LEA proteins prevent protein aggregation due to water stress

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LEA (late embryogenesis abundant) proteins in both plants and animals are associated with tolerance to water stress resulting from desiccation and cold shock. However, although various functions of LEA proteins have been proposed, their precise role has not been defined. Recent bioinformatics studies suggest that LEA proteins might behave as molecular chaperones, and the current study was undertaken to test this hypothesis. Recombinant forms of AavLEA1, a group 3 LEA protein from the anhydrobiotic nematode *Aphelenchus avenae*, and Em, a group 1 LEA protein from wheat, have been subjected to functional analysis. Heat-stress experiments with citrate synthase, which is susceptible to aggregation at high temperatures, suggest that LEA proteins do not behave as classical molecular chaperones, but they do exhibit a protective, synergistic effect in the presence of the so-called chemical chaperone, trehalose. In contrast, both LEA proteins can independently protect citrate synthase from aggregation due to desiccation and freezing, in keeping with a role in water-stress tolerance; similar results were obtained with lactate dehydrogenase. This is the first evidence of anti-aggregation activity of LEA proteins due to water stress. Again, a synergistic effect of LEA and trehalose was observed, which is significant given that non-reducing disaccharides are known to accumulate during dehydration in plants and nematodes. A model is proposed whereby LEA proteins might act as a novel form of molecular chaperone, or 'molecular shield', to help prevent the formation of damaging protein aggregates during water stress.

Key words: aggregation, anhydrobiosis, desiccation tolerance, late embryogenesis abundant (LEA) protein, nematode, water stress.

INTRODUCTION

LEA (late embryogenesis abundant) proteins were first characterized in cotton and wheat ([1], and references therein) and are produced in abundance during seed development, comprising up to 4% of cellular proteins [2]. Their expression is linked to the acquisition of desiccation tolerance in orthodox seeds, pollen and anhydrobiotic plants, but many LEA proteins are induced by cold or osmotic stress, by exogenous abscisic acid, or are even expressed constitutively, e.g. *dhnX* from *Arabidopsis thaliana* [3]. Their precise function is unknown, but they are assumed to protect cellular or molecular structures from the damaging effects of water loss; a number of putative mechanisms have been proposed, including hydration buffering, ion sequestration, direct protection of other proteins or membranes, or renaturation of unfolded proteins [1,4]. However, these suggestions for function are supported by relatively little evidence. However, there seems to be some effect on stress tolerance, since tomato, wheat and barley LEA proteins confer increased resistance to osmotic or freeze stresses when introduced into yeast ([5], and references therein), and a barley LEA protein improves tolerance to water deficit in transgenic rice [6] and wheat [7]; furthermore, *in vitro*, an algal LEA protein diminished freeze damage of the enzyme LDH (lactate dehydrogenase) [8].

Genes of LEA proteins have been identified in many plant species, and at least six different groups of LEA proteins have been defined on the basis of expression pattern and sequence; the major categories are group 1, group 2 and group 3 [1,4,9]. Group 1 LEA proteins, of which the wheat Em protein is the type sequence, have been further subdivided into two superfamilies by Wise [9], and are only found in plants. They are unstructured in solution [10], but contain a conserved 20-residue amino acid motif, most often in one copy [1]. Group 2 LEA proteins, which Wise [9] sug-

gests comprise three superfamilies, are also known as 'dehydrins' [11] and are mainly found in plants, including algae. Group 2 proteins are characterized by up to three sequence motifs, known as the K-domain (lysine-rich), the Y-domain (DEYGNP) and the S-segment (poly-serine stutter). Again, they are largely unstructured, although they show some α-helical content [12–14]. The group 3 LEA proteins, comprising two superfamilies [9], are characterized by a repeated 11-mer amino acid motif whose consensus sequence has been broadly defined as $\Phi\Phi E/QX\Phi KE/QK\Phi XE/D/Q$ (where Φ represents a hydrophobic residue) [15]. Interest in this group has been increased by the discovery of homologues in organisms other than plants, including the nematodes *Caenorhabditis elegans*, *Steinernema feltiae* and *Aphelenchus avenae*, and the prokaryotes *Deinococcus radiodurans*, *Bacillus subtilis* and *Haemophilus influenzae* [15–17]. The group 3 LEA protein from anhydrobiotic nematode *A. avenae* [18], and a putative example from bullrush [19], are natively unfolded in solution, but seem to become more structured on drying. A genetic study [20] in *D. radiodurans* supports a role for group 3 LEA proteins in desiccation tolerance, but, again, their precise function is unknown.

We have previously recognized the need for a mechanism to formulate reasoned hypotheses relating to LEA protein function, which can then be tested experimentally [21]. Novel bioinformatics [9] make predictions for the function of the various LEA protein groups and subgroups (or 'superfamilies', in the nomenclature of Wise), based on similarities in peptide profiles of the LEA proteins and other proteins of known function. For the group 1 LEA proteins of superfamily 6, of which wheat Em is a representative, and the group 3 LEA proteins of superfamily 2, which include the AavLEA1 protein from *A. avenae*, a molecular chaperone activity is suggested by bioinformatics [21]. In the present study, we test these predictions and show that, while neither of

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the LEA proteins behaves like a 'classical' molecular chaperone, they both possess anti-aggregation activity during water stress, and prevent protein inactivation under these conditions.

EXPERIMENTAL

Recombinant LEA proteins

Details of AavLEA1 recombinant protein production and purification have been described previously [18]. For the group 1 LEA protein, the Em cDNA sequence (a gift from Dr A. C. Cuming, University of Leeds) was amplified by PCR from the vector pMTK1b [22] using oligonucleotide primers containing engineered NdeI (5′-AGAGACATATGGCTTCCGGC) and BamHI (5′-TGCTTGCTAGGATCCCGGAT) sites. The sequence was cloned into pCR2.1-TOPO vector, cut out using the above enzymes and ligated into pET15b vector (Novagen, Madison, WI, U.S.A.). The plasmid was transformed into the *Escherichia coli* strain BL21(DE3). Expression of the protein with an N-terminal His$_6$-tag and purification were performed as described for the AavLEA1 protein [18]. Protein concentration was determined by A_{280} using a molar absorption coefficient of 1280/M/cm calculated with EXTCOEFF programme on Biology Workbench (http://workbench.sdsc.edu/).

Thermal aggregation assay

CS (citrate synthase; EC 4.1.3.7) from porcine heart was purchased from Sigma and dialysed against 50 mM Tris/HCl and 2 mM EDTA (pH 8.0). The concentration of CS was determined using a molar absorption coefficient of 1.78 for a 1 mg/ml solution at 280 nm at a 1 cm pathlength [23]. Light scattering was measured in a stirred quartz cell in a Cary Eclipse fluorescence spectrophotometer with a thermostatically controlled cell holder. To determine aggregation kinetics, 15 μM CS was diluted 200-fold in 40 mM Hepes/KOH (pH 7.5) and equilibrated at 43 °C. Both excitation and emission wavelengths were set at 500 nm, with a spectral bandwidth of 2 nm. Where the effect of proteins and other solutes on CS aggregation kinetics was to be determined, these were equilibrated in 40 mM Hepes/KOH (pH 7.5) at 43 °C, before the addition of CS. All experiments were performed three times.

Water-stress aggregation assay

Aggregation of CS or LDH was monitored by reading absorbance A at 340 nm in a DU800 spectrophotometer (Beckman Coulter, High Wycombe, Bucks., U.K.). For vacuum drying, 0.12 mg of porcine heart CS with or without protectant proteins (at molar ratios of 1:1, 1:2 and 1:10) was dried in 100 μl vol. in polystyrene tubes in a Dura-Stop™ microprocessor-controlled tray drier (FTS Systems) at 25 °C, 2000 mT for 1 h. All proteins were first dialysed overnight with several changes of distilled water to remove buffer salts. Before desiccation, samples were degassed by spinning under vacuum for 10 min in an Eppendorf concentrator 5301. Dried samples were rehydrated to a final volume of 170 μl for aggregation assay. For the cryoprotection assay, 170 μl of CS or LDH at a concentration of 200 μg/ml in polystyrene tubes was flash-frozen by immersion in liquid N$_2$ for 30 s and then thawed at ambient temperature. Assays were performed in triplicate and the appropriate buffer solution without a protectant was used as the blank.

CS activity

CS activity was assayed according to the method of Srere [24]. To determine the CS activity, 1 μl of CS/stabilizer (from the samples used in the aggregation assay) was added to 1 ml of
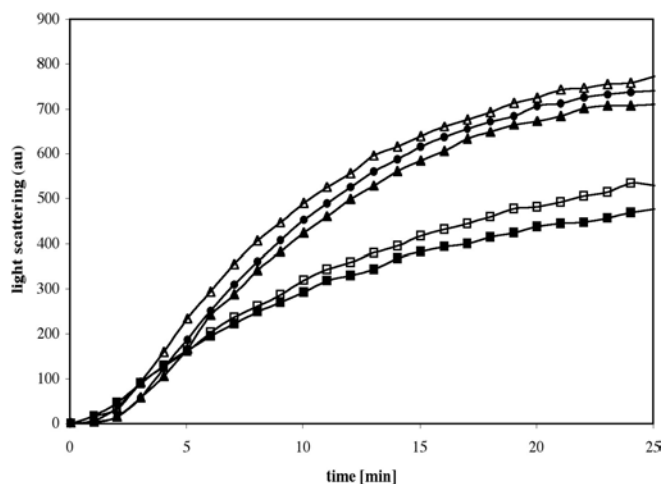
Figure 1 LEA proteins alone do not prevent heat-induced aggregation of CS

Aggregation kinetics of CS at 43 °C in the presence or absence of LEA proteins and a small heat-shock protein p26 are shown, as determined by light scattering (in arbitrary units 'au' on the *y*-axis) assayed by a fluorescence spectrophotometer. Key to symbols: CS (final concentration 75 nM) alone (●), CS plus group 1 LEA (1 μM, △), CS plus group 3 LEA (1 μM, ▲), CS plus p26 (75 nM, □) or CS plus p26 (150 nM, ■).

TE (Tris/EDTA) buffer with 100 μM oxaloacetic acid, 100 μM DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] and 150 μM acetyl-CoA (disodium salt). Change in A_{412} was measured every 1.5 s for 1 min. Enzyme activity was expressed as a percentage of the unfrozen/undried control rate. All samples were assayed in triplicate. Statistical significance was determined by Student's two-tailed t test.

LDH activity

LDH from rabbit muscle was obtained from Roche (Lewes, East Sussex, U.K.) and diluted in 100 mM sodium phosphate buffer (pH 6) following the manufacturer's recommendations. In a microfuge tube, 2 μl of 0.1 mg/ml LDH was added to a total of 50 μl of test stabilizer solution (25 mM Tris/HCl, pH 7) with or without stabilizing agents. Tubes were flash-frozen in liquid N$_2$ and thawed at ambient temperature, or vacuum-dried for 1 h at 25 °C in 2000 mT field in a Dura-Stop™μp freeze dryer and rehydrated immediately in 50 μl of water at ambient temperature. To determine enzyme activity, 2 μl of LDH/stabilizer solution was added to 1 ml of 100 mM sodium phosphate buffer (pH 6) with 100 μM NADH and 2 mM pyruvate. Change in A_{340} was monitored every 1.5 s for 1 min in a DU800 spectrophotometer (Beckman Coulter). All values given are expressed as percentage of rate of reaction measured for unfrozen or undried samples. All samples were assayed in triplicate.

RESULTS

LEA proteins alone do not prevent protein aggregation due to heat stress

The proposal that group 3 LEA proteins behave as molecular chaperones can be tested using a standard unfolding assay, with CS as a model substrate [25]. CS is a dimeric enzyme that is inactivated and forms aggregates at temperatures of 40 °C or above. Irreversible aggregation occurs spontaneously within minutes of incubation at high temperatures and reaches maximum levels after approx. 20 min (Figure 1). This aggregation is decreased

by a molecular chaperone such as the small heat-shock protein p26 from brine shrimp (*Artemia franciscana*) in a concentration-dependent fashion [26]. In contrast, no effect on CS aggregation was observed with either the group 1 LEA protein from wheat or the nematode group 3 LEA protein AavLEA1 at molar ratios up to 100, suggesting that LEA proteins alone are not capable of preventing substrate unfolding due to heat stress (Figure 1). Some molecular chaperones function in an ATP-dependent manner [27], and therefore in subsequent thermal aggregation experiments 2 mM ATP was added together with LEA proteins; other possible cofactors, including GTP, Mg^{2+} and Ca^{2+}, were also tested. However, no effect on CS aggregation was detected (results not shown).

LEA proteins can act synergistically with trehalose to prevent heat-induced protein aggregation

In nematodes undergoing anhydrobiosis, the non-reducing disaccharide trehalose is accumulated in large quantities [28]; desiccation-tolerant brine shrimp cysts are also known to contain high concentrations of this sugar [29]. Trehalose has been described as a 'chemical chaperone' ([30,30a]; also see [31] for comments on this terminology), since it is known to stabilize *in vitro* proteins and other biological structures against damage due to a number of stresses ([32,33]; and references therein). Trehalose has been shown previously to prevent heat-induced CS aggregation [26] and was also effective in our experiments (Figure 2A). Plants do not usually produce significant quantities of trehalose, but desiccation-tolerant seeds typically contain large amounts of another non-reducing disaccharide, sucrose [34]. Therefore in both animals and plants, desiccation tolerance is associated with accumulation of both sugars and LEA proteins. Although LEA proteins by themselves do not prevent CS aggregation at high temperatures, we tested whether they might have some protective activity in the presence of trehalose. When CS is incubated at 43 °C in 400 mM trehalose, aggregation is almost fully repressed; however, lower concentrations of trehalose, e.g. 100 mM, provide partial repression (Figure 2A). Interestingly, when either the group 1 or group 3 LEA protein was added with 100 mM trehalose, a further decrease in CS aggregation was noted. A similar effect was observed when the molar ratio of LEA protein:CS was 100:1, 10:1 or 1:1 (Figure 2B). Thus, LEA proteins enhance the protective effect of trehalose against heat-induced CS aggregation in a synergistic fashion, despite not having any anti-aggregation activity by themselves; this effect of LEA proteins seems to apply at a 1:1 molar ratio with CS.

LEA proteins prevent protein aggregation due to desiccation

It is perhaps not surprising that LEA proteins do not function as classical molecular chaperones; the latter proteins are commonly associated with heat stress, but LEA proteins are not considered to be involved in the heat-shock response [35]. Instead, LEA proteins are linked to water stress by desiccation or cooling, and it is possible therefore that they play a chaperone-like role under these conditions, rather than during heat shock. We therefore examined the ability of LEA proteins to counteract aggregation of CS due to drying or freezing. When subjected to repeated rounds of vacuum drying and rehydration ('cycles of desiccation'), CS shows marked aggregation and associated loss of activity (Figure 3). Aggregation is maximal after two cycles of desiccation, as shown by apparent increase in A_{340} due to light scattering; with increasing number of desiccation cycles, A_{340} apparently decreases, but this is due to the aggregates becoming so large that they no longer scatter light and are often not maintained in suspension, depositing on the vessel walls (Figure 3A). Addition of either Em or AavLEA1 protein reduces CS aggregation to negligible levels, even after
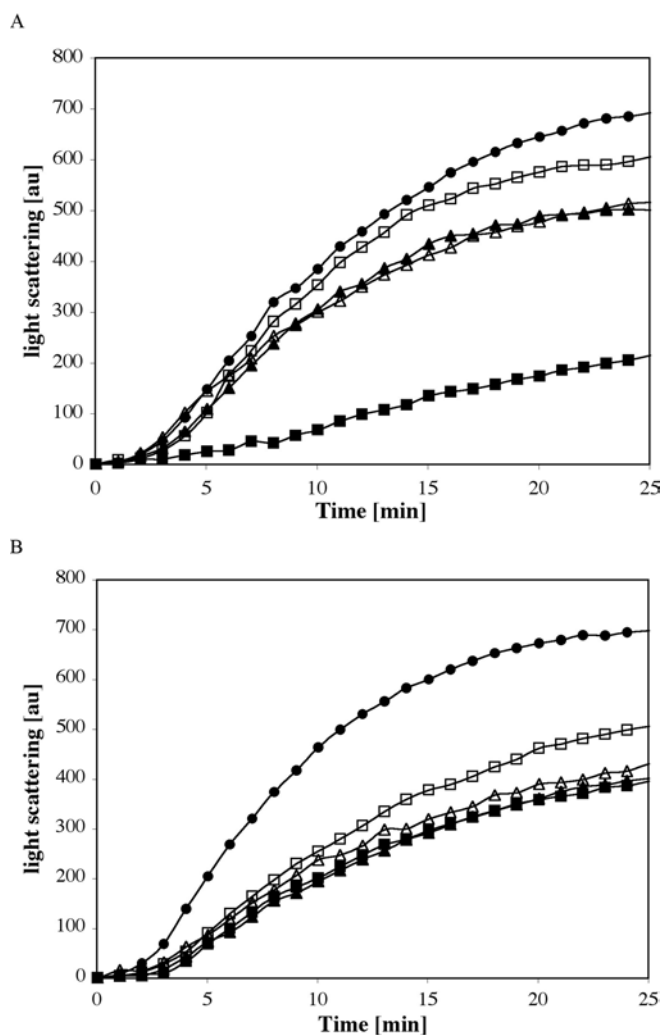

Figure 2   Synergistic effect of trehalose and LEA protein on heat-induced aggregation of CS

(**A**) The aggregation kinetics of CS at 43 °C in the presence or absence of LEA proteins and trehalose were determined as in Figure 1. CS alone (final concentration 75 nM, ●), in the presence of 100 mM (□) or 400 mM trehalose (■), 1 μM group 1 LEA protein and 100 mM trehalose (△) or 1 μM group 3 LEA protein and 100 mM trehalose (▲). (**B**) Aggregation kinetics of CS (final concentration 75 nM) alone (●), in the presence of 100 mM trehalose (□), 100 mM trehalose and 100 nM group 3 LEA protein (△), 100 mM trehalose and 500 nM group 3 LEA protein (▲) or 100 mM trehalose and 1 μM group 3 LEA protein (■).

four cycles of desiccation. Protection against aggregation is not provided in this assay by commonly used stabilizers such as BSA. In accordance with their anti-aggregation activity, both LEA proteins also preserve CS activity, even after four cycles of desiccation. Intriguingly, BSA also seems to preserve enzyme activity, although to a lesser extent than the LEA proteins after multiple cycles of desiccation, suggesting that aggregate formation does not always result in complete loss of CS activity (Figure 3B).

To examine whether LEA proteins can protect enzymes other than CS from desiccation damage, vacuum-drying experiments were performed with LDH, which is known to be sensitive to water loss [36]. Dried LDH lost virtually all activity, but both LEA proteins were able to preserve enzyme function in a concentration-dependent fashion, although apparently similarly to BSA (results not shown). Trehalose is capable of decreasing LDH inactivation in similar experiments: vacuum drying of the enzyme in a solution of 100 mM trehalose, for example, results in maintenance of
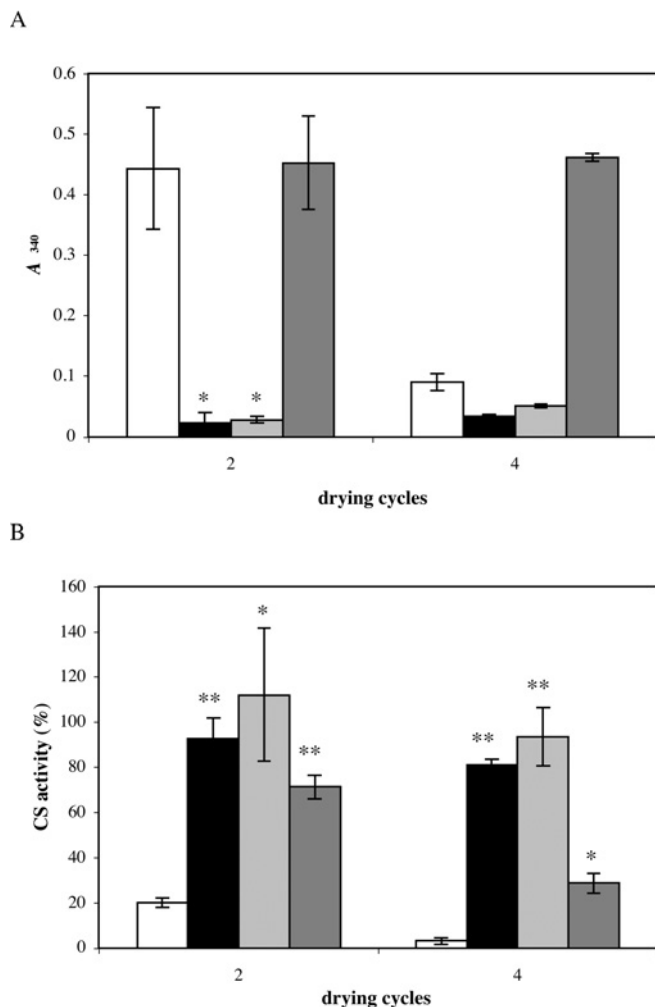
A

B

Figure 3   Effect of desiccation on CS aggregation and activity

(**A**) Aggregation and (**B**) activity of 0.12 mg of CS after desiccation (open bar), in the presence of 0.24 mg of group 1 LEA protein (black bar), 0.24 mg of group 3 LEA protein (grey bar) or 0.24 mg of BSA (dark grey bar). Aggregation is measured by the effect of light scattering giving an apparent A_{340} in the spectrophotometer; enzyme activity is assayed according to standard methods and results are expressed as percentage of control activity. One drying cycle corresponds to vacuum drying (without freezing) for 1 h in a modified tray freeze-dryer followed by immediate rehydration in water to the original volume. Results after two and four drying cycles are shown. *$P < 0.05$ and **$P < 0.01$ shown above the bar represent results significantly different from those for CS alone. Statistical tests were not performed for the four drying cycle data in (**A**) due to the anomalous aggregation of CS, noted in the text.

approx. 20 % activity. When AavLEA1 is used together with 100 mM trehalose to stabilize LDH against drying, a synergistic effect is observed at low LEA protein concentrations (Figure 4). Thus, whereas AavLEA1 alone at concentrations of 1 μM or lower is insufficient to protect LDH, the inclusion of 100 mM trehalose gives greater protection than that seen with trehalose alone. Such a synergistic effect is not seen with BSA, which exhibits a merely additive level of protection when used with trehalose.

LEA proteins can prevent protein aggregation due to freezing stress

CS also suffers marked aggregate formation when subjected to cycles of freezing in liquid N_2 followed by thawing at ambient temperature. Again, both LEA proteins were capable of preventing this aggregation during multiple freeze–thaw cycles, whereas BSA offered only partial protection (Figure 5A). Surprisingly,
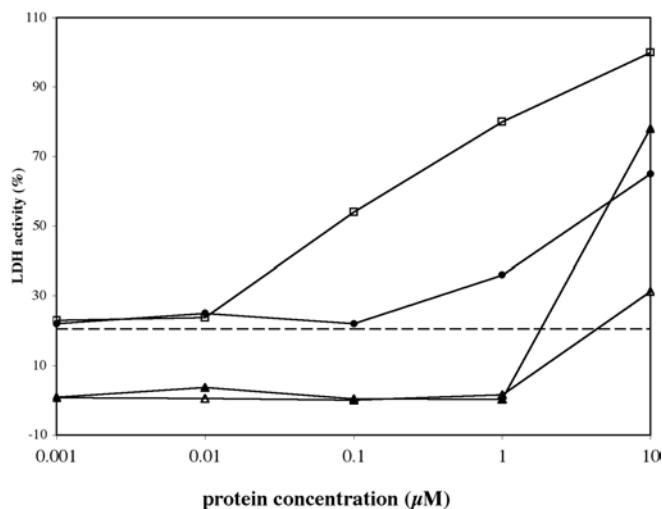

Figure 4   A synergistic effect of LEA proteins and trehalose on protection of LDH during drying

Residual activity of LDH after vacuum drying in the presence of group 3 LEA protein (▲), BSA (△), group 3 LEA protein and 100 mM trehalose (□) or BSA and 100 mM trehalose (●); the dotted line refers to the residual activity due to protection by 100 mM trehalose alone. Trehalose shows a synergistic protective effect with LEA protein, but not with BSA, where an additive effect is seen.

however, aggregate formation (of CS alone) did not seem to destroy its activity, which fell to only approx. 80 % of control levels despite several freeze–thaw cycles. Essentially full CS activity was maintained by both LEA proteins and BSA (Figure 5B). Presumably, these results indicate that denaturation resulting from freeze–thawing is only partial and does not inflict severe damage on the active site of CS.

LEA proteins also prevent LDH aggregate formation due to rapid freezing in liquid N_2, as determined in a light-scattering assay. The degree of aggregation increases with the number of freeze–thaw cycles, but addition of either group 3 or group 1 LEA protein prevents this during successive freeze–thaw cycles to approximately the same extent as BSA, a well-known cryoprotectant protein (Figure 6). Similar protective effects of both LEA proteins were also observed when LDH was subjected to slow freezing by placing at −20 °C overnight (results not shown). Freezing LDH alone abolishes enzyme activity, but the LEA proteins are able to prevent this inactivation, again in a concentration-dependent manner and similarly to BSA (results not shown).

LEA proteins in molar excess prevent aggregation

To determine how the anti-aggregation activity of the LEA proteins depends on their concentration, a vacuum-drying experiment was performed with CS in the presence of LEA proteins or BSA at molar ratios of 1:1, 5:1 and 10:1 (protectant/CS). Figure 7(A) shows that there is little, if any, anti-aggregation activity with equimolar amounts of LEA proteins, but a high level of protection is obtained with a 10:1 molar ratio and a 5:1 ratio gives an intermediate activity. In contrast, BSA does not prevent desiccation-induced aggregation of CS at any molar ratio. Despite this, performance of BSA is indistinguishable from that of LEA proteins in preserving CS activity throughout the drying experiment (Figure 5B). This agrees with the results of Figures 3 and 5, suggesting that the catalytic site of CS is not compromised by aggregation due to desiccation or freeze–thaw, unlike under heat-stress conditions [25].
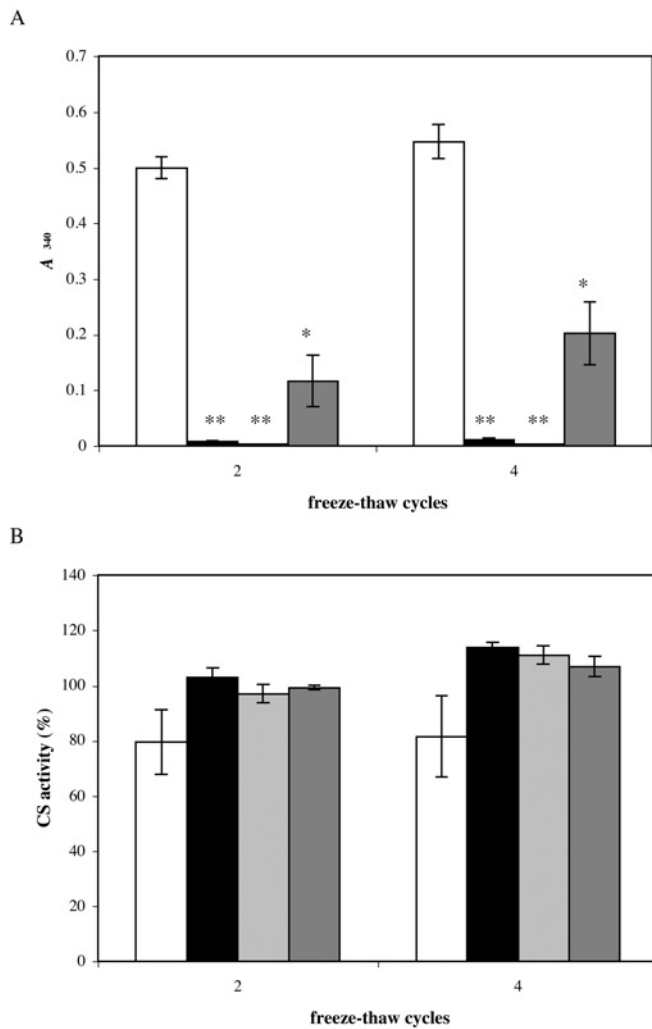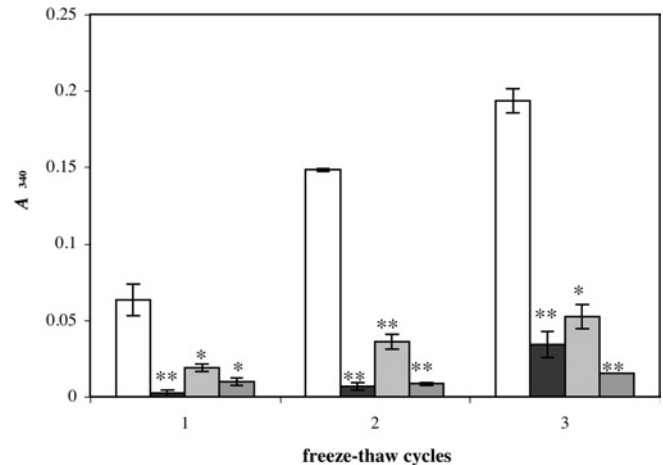
Figure 6   Protection of LDH by protein protectants from aggregation due to freezing and thawing

LDH aggregation on repeated freezing in liquid N_2 and thawing at ambient temperature is indicated by light scattering at A_{340}. Results are shown for 12.5 μM LDH alone (open bar), for LDH in the presence of 25 μM group 1 LEA protein (black bar), for LDH in the presence of 25 μM group 3 LEA protein (grey bar) and for LDH together with 25 μM BSA (dark grey bar). *$P < 0.05$ and **$P < 0.01$ shown above the bar represent results significantly different from those for LDH alone.

Figure 5   Protection of CS by protein protectants from aggregation and inactivation by freezing and thawing

(**A**) Aggregation and (**B**) activity of 0.25 mg/ml CS only after freezing in liquid N_2 and thawing at ambient temperature (open bar), in the presence of 0.5 mg/ml group 1 LEA protein (black bar), 0.5 mg/ml group 3 LEA protein (grey bar) or 0.5 mg/ml BSA (dark grey bar). Aggregation is measured by apparent A_{340} as previously; enzyme activity is assayed according to standard methods and results are expressed as percentage of control activity. One freeze–thaw cycle corresponds to snap freezing in liquid N_2 followed immediately by thawing at ambient temperature. Results after two and four cycles are shown. *$P < 0.05$ and **$P < 0.01$ shown above the bar represent results significantly different from those for CS alone.

DISCUSSION

LEA proteins are widespread in anhydrobiotes, and an appreciation of their function would represent an important advance in our understanding of desiccation tolerance. In the present study, we have tested the hypothesis [21] that LEA proteins might function as molecular chaperones. Chaperones are capable of binding to non-native proteins, to maintain them in a folding competent state and prevent irreversible aggregation [31]. In a standard assay for chaperone activity and prevention of thermal aggregation of CS, neither of the LEA proteins tested was functional. Expression of LEA proteins has not been found to be up-regulated by heat [35], and hence the lack of 'classical' chaperone activity is not unexpected in them. The LEA proteins are also structurally very different from most chaperones, and while other unstructured proteins have been found to possess chaperone activity, e.g. α-casein

[37] and MAP2 (mitogen-activated protein 2) [38], this property was attributed to micelle formation and the presence of hydrophobic patches respectively, neither of which has been observed for the LEA proteins [18]. However, interestingly, we found that LEA proteins were capable of suppressing protein aggregation and inactivation under water-stress conditions.

The behaviour of proteins on desiccation remains relatively poorly understood. It is known that removal of water can cause irreversible structural changes leading to aggregation and inactivation in some proteins (although not all) [39]. Certainly, CS undergoes aggregation on both drying and freezing, and inactivation on drying. However, the pathway of denaturation appears to be different from that found in heat shock. Inactivation of enzyme activity is an early event during thermal stress of CS [25], occurring before significant levels of aggregation. Surprisingly, on freezing, CS forms aggregates that are still largely active. Presumably, the CS molecules in such an aggregate must still be in dimeric form with the active sites undamaged. On desiccation, the aggregates formed show much less activity. Either there is a separate pathway for CS unfolding on desiccation, which results in inactive aggregates, or the pathways are similar initially but the aggregates formed are then inactivated by a further removal of water on desiccation; this would be consistent with desiccation being a more extreme stress. Characterization of the aggregates formed by CS on freezing and drying warrants further investigation. It will also be of interest to assess if a similar relationship between protein aggregation and inactivation exists for other model enzymes.

Other studies of LEA function have looked mainly at inactivation of LDH on freezing ([40], and references therein; [8]) and found various LEA proteins to have protective properties similar to, or in the case of certain dehydrins, better than, BSA. Only one group has addressed enzyme inactivation on desiccation, and found a *Citrus* dehydrin to be approx. 20 % more effective than BSA in protecting malate dehydrogenase activity [41]. Our results are consistent with these and, although it is possible to conclude that the LEA proteins can maintain enzyme activity on dehydration, it is difficult to assign them a specific role in desiccation
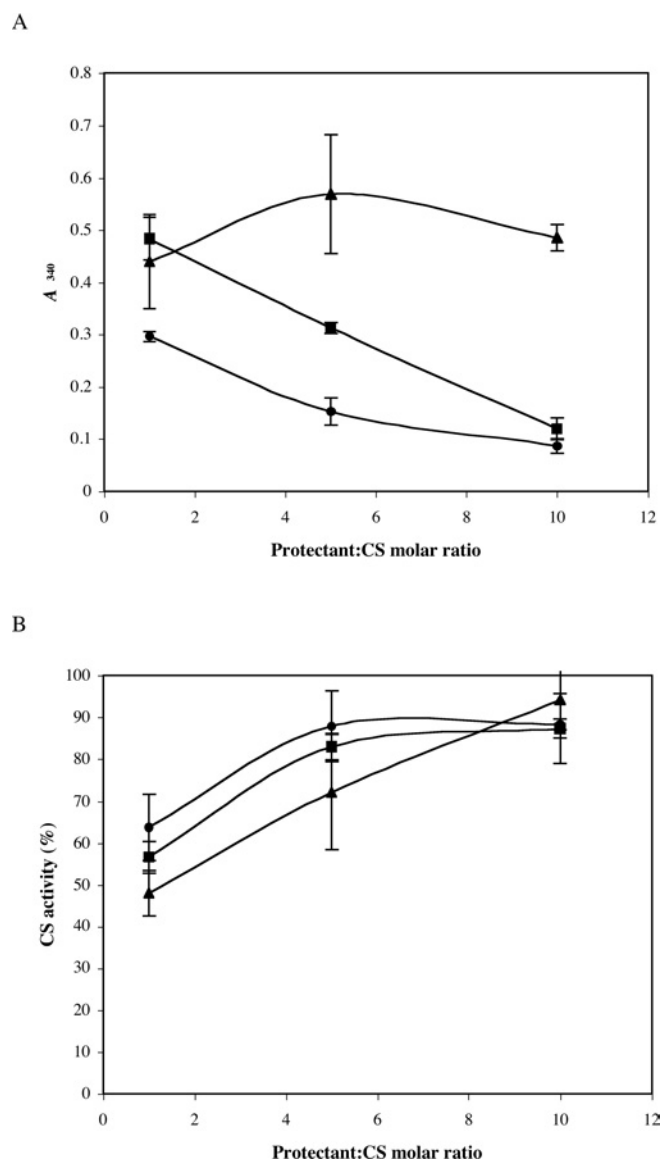
A

B

Figure 7   Effect of different molar ratios of LEA proteins and BSA on CS aggregation and activity after desiccation

(**A**) Aggregation and (**B**) activity of CS, measured as previously, after two cycles of desiccation in the presence of varying concentrations of group 1 LEA protein (■), group 3 LEA protein (●) or BSA (▲). Although BSA is not able to prevent CS aggregation due to drying, even at a 10:1 molar ratio, the LEA proteins partially or completely abolish aggregation at 5:1 and 10:1 molar ratios respectively.

tolerance when BSA, a protein of completely unrelated function, can act in a similar way. However, our results suggest that it is not sufficient to look at enzyme activity alone. If the objective is to stabilize a particular protein *in vitro*, then this approach may be valid; as long as the enzyme is still active, it is perhaps not important whether or not aggregates are formed. But *in vivo*, formation of aggregates, regardless of whether or not the proteins within them are still functional, is probably damaging to the cell. Analysing aggregation results takes into account the formation of potentially harmful 'active aggregates' and has provided much clearer evidence for a role of the LEA proteins in desiccation tolerance: they are very effective at suppressing aggregation on desiccation, and this is not a property shared by all proteins.

Even though there is still much to be learnt about the behaviour of CS on desiccation and freezing, we can nevertheless begin to formulate a hypothesis about the role of the LEA proteins. BSA is known to be an effective stabilizer on freezing [42], although exactly how it functions is not understood. It could be due to macromolecular crowding effects [43], which are known to make unfolding energetically unfavourable. These could also account for the stabilizing effects of the LEA proteins on freezing. However, in the absence of bulk water on desiccation, only the LEA proteins are effective at preventing aggregation. Potentially, their unordered, flexible structure may allow them to act as a kind of 'molecular shield', forming a physical barrier between neighbouring CS molecules and preventing contact between them. Alternatively, they may function as 'chaperones for water stress' and form defined complexes with CS molecules as they unfold in a manner analogous to conventional molecular chaperones. Further investigation of the interactions between the LEA proteins and CS in the dry state is required to define the mechanism of LEA activity, although assessing protein–protein interactions in the dry state could prove technically difficult. It should also be pointed out that, although we are grouping both LEA proteins together in these analyses, in terms of sequence, group 1 and group 3 LEA proteins are clearly different. It has been hypothesized previously that LEA proteins from different groups may have distinct roles [5,21]; further experiments will be necessary to test this idea.

Since, *in vivo*, the LEA proteins are produced in the presence of high concentrations of disaccharides, we also tested the effect of trehalose on the protective properties of the LEA proteins. LEA proteins alone do not seem to have any protective function against heat stress, but interestingly their presence improves the stabilizing properties of trehalose. The reason for this is unclear, but it might be that while the LEA proteins cannot prevent CS aggregation on heat shock, they do have some effect on the unfolding pathway that is not seen unless aggregation can also be prevented, e.g. by trehalose. A similar synergistic effect between trehalose and the group 3 LEA protein AavLEA1 is also seen on desiccation, where they improve the recovery of LDH activity to a level greater than the sum of their individual effects. This might be significant in anhydrobiotic organisms, where both LEA proteins and non-reducing disaccharides are implicated in desiccation tolerance.

In summary, we presented the first experimental evidence that LEA proteins are capable of suppressing desiccation-induced protein aggregation *in vitro*. These results strongly support a role for the LEA proteins in water stress through desiccation or freezing. Further investigation of the behaviour of proteins during extreme water loss will be required to elucidate the function of the LEA proteins in more detail.

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