

Functional dissection of Hydrophilins during *in vitro* freeze protection

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ABSTRACT

In plants, Late Embryogenesis Abundant (LEA) proteins typically accumulate in response to low water availability conditions imposed during development or by the environment. Analogous proteins in other organisms are induced when exposed to stress conditions. Most of this diverse set of proteins can be grouped according to properties such as high hydrophilicity and high content of glycine or other small amino acids in what we have termed hydrophilins. Previously, we showed that hydrophilins protect enzyme activities *in vitro* from low water availability effects. Here, we demonstrate that hydrophilins can also protect enzyme activities from the adverse effects induced by freeze–thaw cycles *in vitro*. We monitored conformational changes induced by freeze–thaw on the enzyme lactate dehydrogenase (LDH) using the fluorophore 1-anilino-naphthalene-8-sulfonate (ANS). Hydrophilin addition prevents enzyme inactivation and this effect is reflected in changes in the ANS-fluorescence levels determined for LDH. We further show that for selected plant hydrophilins, removal of certain conserved domains affects their protecting capabilities. Thus, we propose that hydrophilins, and in particular specific protein domains, have a role in protecting cell components from the adverse effects caused by low water availability such as those present during freezing conditions by preventing deleterious changes in protein secondary and tertiary structure.

Key-words: cryoprotectants; dehydrins; freezing stress; Hydrophilins; lactate dehydrogenase; LEA proteins.

INTRODUCTION

Drought and cold conditions are among the most important adverse factors affecting plant growth and crop production. An array of responses is induced in plants exposed to a variety of stress conditions; however, a significant cross talk has been detected between these responses, particularly in those that share stressful factors. That is the case of drought and cold stress, where a reduction in water availability is

observed (Xiong, Schumaker & Zhu 2002; Yamaguchi-Shinozaki & Shinozaki 2005). At the molecular level, drought and cold treatments induce some of the Late Embryogenesis Abundant (LEA) proteins, a group of proteins first characterized as accumulating during the last stages of embryogenesis (Ingram & Bartels 1996; Bray 1997; Battaglia *et al.* 2008). Most LEA proteins are highly hydrophilic and rich in glycine and other small amino acid residues. Moreover, proteins with similar characteristics are found in other organisms and are also involved in stress responses, suggesting common functions for all of them (Garay-Arroyo *et al.* 2000; Tunnaclyffe & Wise 2007). We have coined the term ‘Hydrophilins’ to describe proteins with these common features (Garay-Arroyo *et al.* 2000).

Different forms of dehydration stress, in particular low and/or freezing temperatures, induce several plant hydrophilins. For instance ERD10, a group 2 LEA protein, was originally identified upon dehydration treatments in *Arabidopsis thaliana*, and subsequently found to be induced by cold treatments as well (Kiyosue, Yamaguchi-Shinozaki & Shinozaki 1994). ERD10 contains a serine-rich region known as S-segment, as well as three 15-amino acid-long motifs known as K-segments, a landmark of LEA type 2 proteins or dehydrins (Close 1997). Over-expression of multiple *Arabidopsis* LEA2 group proteins such as ERD10, RAB18, COR47 and LTI30 resulted in plants, which showed increased freezing tolerance and improved survival under low temperature conditions (Puhakainen *et al.* 2004). A role in stress tolerance for dehydrins is also supported by the co-segregation of a dehydrin gene with chilling tolerance during seedling emergence in cowpea (Ismail, Hall & Close 1999). Similarly, RcDhn5, a 29 kDa acidic dehydrin from the evergreen *Rhododendron* species (*R. catawbiense* Michaux.), containing an S-segment and two K-segments, co-segregates with leaf freezing tolerance in *Rhododendron* F₂ progenies, which correlates with cold-acclimation ability (Lim, Krebs & Arora 1999; Marian, Krebs & Arora 2004). Group 4 LEA proteins, which show a conserved N-amino portion that is predicted to form amphipathic α -helices and a less conserved C-terminal region (Dure 1993), also have been implied in freezing tolerance (Imai *et al.* 1996), again indicating that the hydrophilin response may not be limited to one particular form of water limitation stress.

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We have previously shown that hydrophilins can protect enzyme activities from the adverse effects of partial water loss in an *in vitro* system using protein members from different origins such as plants, fungi and bacteria (Reyes *et al.* 2005). To address whether hydrophilins can also show some protecting activity to an enzyme exposed to low temperatures, we have adopted an *in vitro* assay whereby the activity of lactate dehydrogenase (LDH) or malate dehydrogenase (MDH) is significantly reduced upon freezing. Lin & Thomashow (1992) were the first to show that COR15a from *Arabidopsis thaliana* could protect LDH from freezing effects using the assay developed earlier (Carpenter & Crowe 1988). Later, a number of studies have shown that dehydrins (LEA group 2) from different species can also protect LDH (Kazuoka & Oeda 1994; Houde *et al.* 1995; Wisniewsk *et al.* 1999; Hara, Terashima & Kuboi 2001; Bravo *et al.* 2003; Momma *et al.* 2003; Sanchez-Ballesta *et al.* 2004). Additionally, members of LEA group 3 (Honjoh *et al.* 2000) and more recently a group 1 LEA protein present in wheat and a group 3 LEA-like protein from the nematode *Aphelenchus avenae* (Goyal, Walton & Tunnacliffe 2005) were also shown to protect LDH activity *in vitro*. However, except for the more recent work of Goyal *et al.* (2005), there have been no comparisons of different hydrophilins with respect to their cryoprotective activities and little is known about their mode of action during *in vitro* protection.

Here, we further analyze the effects of *in vitro* freezing on enzyme activity and structure while assessing the protecting activity of different hydrophilins. We show that LDH and MDH activities are severely affected by repeated freeze–thaw cycles and this is also reflected in changes in enzyme structure as revealed by changes in fluorescence due to differential binding of the fluorophore 1-anilino-naphthalene-8-sulfonate (ANS). The pattern of ANS fluorescence during freeze–thaw cycles is distinct to that observed for LDH inactivation during partial water loss, as shown in our earlier study (Reyes *et al.* 2005), indicating differences between the two forms of LDH inactivation. Furthermore, we show that hydrophilins are able to prevent enzyme inactivation because of freeze–thaw. We have used hydrophilins from different organisms, including dehydrins from *A. thaliana* (ERD10) and *Rhododendron* (RcDhn5), the group 4 LEA protein AtLEA4-5 (At5g06760) from *A. thaliana*, YciG from *Escherichia coli* and Sip18 from *Saccharomyces cerevisiae*. Different hydrophilins exhibited various degrees of protection. In addition, we show that for selected cases, hydrophilin protection depends on particular domains of the proteins. Deletion of the amphipathic K-segments of *Arabidopsis* ERD10 and *Rhododendron* RcDhn5 shows a reduction in their cryoprotecting capabilities. In contrast, a deletion strategy that selectively maintains the conserved domain in AtLEA4-5 retains its full cryoprotective activity. These results demonstrate that certain domains present in hydrophilins are necessary for preserving enzyme activity under adverse conditions. Finally, we show that the protective effect of RcDhn5 is also paralleled by changes in enzyme structure as determined by

ANS fluorescence of LDH samples. Our results indicate that hydrophilins utilize distinct protein domains to confer protection from freezing to enzymatic activities *in vitro*, and suggest a mode of action for their *in vivo* function during stress.

MATERIALS AND METHODS

Proteins

Recombinant ERD10 and AtLEA4-5 and their respective deletion constructs for ERD10-2K, ERD10-1K, ERD10-s and AtLEA4-5H were obtained by PCR amplification from *Arabidopsis* cDNA and cloned in the pTrc99A vector as described previously (Campos, Zamudio & Covarrubias 2006). The C-terminus of AtLEA4-5 was cloned but it could not be expressed in *E. coli* to allow its testing in the *in vitro* assay. Recombinant ERD10, AtLEA4-5, YciG and Sip18 and their purification has been described previously (Reyes *et al.* 2005). The *RcDhn5* clone (# CA2D12; Genbank accession # CV015064) was obtained from a cDNA library prepared from cold-acclimated (winter-collected) *Rhododendron catawbiense* leaves (Wei *et al.* 2005). Recombinant dehydrin RcDhn5 was cloned as a glutathione-S-transferase (GST) fusion in the pGex-6P-3 vector (Amersham, Piscataway, NJ, USA). Affinity purification of recombinant RcDhn5 was performed according to the manufacturer's specification. Recombinant RcDhn5 protein was cleaved from the GST affinity tag with the addition of Precision Protease (Amersham). Its deletion version (RcDhn5-del) was sub-cloned by PCR techniques as a His-tagged protein by transferring it into the pDEST17 vector (Invitrogen, Carlsbad, CA, USA) using Gateway technology. Affinity purification of recombinant RcDhn5-del using Ni-NTA resin was performed according to the manufacturer's specification (Qiagen, Valencia, CA, USA). RNaseA, α -crystallin, poly L-Lysine and trehalose were obtained from Sigma (San Louis, MO, USA). LDH and MDH were obtained from Roche (Indianapolis, IN, USA) as 50% glycerol enzyme suspensions. A list of all proteins used here and their relevant features is included as Supporting Information Table S1.

In vitro freeze–thaw assays

Samples contained LDH or MDH at 250 nM final concentration (monomer) in 25 mM Tris pH 7.5. Different proteins tested for protection were added at the same 250 nM final concentration, as determined by light absorbance and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of the purified proteins. The mixture in a final volume of 75 μ L was frozen for 15 min in dry ice and thawed for 15 min in a water bath at 25 °C. This constituted one freeze–thaw cycle, which was repeated up to five times as indicated in the text. At the end of the treatment, the samples were transferred to wet ice until enzyme activity was determined.

Enzyme activity measurements

MDH and LDH enzymatic activities were determined using aliquots of 8 μL and 15 μL , respectively, in a final volume of 600 μL of the reaction assay buffer. MDH enzymatic activity was assayed in 150 mM potassium phosphate buffer pH 7.5 containing 0.2 mM oxalacetate (Sigma) and 0.2 mM NADH (Roche). For LDH, the assay buffer was 25 mM Tris-HCl pH 7.5 containing 2 mM pyruvate (Sigma) and 0.15 mM NADH (Roche). MDH and LDH activities were monitored as the rate of decrease in absorbance at 340 nm for 1 min due to the conversion of NADH into NAD at 25 °C. The rate determined for the untreated samples was considered as 100% in all graphs. Enzyme activity for each sample shown was determined at least in three independent tests, as indicated in the figure legend for each experiment.

ANS-fluorescence assays

Changes in fluorescence due to binding of the fluorescent probe ANS (Sigma) were measured using a DyNA Quant 200 instrument (Amersham) according to manufacturer directions (supplement 5 to Users Manual). LDH or MDH were incubated in 25 mM Tris-HCl, pH 7.5. Final monomer concentration was 0.5 μM for both enzymes, instead of 0.25 μM as for the freeze-thaw assays. Hydrophilins were added at the same ratio used in the freeze-thaw assays. ANS was added to 5 μM final concentration, immediately after the final freeze-thaw cycle. All fluorescence measurements were repeated at least four different times, as indicated in the figure legends.

RESULTS

An *in vitro* assay to measure inhibition of enzymatic activity by freeze-thaw

We have previously shown that the gradual removal of water has adverse effects on enzyme activity *in vitro* (Reyes *et al.* 2005). To explore whether another form of limiting water availability has similar effects, we have used freezing as well. It has been previously reported that freeze-thaw reduces LDH activity, and an assay has been used in the past to measure the protective effect of LEA proteins on LDH activity during such a stress (Lin & Thomashow 1992). Thus, we have modified our partial water-loss assay to introduce a freezing treatment, and in addition to LDH we have also examined whether MDH is sensitive to freeze-thaw as well. Samples containing LDH or MDH were incubated in dry ice for 15 min and then thawed in a water bath at 25 °C for another 15 min (Fig. 1a). To increase the chances of detecting possible adverse effects, this two-step cycle was repeated up to five times before determination of residual enzyme activity. Figure 1b shows the loss of LDH or MDH activity obtained after one to five cycles of freeze-thaw. Both enzymes showed a dramatic decrease in activity already detectable after one freeze-thaw cycle. Although activity of LDH and MDH continued to decrease with

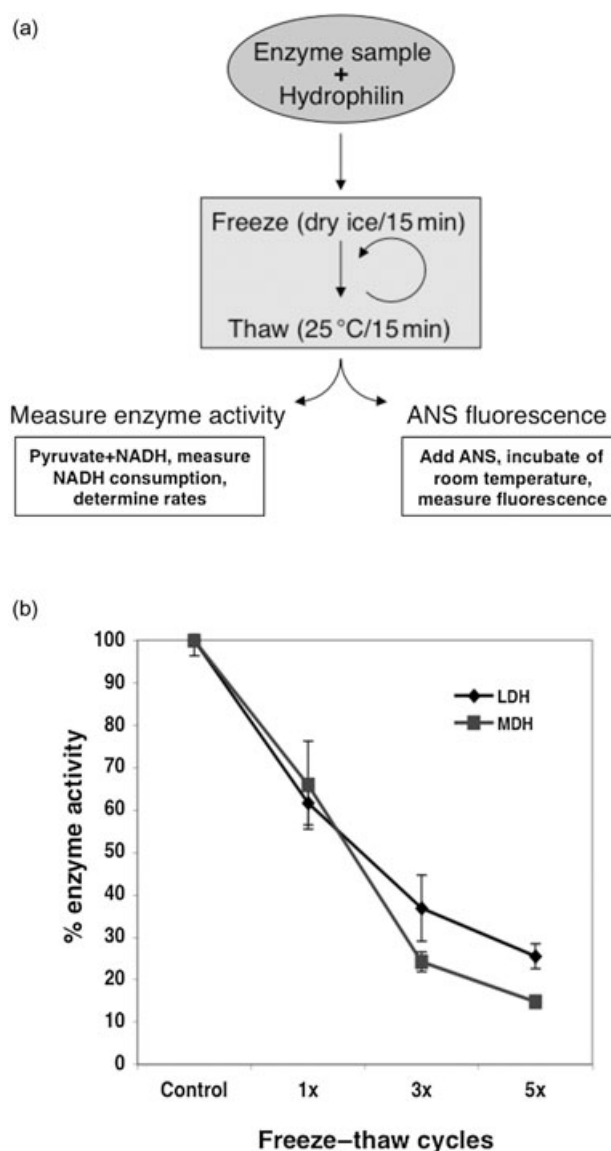


Figure 1. An *in vitro* assay to monitor the effects of freezing on enzyme activity. (a) Flow chart of the experimental design. Samples containing lactate dehydrogenase (LDH) with or without addition of a putative protectant protein were incubated in dry ice for 15 min and then moved to a water bath at 25 °C for 15 min. One, three or five identical cycles were performed and samples were returned to ice. Immediately thereafter, remaining enzyme activity was determined or 1-anilinonaphthalene-8-sulfonate (ANS) was added to determine sample fluorescence. (b) Decreased activity of LDH (diamonds) and malate dehydrogenase (MDH; squares) under freezing conditions. Both enzymes exhibited a continuous decay in activity with the increasing number of freeze-thaw cycles. Enzyme activity of the untreated enzyme is referred as 100%. Values represent the average of three (MDH) or eight (LDH) independent experiments. Error bars represent standard deviation.

further cycles, MDH showed a slightly higher sensitivity to freeze-thaw. Thus, any of the two enzymes can be used to monitor the effect of potential protectants against freezing. We also attempted a more gradual freeze-thaw cycle by

incubating the samples at -20°C for 1 h. Differences in enzyme activity were not as readily visible as with the procedure described above, and moreover, the procedure became considerably longer, raising concerns about the accuracy of the remaining enzyme activity after extended incubation times. For subsequent experiments presented here, we chose to use the shorter protocol utilizing LDH as the model enzyme; however, protection of MDH activity by a selected number of hydrophilins showed similar results (data not shown).

Hydrophilins protect enzymes against *in vitro* freeze–thaw inactivation

As in the partial water-loss assay (Reyes *et al.* 2005), addition of α -crystallin, a molecular chaperone known to protect proteins from heat shock (Sun & MacRae 2005), did not protect LDH from freezing-induced loss of activity, and showed only a slight but significant protection after three freeze–thaw cycles above the activity lost without any additives (Student's *t*-test $P = 0.028$, Fig. 2a). In contrast, addition of different hydrophilins that we tested showed varying degrees of protection. Both *Arabidopsis* hydrophilins, ERD10 and AtLEA4-5 (LEA groups 2 and 4, respectively) recovered up to 90% of the LDH activity of untreated samples after one freeze–thaw cycle. Moreover, after three cycles, ERD10 maintained 80–90% activity while *Rhododendron* RcDhn5 (LEA group 2) was able to protect LDH above levels obtained for α -crystallin and comparable to those of AtLEA4-5 (Fig. 2a). In contrast, addition of a highly hydrophilic amino acid-based polymer, poly L-Lysine, did not protect LDH above background levels (Fig. 2a).

We also tested hydrophilins from other organisms that had been observed to confer protection in the partial water-loss assay (Reyes *et al.* 2005). YciG from *E. coli*, as in the case of the partial water-loss assay, showed the best protection level also in the freeze–thaw assay. After one or three cycles, it maintained around 90% of the initial LDH activity, while the yeast hydrophilin, Sip18, showed limited protection ($P = 0.01$ at three cycles), similar to that of α -crystallin (Fig. 2b). This result is in direct contrast to the protection observed for the partial water-loss assay, where Sip18 appeared to be as a good protectant as ERD10. RNase A, a globular protein heretofore unrelated to stress responses did not show any protection under the same conditions (Fig. 2b). In addition to hydrophilins, we tested the non-reducing disaccharide trehalose, a compatible osmolyte known to accumulate in diverse organisms in response to stress (Elbein *et al.* 2003) and known for its protein-stabilizing properties during stress (Carpenter & Crowe 1989). The addition of trehalose resulted in limited but consistent protection of LDH from freeze–thaw inactivation ($P = 0.002$ and $P = 0.001$ at one and three cycles, respectively; Fig. 2b). However, it should be noted that trehalose was present at 10 mM, while hydrophilins were present at 250 nM in a 1:1 ratio to LDH on a molar basis, the latter at a 40 000-fold lower concentration, suggesting that hydrophilins and trehalose confer protection via different mechanisms. In two

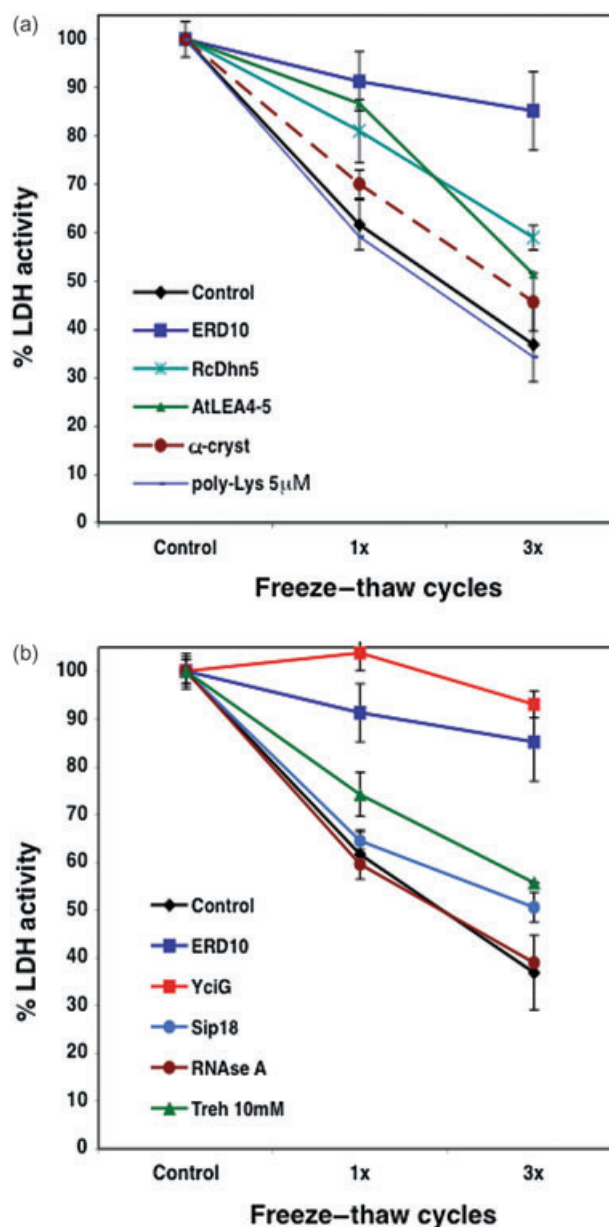


Figure 2. Samples containing lactate dehydrogenase (LDH) with or without hydrophilins were frozen and thawed once (1 \times), three (3 \times) or five (5 \times) times and LDH activity determined immediately after thawing. Symbols indicate hydrophilins added. Percent values are with respect to untreated samples. (a) LDH activity alone (diamonds) or with addition of plant hydrophilins ERD10 (squares), Rc-Dhn5 (asterisks), AtLEA4-5 (triangles). As controls the unrelated α -crystallin (circles) and the homopolymer poly-L-lysine (dots) are shown. Values represent the average of 6–10 independent experiments, except for poly L-lysine which was repeated three times. Error bars represent standard deviation. (b) ERD10 (squares, shown here again for comparison with panel a), or other hydrophilins YciG (from bacteria, squares), Sip18p (*S. cerevisiae*, circles), and the unrelated RNase A (circles) and the reducing sugar trehalose (triangles) are shown. Values represent the average of at least three independent experiments. Error bars represent standard deviation.

different assays where water limitation is imposed in different ways, namely the partial water-loss and freeze–thaw assays, hydrophilins are able to protect enzymatic activity. Together, these results suggest that water limitation is responsible for the loss of enzyme activity in both assays, and furthermore, that hydrophilins protect against the adverse effects of water limitation.

Particular protein domains are involved in the protective activity of hydrophilins

To start exploring the role of recognized domains present in plant hydrophilins, we have produced truncated versions of two dehydrins, ERD10 and RcDhn5, as well as of a group 4 LEA protein, AtLEA4-5 (At5g06760). Dehydrins possess characteristic and defining domains known as K-segments, which have been proposed to conform to amphipathic α -helices (Close 1997). For ERD10, we considered a series of truncated proteins where we removed the three K-segments present one at a time, thus generating ERD10 derivatives containing three, two, one or no K-segments but maintaining the S-segment (Fig. 3a, left panel). For RcDhn5, we removed half of the protein at the C-terminus, which included the two K-segments present in RcDhn5 along with adjoining sequences. As with ERD10, we kept the S-segment present in RcDhn5. Finally, in the case of AtLEA4-5, we have maintained the N-terminus of the protein, which corresponds to the most conserved domain in group 4 LEA proteins and has the potential to conform to an amphipathic α -helix (Dure 1993), but removed the hydrophilic domain present at the C-terminus. This series of deletions is shown diagrammatically in Fig. 3, along with the data showing the protection levels obtained in each case.

For the dehydrins tested, the truncated proteins conferred reduced protection to LDH from a freeze–thaw treatment when compared with their respective full-length proteins. In the case of both dehydrins, ERD10 and RcDhn5, removing K-segments had a deleterious effect on their capacity to protect LDH activity. ERD10-s, lacking all three K-segments, was less effective than full-length ERD10 in preserving LDH activity but still behaved as a good protectant (Fig. 3a). We also tested truncated ERD10 proteins containing one or two K-segments, (ERD10-1K and ERD10-2K, respectively, Fig. 3a). Although both proteins retained protective properties, after five freeze–thaw cycles, ERD10-2K prevented LDH inactivation to levels similar to those of the full-length protein, while ERD10-1K, containing a single K-segment, offered protection to levels comparable to those of ERD10-s, devoid of all K-segments (Fig. 3a). On the other hand, the loss of protection observed for the truncated protein was more dramatic in the case of RcDhn5, where deletion of the two K-segments rendered the mutant RcDhn5-del incapable of offering any protection to LDH above that seen for control samples (Fig. 3b). In direct contrast, the protection behaviour observed for AtLEA4-5 vis-à-vis its shortened version, revealed that the truncation did not affect its protection efficiency. It is noteworthy that the shortened version of AtLEA4-5-H still

possesses the N-terminus, which is the most conserved region among the members of this family in different plant species (Dure 1993). In all three cases analyzed here, the presence of recognized domains in plant hydrophilins correlates with, and appears to be essential for, the protective properties of the proteins. An alternative explanation is that the shortened polypeptides are less efficient in conferring protection because of their reduced mass. To test this possibility, we determined the protection levels of ERD10-s and RcDhn5-del when present at twice their original concentration in the assay and determined that the level of protection was not increased (data not shown). Together, these results suggest that the conserved domains present in plant hydrophilins are important for their protecting activity *in vitro*, and suggest that these domains are important for hydrophilin functions during stress responses *in vivo*, possibly by mediating interactions with their protein targets or by favouring a particular conformation during dehydration that is necessary for optimal activity.

Loss of enzyme activity is associated with protein structural changes during freeze–thaw

Upon partial water loss, alterations in protein structure can be monitored by changes in binding efficiency of the fluorophore ANS (Reyes *et al.* 2005), which binds to hydrophobic residues available in proteins, resulting in a particular fluorescence intensity and thereby reflecting protein structure (Stryer 1965). Because freeze–thaw treatment also results in a decrease in enzyme activity, possible changes in tertiary structure due to freeze–thaw treatments were monitored using ANS. Upon freeze–thaw cycles, the ANS fluorescence of LDH increases around two-fold and this increase is maintained up to three freeze–thaw cycles, while after five cycles ANS fluorescence decreased slightly (Fig. 4a). Assays involving MDH show a similar increase in fluorescence upon freezing, but after five cycles it continues to increase close to three-fold over the value obtained for the untreated enzyme sample (Fig. 4a). These changes in ANS fluorescence can be interpreted as a series of protein structural modifications that gradually expose hydrophobic residues along the polypeptide, reflecting a decrease in enzyme activity.

The ANS-fluorescence change observed for both enzymes is somewhat different from that observed for LDH and MDH during partial water loss in our previous study (Reyes *et al.* 2005). In that study, the ANS fluorescence initially fell to 0.5-fold of that in untreated samples, followed by a gradual increase until it reached 1.7-fold at 99.4% water loss. Such contrasting observations in the two studies indicate that the hydrophobic residues exposed during water removal by dehydration or a freeze–thaw treatment are not equivalent, perhaps because protein–water interactions in each case lead to distinct routes of protein unfolding, resulting in distinct ANS-fluorescence patterns.

We then analyzed the effect of hydrophilin addition to LDH samples during freeze–thaw. Interestingly, RcDhn5 showed a low level of basal fluorescence by itself (data not

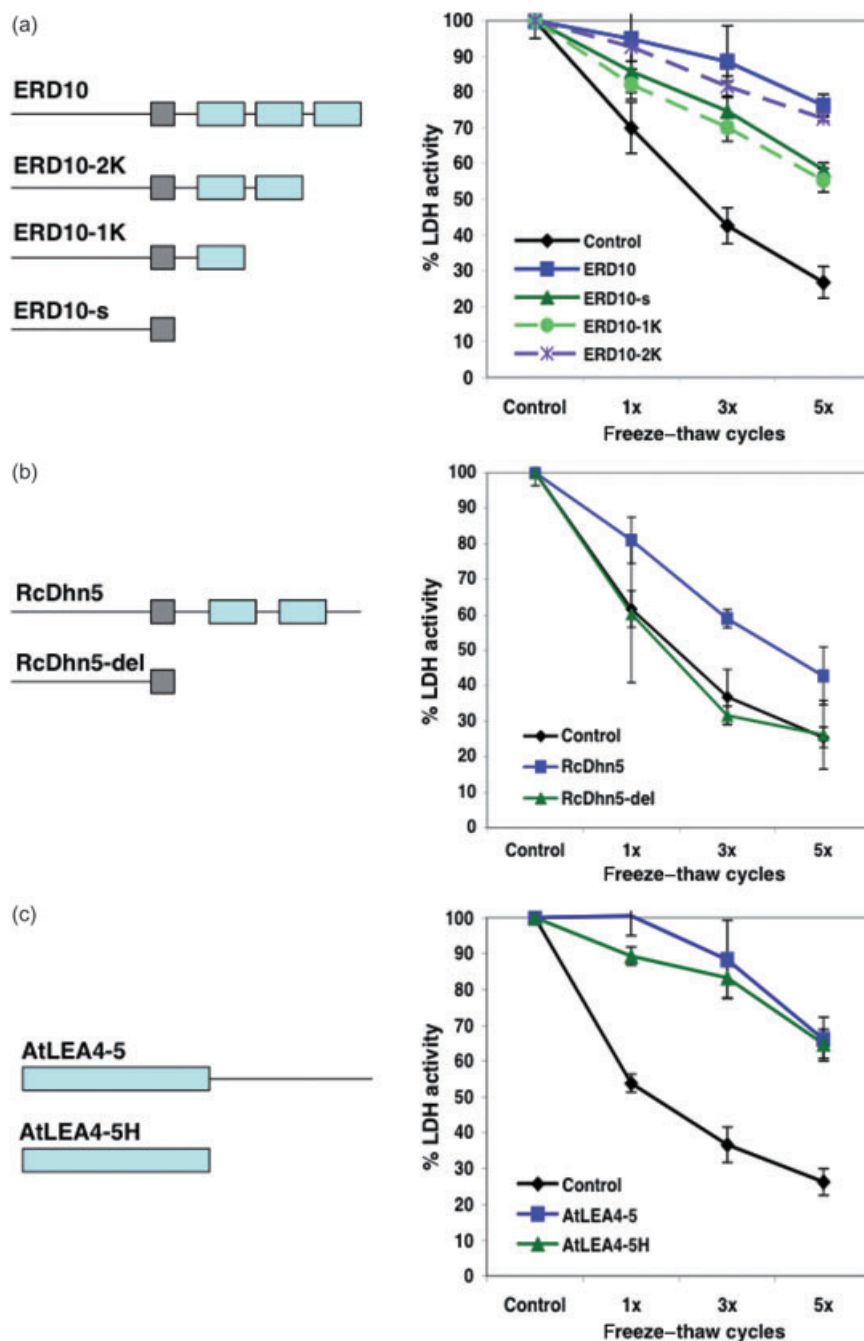


Figure 3. Protein domains involved in preventing freezing effects on lactate dehydrogenase (LDH) activity. Freeze-thaw assays were performed for three proteins including deletion versions of each of them as shown in the diagrams at left. (a) ERD10 (squares) or truncated proteins lacking one K-segment (ERD10-2K, asterisks, dashed line), two K-segments (ERD10-1K, squares, dashed line) or all three K-segments (ERD10-s, triangles). (b) RcDhn5 (squares) or a truncated protein lacking the two K-segments (RcDhn5-del, triangles). (c) AtLEA4-5 (squares) or a truncated protein lacking the hydrophilic domain (AtLEA4-5H, triangles). Values represent the average of four to six independent experiments, except for LEA4-5H, which was repeated three times. Error bars represent standard deviation.

shown) or in the presence of LDH (Fig. 4b), thus allowing us to measure the effect of RcDhn5 addition on ANS-fluorescence changes in LDH upon freeze-thaw treatments. Upon addition of RcDhn5 to LDH and after three freeze-thaw cycles, ANS-fluorescence levels were substantially lower than those obtained for LDH alone under the same conditions ($P = 0.003$, indicated by a dashed line and an

arrow in Fig. 4b); ANS fluorescence increased by ~135% for LDH alone, following three freeze-thaw cycles compared with an increase by only ~56% for the LDH/RcDhn5 mixture. Moreover, the intermediate fluorescence values obtained are more similar to the values obtained for LDH after only one freeze-thaw cycle ($P = 0.08$). This result is in agreement with the values obtained in the LDH activity

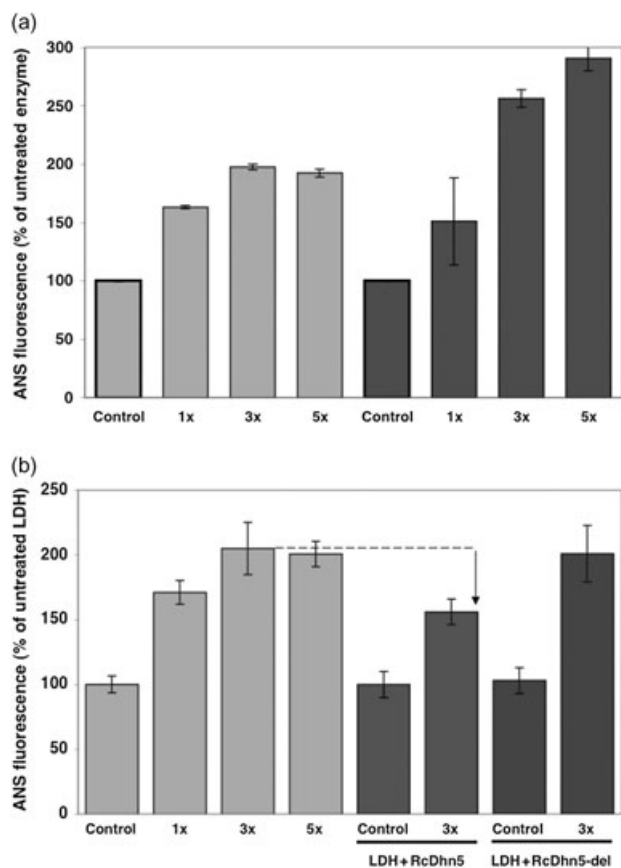


Figure 4. 1-Anilinonaphthalene-8-sulfonate (ANS) fluorescence monitors enzyme changes during freezing and hydrophilin protection. (a) Malate dehydrogenase and lactate dehydrogenase (LDH) show increased ANS fluorescence upon freezing *in vitro*. Samples containing enzyme were subjected to different freeze–thaw cycles as indicated, or left untreated (shown as 100, control). (b) Rh-Dhn5 presence limits the ANS fluorescence increase observed for LDH upon freezing. The untreated sample (corresponding to a value of 100) is used as reference for all other values. Samples containing enzyme with or without addition of RcDhn5 or RcDhn5-del (lacking the two K-segments) were subjected to different freeze–thaw cycles as indicated. A dashed horizontal line and an arrow were added to allow comparison between samples with or without RcDhn5 addition. Results represent the average of four to seven independent experiments and error bars indicate standard deviation.

assay where the remaining LDH activity for the enzyme alone was $61.6\% \pm 5.2$ after one cycle, while that of the LDH/RcDhn5 sample after three cycles was $59\% \pm 2.5$ (Fig. 3b). These results suggest that RcDhn5 helps maintain, at least partially, the native LDH structure and this is reflected in the protection of the LDH activity (Fig. 3b). The truncated version of RcDhn5 lacking the K-segments failed to protect LDH activity in the freeze–thaw assay as shown previously (see Fig. 3b). To test whether this behaviour was also paralleled by protein structural changes, we incubated LDH together with RcDhn5-del and performed three freeze–thaw cycles followed by ANS addition. The resulting fluorescence attained levels comparable to those of LDH

without any additives under the same treatment (Fig. 4b). Our data suggest that the absence of K-segments in RcDhn5-del interferes with its ability to protect LDH activity, resulting in structural changes that lead to enzyme inactivation. We were unable to measure the effect on ANS fluorescence of ERD10 and most other hydrophilins analyzed herein because of the intrinsic fluorescence of these proteins and resultant interference with experimental readings (data not shown). Also, because Sip18 did not show a strong protective behaviour in the freeze–thaw assays, it was not considered a good candidate for the test, in contrast to what we observed before in the partial water-loss assay (Reyes *et al.* 2005).

DISCUSSION

LDH activity is affected by freeze–thaw and can be protected by hydrophilins

We are fully aware that the use of *in vitro* assays implies several limitations to our understanding of protein activities *in vivo*. However, in recent years, significant insight has been gained about the basic mechanisms of action of several molecules, including chaperones, using such approaches (Sun & MacRae 2005; Basha, Friedrich & Vierling 2006).

Prior to this study, an *in vitro* freezing assay using LDH as the reporter enzyme had been reported (Carpenter & Crowe 1988; Lin & Thomashow 1992) and had been used to test the protecting activity of different additives such as reducing sugars and importantly, LEA proteins from different groups and species (Tunnacliffe & Wise 2007). We have adapted the assay to perform multiple freeze–thaw cycles that would allow us to better visualize the somewhat overlapping effects observed for the hydrophilins tested here. For instance, the protection offered by ERD10 and AtLEA4-5 is comparable after one and three cycles; however, after five cycles it is evident that ERD10 is a better protectant than AtLEA4-5 (Fig. 3). Thus, use of repeated cycles can uncover small differences that would be missed otherwise. We also attempted to use a different freeze–thaw regime, where the freezing step was carried out more gradually. However, this setup showed significant disadvantages; firstly, the assay became considerably more time-consuming, causing concerns about the accuracy of the enzyme activity measurements. Secondly, the reduction in activity due to freeze–thaw is less pronounced, thus minimizing and possibly masking the differences seen for different proteins added during the assay. In addition to LDH, we also tested MDH, another enzyme that showed sensitivity to freezing and could be used in our assays. Although only a selected set of experiments was carried out using MDH (data not shown), the results indicated that either LDH or MDH could be used for the assay, reinforcing the strength of our assay.

Changes in LDH or MDH activity after repeated cycles can be monitored at the protein structure level by the use of the fluorophore ANS. Its binding to hydrophobic residues in a given protein results in a quantifiable level of fluorescence (Stryer 1965). Inactivation of LDH or MDH during

freeze–thawing resulted in a steady increase in ANS fluorescence over the freeze–thaw cycles applied. This increase likely reflects an enhanced exposure of hydrophobic residues as the enzyme is further inactivated; however, we cannot rule out that a more complex pathway of enzyme denaturation is taking place and we can only determine the overall increase in fluorescence. Still, ANS fluorescence could be used to monitor changes in protein structure during the freeze–thaw treatment of LDH or MDH. The use of both the enzyme activity assay and the fluorescence assay allowed us to study the effects of hydrophilin addition to prevent enzyme denaturation during freezing *in vitro*.

In our earlier study, hydrophilin protection upon controlled water-removal showed that certain hydrophilins are able to protect LDH or MDH with distinct levels of efficacy (Reyes *et al.* 2005). The protection efficiency for different hydrophilins in a freeze–thaw test as observed in the current study also varied (see Fig. 2), and the degree of protection for each hydrophilin was not always identical to that observed during the partial water-loss assay. For instance, while Sip18 is a good protectant in the partial water-loss assay, it is less so in the freeze–thaw assay (Reyes *et al.* 2005). A similar behaviour was observed for AtLEA4-5 (Reyes *et al.* 2005). Thus, we suggest that conditions by which water limitation affects LDH activity are different between controlled water removal and freezing treatments, leading to distinct protection levels by hydrophilins. On the other hand, RcDhn5, ERD10 and YciG showed good protection in both assays, indicating that these three hydrophilins are able to protect LDH, irrespective of the form of stress (Reyes *et al.* 2005, Fig. 2a and data not shown). We also tested another LEA protein, Pv-LEA18, a group 6 LEA protein from *Phaseolus vulgaris*, which did not offer significant protection in either water removal or freezing assay (data not shown). Pv-LEA18 belongs to a highly conserved LEA protein family, whose members are induced upon water stress and during embryogenesis (Colmenero-Flores *et al.* 1999), thus its protection substrates may be molecules different from proteins, perhaps nucleic acids. We are currently exploring this possibility.

Contribution of protein domains to protective function

Characteristic protein sequence motifs have been recognized in the different families of LEA proteins. ERD10 contains an S-segment as well as three K-segments, while RcDhn5 contains an S-segment and two K-segments. AtLEA4-5 (group 4 LEA protein) contains an N-terminal half predicted to conform as an amphipathic α -helix and corresponds to the most conserved domain among family members, while the second C-terminal half has features of an unstructured random-coil (Dure 1993; Ingram & Bartels 1996; Battaglia *et al.* 2008). Here we have shown that removing specific domains from plant hydrophilins compromises their protective properties. This effect was more pronounced for RcDhn5, where removing both K-segments completely eliminated its ability to prevent freeze–thaw

inactivation of LDH. Although removal of K-segments present in ERD10 also resulted in reduced protection, the residual activity was easily detected. The less effective protection conferred by the RcDhn5 amino-terminal region lacking the K-segments, compared with the corresponding ERD10 region may be explained by the low similarity between these regions and because RcDhn5 amino-terminal region contains labile amino acids such as cysteine residues not present in the equivalent region of ERD10. Significantly, the presence of a single K-segment in the truncated ERD10-1K construct showed protection levels similar to those of the ERD10-s version lacking all three K-segments, while presence of a second K-segment provided ERD10-2K with protective qualities similar to those of the full-length ERD10. In contrast, the truncated version of AtLEA4-5 maintained the proposed amphipathic α -helix, and this shortened fragment consistently maintained the protective properties of the full-length protein (Fig. 3c). It is noteworthy that in all three cases, the conserved protein domains are highly hydrophilic. It is possible that upon decrease in water availability, the induction of a more stable conformation in ERD10, RcDhn5 and AtLEA4-5, and their interaction with protein domains in LDH are – at least in part – responsible for the protection of enzyme activity. In fact, it has been shown for a *Glycine max* LEA4 protein that its structure can change to conform to α -helices upon interaction with sodium dodecyl sulphate or in the dried state (Shih *et al.* 2004). This property could be also reflected in our previous observation that ERD10 and AtLEA4-5 fragments expressing these same domains are detrimental for growth when expressed in *E. coli* (Campos, Zamudio & Covarrubias 2006). In this case, the expression of certain hydrophilin domains in bacteria could be sequestering cellular components needed for optimal growth. From the aforementioned results, we suggest that the recognized domains present in the three hydrophilins tested are important for their protective activity. Although structural analyses had been carried out before for isolated protein domains in dehydrins (Mouillon, Gustafsson & Harryson 2006), our results represent the first analysis of individual domains present in LEA proteins and their contribution to protein function.

LDH protection and associated protein structure changes induced by RcDhn5

The changes in ANS fluorescence observed upon freeze–thaw treatment can be interpreted as a series of protein structural modifications that gradually expose different hydrophobic residues along the polypeptide, reflecting a decrease in enzyme activity. The ANS fluorescence of LDH exposed to freezing is gradually increased, suggesting that hydrophobic residues are being exposed to ANS interaction along the protein. Upon subsequent freeze–thaw cycles, the LDH protein structure must be altered further in a way that now exposes additional hydrophobic residues available to ANS binding (Fig. 4). The intensity or the rate of change in ANS fluorescence alone cannot explain how the structure

changes, as it only reflects the sum of hydrophobic groups available. However, it is a manifestation of a different architecture of the protein from the initial state.

Interestingly, although both the water-removal assay (Reyes *et al.* 2005) and the freeze–thaw assay (current study) result in a decrease in enzyme activity, the pattern of ANS fluorescence shows differences between the two ways of inactivating LDH. During partial water loss, ANS fluorescence initially decreased, followed by an increase upon subsequent water loss and finally reaching a two-fold increase over the level of the unstressed enzyme (Reyes *et al.* 2005). In contrast, freeze–thaw treatment resulted in a constant increase in ANS fluorescence (Fig. 4). We suggest that these particular profiles are indicative of different pathways in protein structural changes occurring due to the two treatments, and thus, water removal and freezing follow alternate routes of protein–water interactions. Still, we have shown that hydrophilins are capable of protecting enzyme function under both forms of stress. These results suggest that hydrophilins are capable of protecting different kinds of structure alteration, a process reminiscent of chaperone protection upon heat stress (Stirling *et al.* 2006). However, hydrophilins do not seem to correspond to cold stress chaperones; in our assay, we have observed that ATP addition does not increase protection efficiency of ERD10 and that its addition after freeze–thaw treatment of LDH does not restore its lost activity (data not shown), indicating that ERD10 does not act as a chaperone by reversing the effects of protein denaturation induced by freezing. However, other hydrophilins used in this study were not included in this test. On the contrary, we suggest that hydrophilins would prevent protein changes leading to enzyme inactivation during different stress treatments and that important components of this property reside in their flexible structure and highly hydrophilic and conserved domains. These domains would be involved in establishing particular interactions with the residual water and distinct proteins or other macromolecules, and we propose that this is a main component of their function *in vivo*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Proteins used in this study and their relevant features.

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