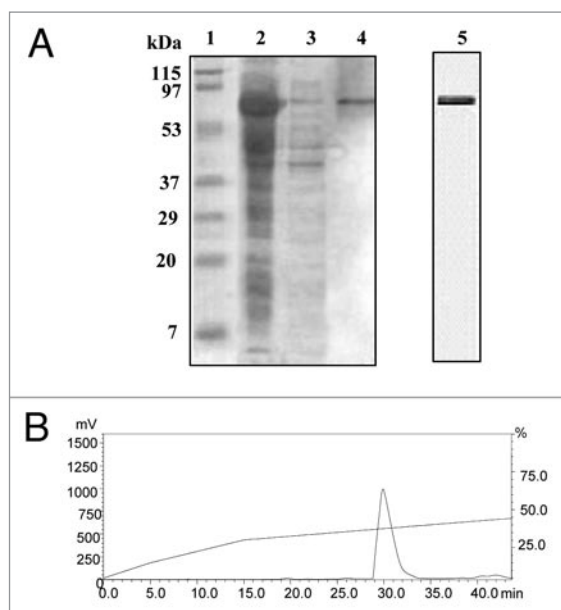
Figure 4. (A) SDS-PAGE analysis of purified recombinant PgHsp70 protein. PgHsp70 carrying a N-terminal hexahistidine tag was overproduced in pET-28a(+) and purified by Ni^{2+} NTA affinity chromatography. Lane 1 is molecular mass markers; the sizes (in kDa) are indicated adjacent (left side) to the gel. Lane 2 IPTG-induced supernatant fraction containing enriched PgHsp70 protein (70 kDa). Lane 3 is un-induced protein PgHsp70, Lane 4 is Ni$_{2+}$ NTA purified PgHsp70. The gel containing lanes 1–4 is stained with Coomassie Brilliant Blue. Lane 5: the purified PgHsp70 protein was run on another gel and silver stained. (B) Reconfirmation of PgHsp70 purity by the reverse-phase-HPLC analysis (detector: 280 nm) on C18 (Phenomenex, C18, 5 µM I.D. 250 x 4.6 nm) using aceto-nitrile (0.1% TFA)/water (0.1% TFA) gradient 0–15 min at the rate of 2% min^{-1} solvent; 15 to 45 min 0.5% min^{-1} solvent at a flow rate of 1.0 ml min^{-1}.
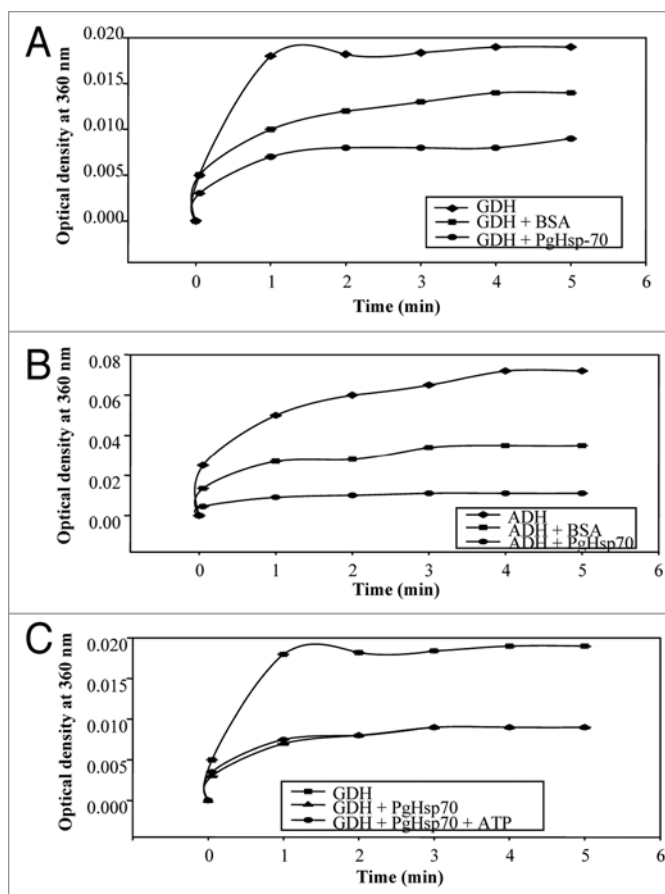

Figure 5. Thermally induced aggregation of model proteins such as glutamate dehydrogenase/or alcohol dehydrogenase with/without PgHsp70 at 50°C was Light scatter assayed by incubating the reaction mixture (1 ml) containing 20 mM Tris buffer (pH 8.0), 100 mM sodium chloride, 0.2 mg ml^{-1} glutamate dehydrogenase/or alcohol dehydrogenase and 0.2 mg ml^{-1} of the recombinant heat shock protein PgHsp70 (with or without ATP 5 mM) or BSA (0.2 mg ml^{-1}) aggregation was monitored by the measuring the O.D. at 360 nm. (A) Light scatter assay was done using substrate protein GDH to assess the chaperone activity of PgHsp70 in the presence and absence of BSA (B) Light scatter assay was done using substrate protein ADH to assess the chaperone activity of PgHsp70 in the presence and absence of BSA. (C) Light scatter assay was done using substrate protein GDH to assess the chaperone activity of PgHsp70 in the presence and absence of ATP. The error bars have not been added in this figure because the SD in all the cases was less than 10%.

hydrophobic aromatic residues, located in the protein-binding site may be involved in maintaining the secondary structure as observed in other proteins of Bacillus sp.[48] The CD spectra in the presence of SDS clearly revealed that the PgHsp70 is slightly changed in conformation (80% α-helix, 20% unordered) (**Fig. 10A**) and its chaperone activity (**Fig. 10B**).

The excitation spectrum of native PgHsp70 showed λ_{max} excitation at 325 nm, when emission was fixed at 340 nm (data not shown). When the fluorescence spectra of PgHsp70 were recorded as a function of pH, the fluorescence intensity did not change with shifting of pH from neutral to acidic and basic range (**Fig. 11**). As the pH was changed from 8 to 10 and 8 to 4, there was no significant change in the α-helix content (**Table 2**) and further the chaperone activity of the PgHsp70 was not affected at extreme pH. These observations also suggested that by preserving the secondary structure the chaperone activity can be retained.

The PgHsp70 chaperone activity enhanced with an increase in temperature from 35 to 45°C, and thereafter it changed slightly (**Fig. 6C**). This suggested that the chaperone activity of PgHsp70 is retained even at elevated temperatures.

PgHsp70 helps solubilization of recombinant carbonic anhydrase. Carbonic anhydrases are zinc-containing enzymes catalyzing the reversible hydration of CO_2. They are abundant in plants and algae, where they are essential for photosynthetic CO_2 fixation.[49] When we expressed carbonic anhydrase in *E. coli*, the partially folded protein intermediates were found to aggregate into inclusion bodies. Inclusion bodies produced in cytosol of all bacterial expression systems are composed of densely packed denatured protein molecules in the form of particles.[27] Refolding of inclusion body proteins into biologically active forms requires treatment with some conventional chemical agents such as Tween-20, 40, 60 and 80; high concentration of urea and Triton X-100.[27] However, these treatments are highly costly, time consuming, and the recovery is low. We were able to obtain the recombinant carbonic anhydrase in soluble fraction when it
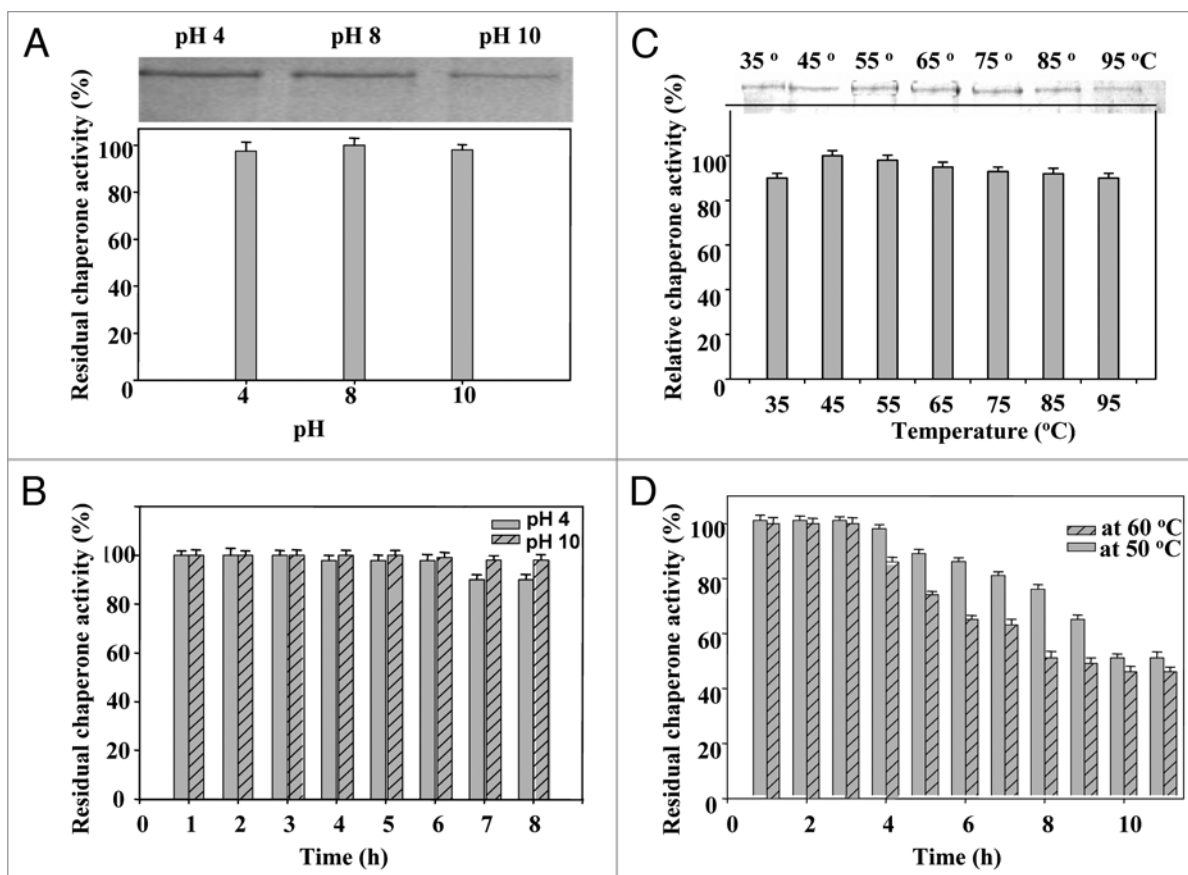
Figure 6. Effect of pH (A) on chaperone activity of PgHsp70; (B) on stability of PgHsp70, (C) effect of temperature on chaperone activity (experimental conditions as mentioned in Fig. 5), and (D) temperature stability of PgHsp70.

was co-expressed along with a molecular chaperone PgHsp70 (**Fig. 12**). These results clearly suggest that simultaneous co-expression of molecular chaperones like PgHsp70 can meet the additional demand for protein folding support and protein quality control system, under conditions when *E. coli* molecular chaperone system alone is unable to cope with the rate of recombinant carbonic anhydrase protein synthesis under IPTG induced condition. The recombinant carbonic anhydrase protein was available as a soluble fraction from 100 µl of *E. coli* lysate. It is shown to have conserved enzymatic activity determined at 220 U ml^{-1}.

Materials and Methods

Substrates and components for the chaperone assay. Glutamate dehydrogenase, alcohol dehydrogenase, KI, CsCl, acrylamide, urea, SDS, p-nitrophenylacetate and Gdn.-HCl were purchased from Sigma (USA), Ni^{2+}-NTA resin (Qiagen). Buffer solutions were prepared with ultra-high quality water (ELGA UGQ, UK). All other chemicals were of analytical grade. *Pennisetum glaucum* seeds (line 863B; a drought tolerant parental line) were procured from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. Seeds were surface sterilized and then germinated as described earlier.[28]

Construction of *P. glaucum* cDNA Library. A cDNA library was constructed from 5 µg of Poly A RNA in Uni-ZAP XR vector

using Zap-cDNA synthesis kit (Stratagene, La Jolla, CA) following the manufacturer's protocol. The resulting phage library contained 1 x 10^9 plaque forming units per milliliter. Sequence analysis of one of the clones identified a full-length cDNA that showed a highest (99%) with BLASTx chloroplast-Hsp70 of *Cucumix sativus* [Cs] (Acc. No. **ABM92419**) homology to the PgHsp70 (Acc.No. **CD726127**).

Cloning, expression and purification of recombinant PgHsp70. To clone and express PgHsp70 in *E. coli* as a N-terminal His-tagged recombinant protein of a prokaryotic expression vector, pET-28a(+) was used for PCR amplification of coding region. Two oligo nucleotides were designed; one for the N-terminus region, 5'-CGA GTC CAT ATG GCC GCG AAG GGA GAC GG-3' and the other for the C-terminus region, 5'-CTA GGA TCC TTT AGT CGA CTT CCT CGA TCT TG-3' of PgHsp70. The 5' and 3' untranslated regions were removed and an *Nde I* site at the translation initiation site and a *Not I* site just downstream of the translation termination codon were introduced. Using these primers the complete coding sequence for the PgHsp70 was PCR amplified (150 ng of each primer, 200 mM dNTPs, 2.5 units Taq DNA polymerase in a 50 µl reaction; 94°C 1 min; 55°C 1 min and 72°C 1 min; 30 cycles) using total cDNA as template. The amplified product was gel purified, digested with *Nde I* and *Not I* and cloned into *Nde I* and *Not I* sites of pET-28a(+) expression vector.
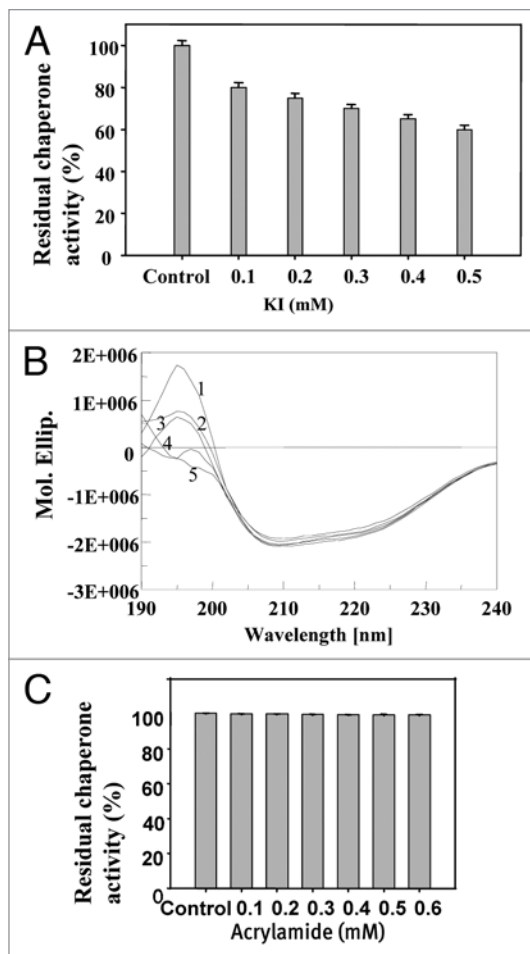
Figure 7. Effect of anion charger KI: (A) on chaperone activity of native PgHsp70 in 0.1 M phosphate buffer (pH 8.0) at 50°C for 5 min. (B) on secondary structure of PgHsp70 [spectra 1 & 2–5 represents native PgHsp70, different concentration of KI (0.2–0.5 mM) with native PgHsp70] (C) Effect of acrylamide on chaperone activity experimental conditions as mention in Figure 5. The error bars have not been added in this figure because the SD in all the cases was less than 10%.

Figure 8. Effect of inhibitor Gdn.HCl: (A) on secondary structure of PgHsp70 [spectra 1–5 represent PgHsp70, PgHsp70 with different concentrations of Gdn.HCl (0.2 to 0.5 mM)], (B) on chaperone activity of PgHsp70 (experimental conditions as mentioned in Fig. 5).

Overnight bacterial culture of *E. coli* BL21 transformed with the respective expression plasmids were inoculated to fresh LB broth containing 50 μg ml⁻¹ kanamycin and incubated at 200 rpm and 37°C to obtain an optical density of 0.7. at 600 nm. The production of recombinant protein PgHsp70 was induced in the bacterial cells by the addition of IPTG to a final concentration of 1 mM in the culture broth. After 3 h of incubation the bacterial cells were harvested by centrifugation at 8,000 xg for 10 min at 4°C. The bacterial cell pellet was resuspended in lysis buffer containing 20 mM Tris (pH 8.0), 100 mM sodium chloride and lysed by treatment with 0.5 mg ml⁻¹ lysozyme for 30 min at 37°C followed by sonication. The lysate was cleared by centrifugation at 8,000 xg for 20 min and then supernatant loaded onto Ni-NTA column pre-equilibrated with lysis buffer containing 5 mM imidazole. The column was washed with lysis buffer, followed by elution of protein fractions with increasing concentrations of imidazole (50, 100, 150 mM) in the lysis buffer (50, 100 an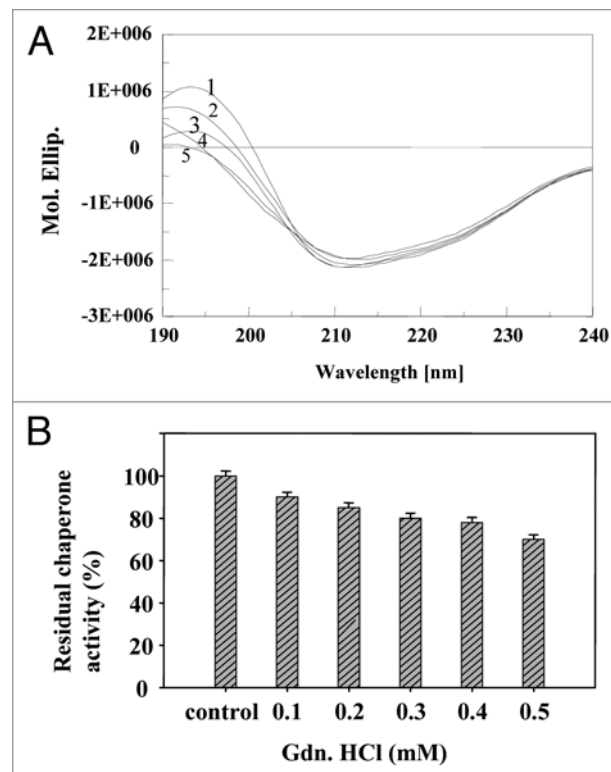d 150 mM). The collected fractions were analyzed by measuring the optical density at 280 nm and this was followed by SDS-PAGE analysis (we have loaded the same amount (0.1 mg ml⁻¹) of protein loaded on each lane). The purified PgHsp70 was dialyzed extensively, with at least four or five changes of dialysis buffer (0.1 mM phosphate buffer, pH 8.0) to remove Tries. The concentration of PgHsp70 protein was estimated by using bovine serum albumin (BSA) as the standard according to the standard method of Bradford.

Real-time PCR analysis of Pghsp70 transcript. The real time quantitative PCR amplification of Pghsp70 along with Pg-β tubulin as an internal housekeeping standard was performed with specific oligonucleotide primers (FORWORD-5'-CGC ATT GAA CCA GGA AGT CAT G-3', REVERSE-5'-CCC CTC ATT TGC TGT CGG TAA-3'), using the first strand cDNA synthesized from RNA samples collected from 12-d old Pennisetum seedlings exposed to different stress [heat (48°C), salt (250 mM), drought (withholding water up to 34 h), cold (4°C), ABA (100 μM) and SA (1 mM)] along with control seedlings, in the presence of SYBR-GreenR using Icycler (BioRad, USA). At the end of the PCR cycles, the products were put through a melt curve analysis to check the specificity of PCR amplification. The experiments were repeated at least five times independently and the data were averaged. The relative change in expression levels of Pghsp70 in response to different stress conditions and application of ABA and heat stress was estimated using REST software.[29] with β-tubulin as a reference gene.

Table 2. Effect of different physical (temperature and pH) and chemical agents (denaturants and inhibitors) on secondary structure of PgHsp70

Effect	α-helix	3/10 helix	Parallel β-sheet	Anti-parallel β-sheet	Beta turn	Unordered	Total
Control (PgHsp 70)	0.582	0.222	-0.001	0.000	0.051	0.144	0.996
Temp. 35°C–85°C	0.582	0.222	-0.001	0.000	0.051	0.144	0.996
pH (4, 8 and 10)	0.582	0.222	-0.001	0.000	0.051	0.144	0.996
PgHsp 70 + Gdn.HCl (0.1 mM)	0.069	0.138	0.172	0.069	0.190	0.363	1.000
PgHsp 70 + Gdn.HCl (0.1 mM) + glycerol (0.001%)	0.582	0.222	-0.001	0.000	0.051	0.144	0.996
PgHsp 70 + KI (0.1 mM)	0.006	0.086	0.300	0.161	0.110	0.340	1.000
PgHsp 70 + acrylamide (0.1 mM)	0.542	0.262	-0.001	0.000	0.051	0.144	0.996
PgHsp 70 + Urea (0.1 mM)	0.059	0.238	0.223	0.223	0.044	0.214	1.000

HPLC analysis. The protein PgHsp70 (0.1 mg ml^{-1}) sample was filtered through 0.22 μm filter (Pall Gellman Lab, Ann Arbor, MI) before applying onto the column with an auto sampler. The reverse-phase-HPLC analysis (detector: 280 nm) on C18 (Phenomenex, C18, 5 μμ 1.D. 250 x 4.6 μm) using solvent of aceto-nitrile (0.1% TFA)/water (0.1% TFA) G gradient column for 0–15 min at the rate of 2% min^{-1} solvent; 15 to 45 min at the rate of 0.5% min^{-1} flow rate of 1.0 ml min^{-1}.

Chaperone activity assay of PgHsp70 by spectrophotometry and densitometry. The ability of PgHsp70 to prevent the thermal aggregation of protein substrates such as glutamate dehydrogenase and alcohol dehydrogenase at 50°C temperature was assayed by incubating the reaction mixture (1 ml) containing 20 mM Tris buffer (pH 8.0), 100 mM sodium chloride, 0.2 mg ml^{-1} glutamate dehydrogenase or alcohol dehydrogenase and 0.2 mg ml^{-1} of the recombinant heat shock protein PgHsp70 (with or without ATP 5 mM) or BSA (0.2 mg ml^{-1}). The assay was carried out according to the method of Jaenicke and Rudolph[30] using a Peltier-controlled cell and the aggregation of the protein substrates monitored by measuring the light scatter over different time intervals at 360 nm in a JASCO-810 Spectropolarimeter. Protein aggregation was also measured by determining the amount of protein that remained soluble or pelleted at 10,000 xg for 30 min at 50°C, followed by SDS-PAGE to determine the relative amounts of the GDH or ADH that remained in solution or aggregated in the presence or absence of the recombinant PgHsp70.

100% chaperone activity means: The activity which was performed at 50°C by incubating reaction mixture 1 ml containing of 20 mM Tris buffer (pH 8.0), 100 mM sodium chloride, 0.2 mg ml^{-1} glutamate dehydrogenase and 0.2 mg ml^{-1} of the recombinant heat shock protein PgHsp70 (without ATP) or BSA and any denaturants considered as control of 100% chaperone activity.

Effect of temperature and pH on chaperone activity of PgHsp70. Effect of temperature on the recombinant PgHsp70 chaperone activity was assayed at pH 8.0 (phosphate buffer) by pre-incubating PgHsp70 at various temperatures (35–95°C) for 2 h, followed by relative PgHsp70 chaperone activity, was determined by using the reaction mixture incubated at 50°C. The effect of pH on chaperone activity of PgHsp70 was evaluated by pre-incubating the PgHsp70 in different 0.1 M buffers [Glycine-
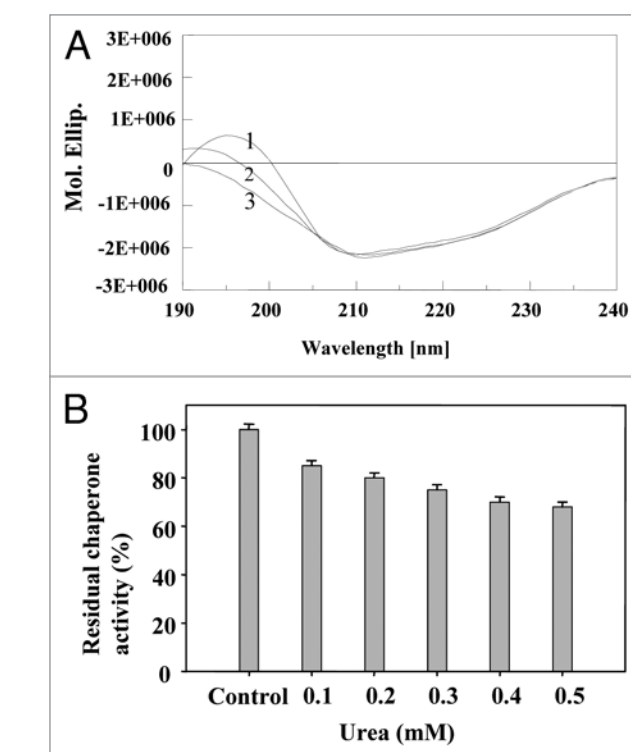
Figure 9. Effect of urea: (A) on secondary structure of PgHsp70 spectra 1–3 represents different concentrations of urea (0.2 to 0.4 mM) with PgHsp70 (B) on Chaperone activity of native PgHsp70 (experimental conditions as mentioned in Fig. 5).

HCl, pH 4.0; phosphate (K_2HPO_4-KH_2PO_4), pH 8.0; Glycine-NaOH, pH 10.0] for 2 h and relative PgHsp70 chaperone activity was determined by using the reaction mixtures at pH 8.0 for 5 min.

Thermostability, half-life and pH stability of PgHsp70. Thermostability and half-life of PgHsp70 were determined by incubating 5 ml of the suitably diluted (dilutions of 0.2 mg ml^{-1} were prepared in 0.1 M phosphate buffer at pH 8.0) protein sample at 50°C (with/without glycerol 0.001%), 90 and 80°C over a period of 10 h, and subsequently assayed for chaperone activity at 50°C. The pH stability of PgHsp70 (0.2 mg ml^{-1}) was
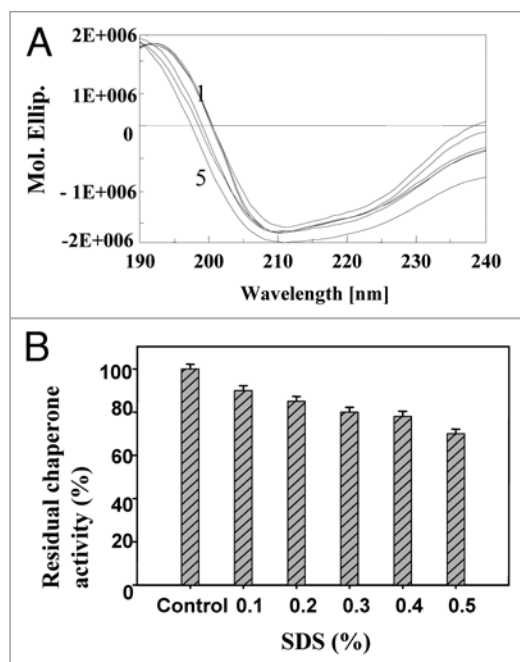
Figure 10. Effect of SDS: (A) on secondary structure of PgHsp70 [spectra 1–5 represents PgHsp70 and different concentration of SDS (0.2 to 0.5%) with PgHsp70 (B) on chaperone activity (experimental conditions as mentioned in Fig. 5).
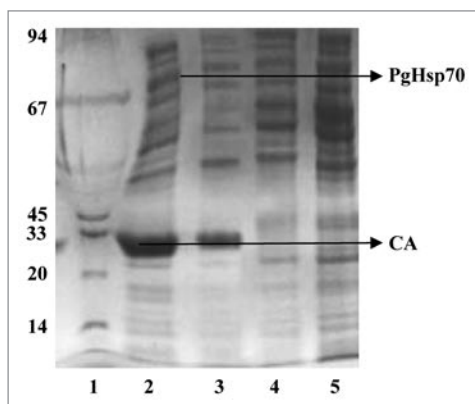

Figure 12. Coomassie brilliant blue-stained SDS-PAGE gel. Electrophoretic fractionation of simultaneous overexpression of PgHsp70 along with CA prevents the aggregation and accordingly prevents the formation of inclusion bodies in *E. coli*: Lane 1 is molecular weight of marker lane; the sizes (in kDa) are indicated adjacent (left side) to the gel. Lane 2 is co-expression of CA with PgHsp70 showing the high level of overproduction of CA in supernatant after after 3 h induction with IPTG (IPTG induced supernatant fraction). Lane 3 is induced CA in the pellet fraction (without PgHsp70); i.e., without co-expression the CA protein was going to pellet fraction. Lane 4 is a pellet fraction of only PgHsp70. Lane 5 is pellet fraction after co-expression of CA with PgHsp70; the CA and PgHsp70 proteins are not appearing. Lanes 4 and 5 were run in another gel and Coomassie Brilliant Blue stained.
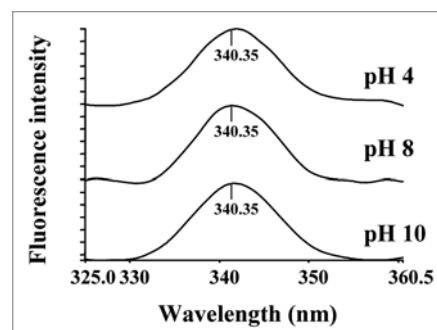
Figure 11. Effect of pH (A) on fluorescence emission spectra of PgHsp70 at 325 nm excitation.

Secondary structure determination of PgHsp70. The secondary structure of the purified PgHsp70 (0.1 mg ml^{-1} prepared in 0.1 M phosphate of pH 8.0) was determined by CD spectroscopy carried out at 45°C on a JASCO-810 Spectropolarimeter equipped with Peltier controlled thermostat cell holder (PTC-423S). The path length of cuvette used was 0.2 cm. Temperature scans were performed by exposing the PgHsp70 to different temperatures (35 to 85°C) and the changes in the structural conformation were recorded at a scanning rate of 20 nm min^{-1} from 190 to 240 nm (the significant changes in the CD spectrum were analyzed in the region of 207 to 222 nm). The CD signal was converted into total molar ellipticity [θ], and the secondary structure of the purified PgHsp70 was determined by using the average of 10 scans with blank-subtraction and plotting as per Sreerama and Woody[31] (Dichroweb software; London, UK).

Effect of KI, urea, SDS, Gdn-HCl and pH on the secondary structure and chaperone activity of PgHsp70. The purified PgHsp70 (The concentration of protein 0.1 mg ml^{-1} prepared in 0.1 M phosphate of pH 8.0) was titrated at room temperature against different concentrations of KI, urea, Gdn.HCl and SDS. The changes in the structural conformation by CD and chaperone activity of PgHsp70 were recorded. The effect of pH on PgHsp70 was studied by using different pH buffers (Glycine-HCl for pH 4.0; phosphate for pH 8.0; Glycine-NaOH for pH 10.0) and changes in the secondary structural conformation of the protein and its in-vitro chaperone activity were recorded.

Effect of pH on PgHsp70 by fluorescence measurements. The steady-state fluorescence measurements were performed in a Hitachi F-4500 fluorescence spectrofluorometer at room temperature using a slit width of 5/5 nm (exc/emi) with a slow scan of 15 nm min^{-1} and quartz cells of 1 cm path length. Fluorescence emission spectra were recorded at 340 nm by using excitation wavelengths of 325 nm for the tryptophan (W) and tyrosine (Y) fluorophore. The protein samples of PgHSP70 were prepared in different 0.1 M buffer systems (Glycine-HCl, pH 4; phosphate [K_2HPO_4-KH_2PO_4] pH 8.0; Glycine-NaOH pH 10.0) and changes in their intrinsic fluorescence intensity were recorded at 280 nm.

Co-expression of PgHsp70 with carbonic anhydrase. Initially the carbonic anhydrous coding region was cloned in *Nde* I and *Bam*H I sites of pET14b the resultant construct expressed the recombinant carbonic anhydrase in *E. coli* which completely

determined by subjecting it to different pH values (0.1 M acetate buffer, pH 4.0 and 5.0; 0.1 M phosphate buffer, pH 8.0; and 0.1 M Tris-glycine buffer, pH 10.0) over a period of 7 h, and subsequently assayed for chaperone activity at 50°C and pH 8.0.

partitioned into inclusion bodies. The same recombinant carbonic anhydrase expression cassette from pET14b was PCR amplified along with T7 promoter and T7 terminator using the following oligonucleotide forward 5'GCG GCC GCA TCA TGG CGA CCA CAC CCG TCC-3' and reverse primer 5'GAG CTC CGA AGT GGC GAG CCC GAT CT3' synthesized based on the sequence information of pET14b vector. The *Not* I and *Sac* I restriction enzyme sites were introduced in the primers. The PCR amplified carbonic anhydrous expression cassette was re cloned into *Not* I and *Sac* I sites of pET28a in which PgHsp70 gene was already cloned into *Nde* I and *Not* I sites as mentioned earlier. The recombinant carbonic anhydrase expression cassette from pET14b starting from T7 promoter to T7 terminator was PCR amplified using the following oligonucleotide primers with *Not* I and *Sac* I restriction enzyme sites (*Not* I: 5'GCG GCC GCA TCA TGG CGA CCA CAC CCG TCC-3' and *Sac* I: 5'GAG CTC CGA AGT GGC GAG CCC GAT CT3') and re cloned into *Not* I and *Sac* I sites of pET28a in which PgHsp70 gene was already cloned into *Nde* I and *Not* I sites as mentioned earlier. The simultaneous overexpression of both recombinant proteins was induced in *E. coli* for two hours in the presence of 1 mM IPTG. The presence of both recombinant carbonic anhydrase as well as recombinant HSP70 proteins was analyzed both in the soluble and insoluble fractions of the *E. coli* lysate.

Assay for carbonic anhydrase. The carbonic anhydrase activity was determined by the method of Armstrong et al.[32] by incubating the reaction mixture containing 1.0 ml of 5 mM p-Nitrophenyl acetate prepared in 0.1 M phosphate buffer (pH 8.0) with 0.2 ml of appropriately diluted carbonic anhydrase (0.2 mg ml^{-1}) for 5 min at room temperature, and determining the hydrolyzed p-Nitrophenylacetate at 348 nm. One carbonic anhydrase unit is defined as one m mole of p-nitrophenylacetate hydrolyzed ml^{-1} min^{-1} under the assay conditions.

Homology modeling structure determination. The 3D structure of PgHsp70 was generated using first approach mode of the comparative modeling tools available at the Swiss model server. The PDB crystal structure (1YUW, 3) of a bovine HSC70 (amino acids: 1–554, resolution 2.6 Å). Jiang et al.[33] was used as template to generate the comparative protein model. The molecular graphics were generated using MOLMOL.[34] All the experiments were performed in three times and mean of the three values with SD within 10% was taken.

Conclusions

The data presented in this study clearly suggests that Hsp70 from *Pennisetum glaucum* is a thermostable protein and retains its chaperone activity at higher temperatures. By comparing specific structural property in relation to the chaperone activity of PgHsp70, it was found that there was no change in the α-helix content or the chaperone activity with the increase in temperature (45–85°C); this hinted a critical role of α-helix in maintaining its chaperone activity. Though thermostable in nature, PgHsp70 showed an overall 3-D structure similar to that of bovine's HSC-70 and exhibit positive ability to refold and help express recombinant protein (e.g., CA) in soluble form rather than in the inclusion bodies in *E. coli*. Finally the chaperone activity of PgHSP70 on different protein substrates, high thermostability, response to broader pH range and ATP-independent in-vitro chaperone activity reflect on its novel properties and suggest that it may be a good candidate for cell homeostasis at high temperature stress. Overall, these features will have important implications on the expression of recombinant proteins in soluble and active form.

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