Screening of Neem extracts for microbial anti-chaperone activity by employing in vitro enzyme refolding assay

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Abstract Microbial heat shock proteins (Hsps) play an important role in pathogenesis and development of resistance to existing drugs. New compounds that target microbial molecular chaperones have the potential of combating the challenge of anti-microbial resistance. The present study was aimed at assessing the employment of in vitro enzyme refolding assay to detect anti-chaperone activity of Neem (*Azadirachta indica*) extracts. Protein extracts of thermotolerant *Escherichia coli* cells were used as a source of Hsps or chaperones. Thermotolerance was found to be induced by pre-treating *E. coli* cells at 47 °C before subjecting them to a lethal temperature of 55 °C. This thermotolerance correlated with over-expression of specific proteins and reduced aggregation as evident from the SDS-PAGE profiles. Refolding assays of denatured enzymes exhibited 45% activity regain in presence of cell protein extracts containing chaperones compared to less than 5% regain in BSA negative controls. The chaperone activity was found to be ATP dependent. Addition of Neem extracts to refolding reaction mixtures distinctly reduced the activity regain (20%) in a dose dependent manner (500 and 1000 ppm). The negative influence of plant extract on refolding of the enzyme in the presence of chaperones gives evidence to its anti-chaperone activity. We propose that the employment of in vitro enzyme refolding assays will help not only to analyze the activity of known and putative chaperones but also to screen natural compounds for anti-microbial-Hsp activity.

Keywords Chaperones · Hsps · Inhibition · Thermotolerance · *Azadirachta indica* · β -Galactosidase · Enzyme refolding

Introduction

The heat shock response is a universal phenomenon found in all living organisms and it is characterized by induction of heat shock proteins (Hsps), a class of proteins that includes chaperones, proteases and regulatory factors (Segal and Ron 1998). Molecular chaperones are involved in catalyzing the refolding of denatured proteins, assisting maturation of newly synthesized proteins and suppressing protein aggregation (Laksanalamai et al. 2001). Synthesis of Hsps is correlated with the development of acquired thermotolerance as well as tolerance to stresses other than heat in bacteria. Acquired thermotolerance refers to increased survival of organisms exposed to lethal temperatures on pre-exposure to near lethal temperatures for a short time (Trent 1996). It is interesting to note that the heat shock response plays a very important role in microbial pathogens. The induction of Hsps during the infection process has been reported in bacteria, protozoans and viruses. Hsps are said to constitute the first line of attack and consolidate pathogen virulence. Not only is the molecular chaperone machinery exploited in nearly all stages of pathogenesis, it is also involved in the development of anti-microbial drug resistance (Neckers and Tatu 2008). This substantiates the use of Hsps as targets for antimicrobial therapy. Novel small molecules that specifically target microbial chaperones could prove to be promising new generation therapeutic agents that can also address the problem of drug resistance. There have been

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great advances in the discovery of eukaryotic Hsp90 inhibitors in the past two decades and nearly 20 of these have entered clinical trials in the past few years (Patki and Pawar 2013). Not only the eukaryotic but the prokaryotic and protozoan homologue of Hsp90 has also been proposed as a potential drug target in several studies (Neckers and Tatu 2008; Garcie et al. 2016). However, studies exploiting the functional inhibition of other bacterial Hsps for therapeutic purposes are few. Although a lot is being discovered about chaperone biology and chaperone inhibition the potential of molecular chaperone inhibitors in the treatment of infectious diseases is yet unexplored. Recent proof-of-principle findings are reported by Kumar and Balbach (2017). In the present study, we have attempted to analyze the extracts of locally known medicinal plant, Neem (*Azadirachta indica*) for anti-Hsp activity in laboratory strains of *Escherichia coli*. Since acquired thermotolerance correlates with the elaboration of Hsps we pre-treated the bacterial strains to near lethal temperatures. The presence of Hsps in cell extracts of thermotolerant cultures was confirmed by SDS-PAGE. The crude protein extracts were then used in in vitro enzyme refolding assays along with plant extracts to assess the Hsp inhibitory potential of the plants.

Materials and methods

Bacterial strains

The bacterial strains were in-house cultures originally obtained from MCC, Pune and stored at -80°C in BHI broth supplemented with 10% glycerol.

Reagents

All chemicals were analytical grade or ultra-pure grade. Tris-HCl, PMSF (Phenylmethylsulfonyl fluoride), lysozyme, Glycine, Ammonium sulphate, ONPG (*o*-nitrophenyl β -D-galactopyranoside), RNaseI, DNaseI, lactose, BSA and ATP were purchased from SRL Pvt. Ltd. Neem tablets were purchased from Himalaya Pharmaceuticals. Geldanamycin was purchased from Sigma-Aldrich.

Thermotolerance and survival assays

Initial growth curve studies over a broad temperature range (30 – 47°C) indicated 37°C as optimum incubation temperature for the in house *E. coli* strains. Complete decline in survival was observed at 55°C which was considered as lethal temperature (data not shown).

Escherichia coli cells were grown aerobically at 37°C to an OD_{600} of 0.5 in LB broth. One ml of culture was

pipetted into Eppendorf tubes and placed at 55°C for desired time intervals. After each time point the tube was withdrawn and Colony forming units (cfu) were determined by serial dilution and plating on LB agar plates. For thermotolerance studies, the tubes were pre incubated at 47°C for 15 min followed by recovery at 37°C for 0, 1 and 2 h before being placed at 55°C . Survival was determined as the ratio of the cfu after treatment to the number of cfu at the zero time point (Delany 1990; Takai et al. 1998; Runde et al. 2014).

Preparation and SDS-PAGE analysis of crude heat shock protein extract

Heat treatment was carried out as described above. The protein extract preparation was entirely carried out as described by Runde et al. (2014). The cells were harvested after heat treatment, washed and then lysed in buffer A (50 mM Tris-HCl, 150 mM NaCl, pH 8). For lysing, buffer A was supplemented with 0.33 mg/ml lysozyme, 20 $\mu\text{g/ml}$ DNase I, 20 $\mu\text{g/ml}$ RNase I, 1 mM PMSF and 1% Triton X-100 and shaken vigorously at 750 rpm for 90 min at 37°C . A part of the supernatant of first centrifugation at $1000g$ (2 min 4°C) was separated and stored as Total cell extract (TCE). Second centrifugation of the remaining at $10,000g$ for 30 min at 4°C yielded the soluble protein fraction (SP). The pellets were washed again in buffer A with reducing concentration of Triton X-100 (1% at first wash and 0.5% at second) under constant rotation at 18 rpm at 4°C for 2 h. Following a centrifugation at $10,000g$ the insoluble protein aggregates were solubilised in rehydration buffer and used as protein aggregate fraction (PA).

The protein concentration in the three (TCE, SP and PA) fractions was determined by Lowry's method. Accordingly 500 ng of protein was loaded in each well. SDS-PAGE analysis of the TCE, SP and PA was carried out in 13% gel in standard Tris/glycine tank buffer (0.025 M Tris-HCl, 0.129 M Glycine, 0.1% SDS) at 100 V in Mini Protean II apparatus (Bio-Rad) (Laemmli 1970). Broad range (30–200 kDa; Sigma Aldrich) molecular weight markers were used as standard (Urban Chmiel et al. 2013).

Enzyme extraction and crude purification

Cells were harvested after 18 h of incubation in broth supplemented with 1% lactose (presence of a substrate like lactose optimizes growth conditions and the production of β -galactosidase enzyme by the bacteria) by centrifuging at $10,000g$ for 10 min at 4°C . The cell pellet was crushed and washed twice in 0.03 M Sodium phosphate buffer (pH 7), centrifuged at $10,000g$ for 10 min at 4°C . The pellets were re-suspended in 5 ml 0.2 M Sodium phosphate buffer

(pH 7). The cell suspensions were sonicated for 30 min using sonicator (100 watts; 33 kHz) (Dakshin Ultra-sonicator) in ice cold water. The extract was then centrifuged at 15,000g for 10 min at 4 °C. The supernatant obtained was stored at −20 °C to be used as enzyme extract for assay (Prasad et al. 2013). The cell extract was precipitated at 4 °C with ammonium sulphate (60–90%), dialyzed and suspended in Z buffer (40 mM Na₂HPO₄, 60 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM Mercaptoethanol, pH 7) (Miller 1972).

Enzyme assay

Protein concentration of the extract was determined by Lowry's method. β -galactosidase activity was determined by the method described by Hsu et al. (2005). 0.5 ml of crude enzyme extract was added to 0.5 ml (15 mM) ONPG (*o*-nitrophenyl β -D-galactopyranoside) in 0.05 M Sodium phosphate buffer (pH 7). Following an incubation period of 10 min at 37 °C, 2 ml of 0.1 M Sodium carbonate was added to stop the reaction and absorbance was measured at 420 nm (Shimadzu UV-1700 UV-Vis Spectrophotometer). One unit of β -galactosidase is defined as the amount of enzyme that produces one micro-mole (μ M) of *o*-nitrophenol per minute under assay conditions (Akolkar et al. 2006).

Preparation of plant extracts

Pure herb leaf extract of Neem (*A. indica*) in the form of tablets (250 mg per tablet) was purchased from Himalaya Pharmaceuticals. The tablets were crushed. The powder (100 g) was packed into a thimble of Whatman filter paper no. 1 soaked in 80% ethanol and subjected to Soxhlet extraction using the same solvent for three cycles, filtered and evaporated in rotary evaporator (Othman et al. 2012).

β -galactosidase denaturation

Extracted β -galactosidase was denatured by heat treatment at 60 °C for 20 min. Equilibrium or reversible unfolding can be achieved using this treatment (Ayling and Baneyx 1996; Kishore et al. 2012).

Enzyme refolding assay

The refolding assay was performed as described by Ayling and Banyex (1996) with modifications. Denatured and native β -galactosidase was diluted 100-fold in ice cold refolding buffer (100 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 10 mM KCl, 2.5 mM DTT and 0.01% Tween 20) in siliconized Eppendorf tubes containing buffer supplemented with BSA and ATP; protein extract of heat stressed

cells (Hsp) and ATP; protein extract of heat stressed cells (Hsp), ATP and Geldanamycin (GA); protein extract of heat stressed cells (Hsp), ATP and Neem extract (500 ppm); protein extract of heat stressed cells (Hsp), ATP and Neem extract (1000 ppm), protein extract of heat stressed cells (Hsp) without ATP; protein extract of unstressed cells (incubated at 37 °C) with ATP. The samples were kept on ice for 5 min and then transferred to room temperature. Aliquots were taken at various time points (0, 30, 60, 90, 120, 150, 180 and 210 min) and β -galactosidase activity assay was performed as described before. Results are expressed as activity relative to native β -galactosidase enzyme. Refer to Freeman et al. (2000) for refolding assays of other enzymes for the analysis of molecular chaperones.

Statistical analysis

In case of thermotolerance experiments the cfu were determined at each time point and survival was determined as a ratio of cfu value at each time point and the cfu value before transfer to lethal temperature (i.e. 0 time point). Arithmetic mean, standard deviation and standard error of survival were calculated. Mean values were plotted on logarithmic scale with the error bars displaying standard error.

For enzyme refolding assays percentage activity regain of denatured enzyme was calculated at different time points. Arithmetic mean, standard deviation and standard error were calculated. Mean values were plotted on linear scale with error bars showing standard error.

Differences in survival rates and percentage enzyme activity between the groups were tested for statistical significance by one way ANOVA test followed by post hoc LSD. All statistical values were considered statistically significant at a *p* value ≤ 0.05 . The statistical package used is SPSS, version 19 IBM Corporation, Somers, NY, USA.

Results and discussion

Pre-conditioning protected *E. coli* cells and improved survival at lethal temperatures: this thermotolerance correlated with reduced protein aggregation

To investigate development of acquired thermotolerance in *E. coli* we examined the survival of exponentially growing cells (37 °C) after a shift to lethal temperature (55 °C) with or without a 15 min pre-treatment (47 °C). The pre-shock protected the cells significantly against subsequent lethal temperature shock. The cells grown at the optimum temperature of 37 °C demonstrated an exponential decrease in

survival at 55 °C. However, cells that received pre-conditioning heat stress at 47 °C demonstrated reduced rate of decrease in survival at 55 °C exhibiting transient thermotolerance. The rate of decline in survival increased after 30 min at 55 °C. Highest thermotolerance was observed in cells that were pre-treated at 47 °C and allowed to recover at 37 °C for a period of 1 h (Fig. 1). Heat inducible thermotolerance has been described in several microorganisms. A similar improvement of resistance of cells to lethal temperature due to heat pre-treatment has been reported in other bacteria such as *Lactobacillus bulgaricus* (Teixeira et al. 1994), *Bacillus cereus* (Periago et al. 2002) and *Bacillus subtilis* (Völker et al. 1992).

There is strong evidence suggesting the involvement of Hsps in the induction of thermotolerance especially during heat stress. It was demonstrated that acquired thermotolerance in *L. bulgaricus* disappeared when protein synthesis was blocked (Gouesbet et al. 2001). Hombach et al. (2014) showed that small heat shock proteins are essential for thermotolerance in *Leishmania donovani*. In the present study it was found that heat shock clearly altered the protein profile pattern in *E. coli* compared to the control. Direct incubation at 55 °C led to increased protein aggregation as is evident in the protein aggregate fraction in SDS-PAGE (Fig. 2a). Heat shock at sublethal temperature at 47 °C followed by recovery at 37 °C for 1 h or more led to increased intensity of 90, 67, 44, 40 kDa bands and appearance of a 29 kDa band. This pre-treatment also clearly led to reduced protein aggregation (Fig. 2b). The temperature and recovery period also corresponds with the

Fig. 1 Survival of *E. coli* cells in thermotolerance experiments. A (45 °C-1 h); B (45 °C-2 h); C (45 °C-0 min); D (55 °C). Data expressed as normalized cfu values at each treatment. Each plotted point represents three trails expressed as mean $\pm$ SEM. Some error bars may be covered by plot symbols

Fig. 2 SDS-PAGE protein profiles of *E. coli* cells. Gels were stained with Silver stain. M molecular mass standard 30–200 kDa, TCE total cell extract, SP soluble protein fraction, PA protein aggregate fraction. **a** Protein profile of cells treated directly at 55 °C for 0, 15 and 30 min. **b** Protein profile of cells pre-conditioned at 47 °C for 15 min and allowed recovery at 37 °C for 1 h before placing at 55 °C for 0, 15 and 30 min

acquisition of thermotolerance as observed in the survival studies. The heat shock response of bacteria has been best characterized in *E. coli* (Lindquist 1986). The major heat shock proteins of *E. coli* include HtpG (approximately 98 kDa), DnaK (70 kDa), DnaJ (40 kDa), GroEL (60 kDa), GroES (11 kDa), GrpE (38 kDa) and proteases (Arsène et al. 2000). DnaK (Hsp70) best correlates with the development of thermotolerance (Lindquist 1986). Based on the molecular masses, the protein bands with increased intensity observed in this study could represent the heat shock proteins of *E. coli*. These proteins may have an important role to play in preventing aggregation of heat

unfolded proteins. Expression studies have clearly demonstrated overexpression or induction of most of these proteins during stresses (Maleki et al. 2016). Since exhibition of acquired thermotolerance correlates well with overexpression or induction of molecular chaperones in bacteria, chaperone proteins like Hsps should dominate crude cell extracts of thermotolerant cells. More so because Hsps are involved in the recovery of cells from applied stress. Therefore, in this study cellular extracts of thermotolerant cells that received pre-treatment at 47 °C and recovery at 37 °C for 1 h prior to lethal heat shock, were used in the enzyme refolding assays.

Presence of protein extracts of pre-conditioned *E. coli* cells caused refolding of heat denatured β -galactosidase indicating chaperone activity

Enzyme fraction precipitated at 40–60% ammonium sulphate showed maximum activity after dialysis (Table 1). Enzyme refolding assays have been commonly used to assess protein refolding properties of molecular chaperones in vitro (Freeman et al. 2000). As previously described, in vitro β -galactosidase refolding assay was employed to assess the activity of heat induced Hsps in cellular protein extracts. Time dependent recovery of enzyme activity was determined following denaturation. Use of Tris-HCl (pH 7.4) buffer for refolding may influence the β -galactosidase activity. Both dilution and change of buffer lead to lower activity range as compared to purified enzyme. Heat denaturation lead to complete loss of enzyme activity. Thermal denaturation studies of β -galactosidase indicate almost 68% loss of enzyme activity when placed for 5 min at 60 °C and less than 5% activity regain on incubation with 1.2 M GuHCl for 15 min (Kishore et al. 2012). Incubation of heat denatured enzyme with total cell extract assumed to contain Hsps caused 45% activity regain at 210 min. Less than 5% activity revival was observed in BSA controls (Fig. 3). Incubation with extracts of unstressed cells showed 20% activity regain. This could be due to certain baseline constitutive Hsps in the extract (Hristozova et al. 2016). Absence of ATP in the reaction

Fig. 3 Reactivation of heat denatured β -galactosidase enzyme measured against time. A (ATP + BSA); B (ATP + Protein extracts of thermotolerant cells-Hsps); C (ATP + Protein extracts of thermotolerant cells-Hsps + Geldanamycin); D (ATP + Protein extracts of thermotolerant cells-Hsps + Neem extract 500 ppm); E (ATP + Protein extracts of thermotolerant cells-Hsps + Neem extract 1000 ppm); F (ATP + Protein extract of unstressed cells incubated at 37 °C); G (Protein extracts of thermotolerant cells-Hsps without ATP). Each point represents three replicates presented as mean $\pm$ SEM. Some error bars may be covered by plot symbols

mixture seemed to stymie the refolding process with less than 6% activity regain. This indicates the predominant role of ATP dependent chaperones in refolding.

A recent study has demonstrated the application of in vitro enzyme folding assays to assess the activity of putative chaperones using total protein extracts of heat shocked *E. coli* cells (Hristozova et al. 2016). It has been demonstrated previously in *E. coli* that Hsps like ClpB and HtpG participate in cellular protein folding by assisting the DnaK-DnaJ-GrpE chaperone system (Thomas and Banyex 2000). Maintaining the integrity of cellular proteins during stress is a function attributed to HtpG in *E. coli* (Sato et al. 2010; Genest et al. 2011). Similarly GroEL has been

Table 1 Crude purification of β -galactosidase from *E. coli*

Purification step	Total enzymatic activity (U/ml)	Total protein (mg/ml)	Specific enzymatic activity (U/mg)	Recovery (%)	Purification fold
Crude extract	5.2	4.6	1.13	100	1
(NH ₄) ₂ SO ₄ precipitation					
10–20%	3.6	3.0	0.98	69.23	0.86
20–40%	4	2.8	1.42	76.92	1.25
40–60%	4.8	2.1	2.28	92.30	2.01
60–90%	3.8	2.5	1.52	73.07	1.34

reported to cause enzyme reactivation in in vitro studies using β -galactosidase as a model substrate (Ayling and Baneyx 1996). Other studies using MDH refolding assays have also shown chaperone dependent refolding (Mogk et al. 2003). In the present study the significant increase in enzyme activity regain observed in the reaction mixtures supplemented with cellular protein extracts (Hsps) relative to the BSA controls indicates that heat shock proteins are instrumental in enzyme refolding. Also all the chaperones such as HspG, DnaK, GroEL, ClpA and ClpB are known to be ATP dependent (Saibil 2013).

Neem extracts led to inefficient refolding of β -galactosidase in a dose dependent manner with results comparable to Geldanamycin

A few recent studies describe microbial heat shock proteins as drug targets. Hsp90 has been described as a drug target against protozoan infections (Pallavi et al. 2010). Rajan et al. (2014) modeled and characterized Hsp60 from *Chlamydomonas reinhardtii* as drug target. *In silico* identification of hot spots for inhibition of DnaK (Hsp70) which is involved in the pathogenicity and antibiotic resistance of *Acinetobacter baumannii* has been recently reported (Chiappori et al. 2015). However, not many studies have been reported on the analysis of therapeutic potential of Hsp/chaperone inhibitors in microbial infections. Microorganisms with well characterized molecular chaperone systems like *E. coli* can be used in the screening assays for chaperone inhibitors. In the present study β -galactosidase refolding assay was employed to test the anti-Hsp potential of Neem (*A. indica*) extracts. Gedunin a tetranortriterpenoid present in the Neem tree extract has been recently identified as Hsp90 inhibitor (Amolins and Blagg 2009). Geldanamycin was used in this study as it is a well-established inhibitor of eukaryotic Hsp90 and is known to interact with the ATP binding pocket of Hsp90. Gedunin differs in its mode of action from Geldanamycin. Patwardhan et al. (2013) have reported that Gedunin inactivates p-23 a co-chaperone of Hsp90. Neem is previously reported to have anti-microbial anti-diabetic, anti-inflammatory, anti-fungal, anti-oxidant, anti-cancer and hepato-protective properties. Safety analysis of Neem extracts in animal model and clinical studies report none or minimum toxicity, safe usage and good therapeutic potential at controlled dosage (Alzohairy 2016). In this study, 500 and 1000 ppm of Neem leaf extract was added to the refolding buffer in β -galactosidase refolding assay to test its potential to interfere with the chaperone activity. Effect of Neem on % activity regain of the enzyme was comparable with that of Geldanamycin and seemed to be dose dependent. The results indicate that the anti-microbial activity of Neem

could be attributed to its chaperone inhibitory effect. While the influence of Gedunin on eukaryotic Hsp90 is known its effect on microbial Hsp90 is yet to be elucidated. The effect of active components extracted from leaves of Neem on molecular chaperone proteins other than Hsp90 in microbes is not clear. Identification and characterization of the active components and study of its exact mode of action in chaperone inhibition will allow extended use of this traditionally known medicinal plant in treatment of infectious diseases.

Conclusions

The present study reports that the extract of the Indian Neem plant *A. indica* exhibits anti-Hsp property and holds potential to be a microbial Hsp inhibitor. Neem is already known to have medicinal value but its prospective as a chaperone inhibitor is not clear. Inhibition of Hsp90 by Neem extract seems to be possible as one of the active components of Neem namely Gedunin is a known Hsp90 inhibitor. However, discovery of other active components that modulate DnaK (prokaryotic Hsp70) activity or any other microbial chaperone activity would be interesting findings. Enzyme refolding assays can be rapid and high throughput preliminary screens for identifying potential drug candidates. Knowledge of bacterial chaperone functions other than enzyme refolding can also can be exploited to design similar screening assays.

The growing concern of antimicrobial resistance has compelled us to turn towards exploring novel drug targets and bacterial chaperones hold enormous potential considering their involvement in stress management and more importantly pathogenesis in microbes. Medicinal plants can be a good source of active compounds with anti-Hsp activity. The present report is an exploratory initiative into analyzing the utility of enzyme refolding assays in screening of natural compounds for Hsp inhibition.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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