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Hydrophilins from distant organisms can protect enzymatic activities from water limitation effects *in vitro*

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ABSTRACT

Hydrophilins are a wide group of proteins whose defining characteristics are high hydrophilicity index (> 1.0) and high glycine content (> 6%). The transcripts of most hydrophilins accumulate in response to water deficit in organisms such as plants, fungi and bacteria. In plants, most of the known Late Embryogenesis Abundant (LEA) proteins belong to this group (Garay-Arroyo *et al*., *Journal of Biological Chemistry* 275, 5668–5674, 2000). To gain insight into the function of hydrophilins, an *in vitro* assay was developed in which the enzymes malate dehydrogenase (MDH) or lactate dehydrogenase (LDH) are subjected to controlled partial water removal. Subtle changes in conformation during partial water removal were detected using 1-anilinonaphtalene-8-sulphonate (ANS), a fluorescent probe, whose emission at 460 nm increases when bound to hydrophobic groups. The results show that water limitation conditions imposed in this *in vitro* assay induce changes in MDH or LDH protein structures, which correlate with enzyme inactivation. It is also shown that plant, fungal and bacterial hydrophilins are able to protect enzymatic activities from water-loss effects in this *in vitro* system, in a wide range of water potentials. In addition, the data in this work indicate that the presence of hydrophilins also avoids the MDH and LDH conformational modifications caused during the assay. These results show that hydrophilins are able to protect enzymatic activities from inactivation due to *in vitro* partial water limitation and thus suggest a function for these proteins *in vivo*.

Key-words: dehydrins; enzyme protection; hydrophilic proteins; hydrophilins; LEA proteins; protein stabilization; water deficit.

INTRODUCTION

Water stress may affect all types of organisms at some stage of their life cycle. Hence, they have developed a number of strategies to cope with water deficit, including changes in enzyme activities and gene expression patterns. In plants, hydrophilic proteins, known as Late Embryogenesis Abundant (LEA) proteins accumulate to high levels during the last stage of seed formation (when a natural desiccation of the seed tissues takes place) and during periods of water deficit in vegetative organs, suggesting a protective role of these proteins during water limitation (Bray 1997). LEA proteins have been grouped into at least six families on the basis of sequence similarity (Ingram & Bartels 1996; Colmenero-Flores *et al*. 1997, 1999). Although significant similarity has not been detected between the members of the different classes, a unifying and outstanding feature of these proteins is their high hydrophilicity and high percentage of glycine residues (Baker, Steele & Dure 1988; Dure 1993).

We have previously shown that most LEA proteins are comprised in a more widespread group, which we call 'hydrophilins'. Their defining characteristics are a glycine content >6% and a hydrophilicity index >1 (Anchordoguy & Carpenter 1996). By database searching, we showed that this criterion selects most known LEA proteins, as well as additional proteins from different taxons, suggesting that hydrophilins represent a functionally conserved adaptation to osmotic stress (Garay-Arroyo *et al*. 2000). Consistent with this idea, we found that within the genomes of *Escherichia coli* and *Saccharomyces cerevisiae,* the 5 and 12 proteins, respectively, that meet our criteria have transcripts that accumulate in response to osmotic stress (Garay-

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Arroyo *et al*. 2000; Posas *et al*. 2000; Yale & Bohnert 2001; Comadurán, G., unpublished results).

Although the functional role of hydrophilins remains speculative, there is evidence supporting their participation in acclimation and/or in the adaptive response to stress. In the case of some plant hydrophilins (LEA proteins), their ectopic expression in plants and yeast confers tolerance to water-deficit conditions (Imai *et al*. 1996; Xu *et al*. 1996; Swire-Clark & Marcotte 1999; Zhang *et al*. 2000), and their presence has been associated with chilling tolerance (Danyluk *et al*. 1998; Ismail, Hall & Close 1999; Rinne *et al*. 1999). Furthermore, deletion of the RMF hydrophilin gene of *E. coli* results in an osmosensitive phenotype (Garay-Arroyo *et al*. 2000).

To gain insight into the function of hydrophilins, we developed an *in vitro* partial water loss assay where the activity of malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) is measured in the presence or absence of a putative protecting protein. These enzymes were chosen as models to test enzyme inactivation during partial water removal because of their sensitivity to different stress conditions such as heat, freeze–thaw cycles and lyophilization (Carpenter, Prestrelski & Arakawa 1993; Dong *et al*. 1995; Anchordoguy & Carpenter 1996), as well as the ease to assay their enzymatic activity. Importantly, in this assay, the amount of water remaining after controlled evaporation is significantly larger than that attained during protein lyophilization or complete dehydration. However, the inhibitory effects of such treatment on enzyme activity were self-evident. Given that MDH and LDH activities were sensitive to different degrees of *in vitro* water removal, we were able to test the putative protective role of hydrophilins. To this end, we selected LEA proteins from different groups: dehydrins DSP16 and ERD10 (group 2), AtLEA76 (group 3), AtD113 (group 4) and PvLEA18 (group 6) (Ingram & Bartels 1996; Colmenero-Flores *et al*. 1997). In addition, we included hydrophilins from *E. coli*, YCIG, and from *S. cerevisiae*, Sip18, and tested the sensitivity of MDH and LDH activity to partial water loss (low water potentials) in the presence or absence of a putative protecting protein.

Our results show that, under the conditions tested, all hydrophilins, except PvLEA18, are more efficient protectants of enzyme activities during *in vitro* partial water loss than other unrelated proteins (RNAse A, βLactoglobulin and αCrystallin, a molecular chaperone). We also show that inactivation of MDH and LDH due to *in vitro* water removal correlates with changes in enzyme exposure of hydrophobic surfaces, as determined by 1-anilinonaphtalene-8-sulphonate (ANS) fluorescence assays. Furthermore, such changes are partially prevented by addition of DSP16 or SIP18. The overall results indicate that hydrophilins protect enzyme activities *in vitro* by a mechanism that prevents modification of the enzyme structure, which is affected upon partial water removal in the absence of a hydrophilin. These data show for the first time a protective *in vitro* function/activity for hydrophilins (LEA proteins included) against water limitation effects.

MATERIALS AND METHODS

Proteins and other chemicals

Bovine serum albumin (BSA) (initial fraction obtained by cold alcohol precipitation, globulin free), αCrystallin from bovine eye lens, βLactoglobulin from bovine milk, and trehalose were purchased from Sigma (St Louis, MO, USA). MDH (EC 1.1.1.37) from pig heart (mitochondrial) and LDH (EC 1.1.1.27) from hog muscle were purchased from Boehringer, (Mannheim, Germany). The commercial preparations of MDH and LDH were supplied in 50% glycerol, and their purity was confirmed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). The hydrophilins used were the dehydrins (group 2) DSP16 from *Craterostigma plantagineum* and ERD10 from *Arabidopsis thaliana;* AtLEA76, a group 3 LEA protein from *A. thaliana*; AtD113, a group 4 LEA protein from *A. thaliana*; PvLEA18, a group 6 LEA protein from *Phaseolus vulgaris.* In addition, we have included hydrophilins from *E. coli*, YCIG, and from *S. cerevisiae*, Sip18. The recombinant proteins PvLEA18, Sip18, YCIG, and DSP16 were expressed as soluble polypeptides containing an N-terminal His-tag carried in suitable pQE vectors (Qiagen, Hilden, Germany) and affinity purified on Ni-NTA Agarose resin (Qiagen) under native conditions. LEA proteins ERD10, D113 and LEA76 were expressed in *E. coli* as untagged proteins, and purified by boiling of a cell extract (Jepson & Close 1995) and subsequent ammonium sulphate fractionation followed by ionic exchange chromatography and dialysis. All purified proteins (up to 95–98% purity) were re-suspended in 150 mM potassium phosphate buffer, pH 7.5. Protein concentrations were determined by the Bradford protein assay and verified by SDS-PAGE.

Partial water loss assays

MDH and LDH were used to test the effect of additives on their activity upon exposure to *in vitro* partial water loss. Experiments were performed in Eppendorf tubes to avoid protein adsorption to glass. For MDH, the enzyme and the additive were dissolved in 50 mM potassium phosphate buffer, pH 7.2. For LDH, experiments were carried out in 25 mM Tris-HCl, pH 7.5. In both cases the final (monomer) enzyme concentration was 250 nM corresponding to 10 μg mL^{-1} of MDH and 8.3 μg mL^{-1} of LDH. The choice of enzyme concentration was based on the following considerations: (1) LDH concentrations higher than 10 μg mL^{-1} may induce self-protection (Carpenter *et al*. 1993; Anchordoguy & Carpenter 1996; Miller, Anderson & de Pablo 1998), and (2) stabilizers present in the commercial preparation may interfere with the assay. We therefore chose the lowest concentration compatible with the sensitivity of the enzyme assay.

Molar ratios of hydrophilin to enzyme were established as the minimum molar ratio at which a specific protein preserved full enzyme activity at a fixed dehydration level (99.5% water loss for MDH and 98.5% water loss for

LDH). The proteins tested in the MDH assays were added at a monomer concentration of 625 nM, corresponding to a protein:enzyme molar ratio of 2.5 : 1. For LDH assays, proteins tested were at 250 nM (monomer) and the molar ratio of protein : enzyme was 1 : 1. Aliquots (75 μL) of the mixtures were placed in a Speed-Vac concentrator (Savant Instruments, Holbrook, NY, USA) and water evaporated for various times to achieve the degree of water loss required. Evaporative cooling prevented heat denaturation of the sample, so freezing could be dispensed during vacuum-drying. The percentage of partial water loss was defined as the amount of water evaporated from the samples, determined by weighting the sample tubes in an analytical balance (Sartorius, Goettingen, Germany) before and after protein mixtures were added and after the evaporation period. To have a reference of the water status of the solutions in these assay, the osmolality of some samples was determined using a cryoscopic osmometer, Osmomat 030 (Gonotec, Berlin, Germany). An osmolality of 0.116 mol kg^{-1} (–2.83 bars) corresponded to a water loss of approximately 50%; 0.228 mol kg^{-1} (–5.07 bars) to approximately 75% of water loss; 0.398 mol kg^{-1} (–9.71 bars) to a water loss of approximately 87.5%; 0.765 mol kg^{-1} (–21.41 bars) was equivalent to approximately 93.75% water loss; and 1241 mol kg^{-1} (–30.24 bars) corresponded to a water loss of 96.87%.

The partially evaporated samples were restored to the initial weight with water either immediately or after 3 d of incubation at 25 °C. After water addition, samples were kept on ice, and care was taken to ensure that all solutes were completely re-suspended before determining enzyme activity. The initial activity was determined on aliquots that had been kept at 4 °C until all samples were ready to assay.

To evaluate the aggregation of MDH during partial water loss, 0.4 mL aliquots of MDH at the concentration of 630 nM monomer (the minimum concentration required for aggregation) in 50 mM potassium phosphate buffer, pH 7.2 were pipetted into Eppendorf tubes and water evaporated in a Speed-Vac concentrator. After partial water removal, samples were restored to the initial volume with water, and the degree of aggregation of MDH was determined by measuring the absorption due to increased turbidity from light scattering at 360 nm in a Beckman DU 600 spectrophotometer (Beckman-Coulter, Fullerton, CA, USA), as previously described (Lee 1995).

Enzyme activity measurements

MDH and LDH enzymatic activities were determined using aliquots of 8 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 (Boehringer). For LDH, the assay buffer was 25 mM Tris-HCl pH 7.5, 100 mM KCl containing 2 mM pyruvate (Sigma) and 0.15 mM NADH (Boehringer). 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.

ANS fluorescence assays

Changes in fluorescence due to binding of the fluorescent probe 1-anilinonaphtalene-8-sulphonate (ANS, Sigma) were measured using a DyNA Quant 200 instrument (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to manufacturer directions (supplement 5 to users manual). LDH or MDH were incubated in 25 mM Tris-HCl, pH 7.5. The final monomer concentration was 500 nM for both enzymes, instead of 250 nM as for the partial water loss assays. Hydrophilins were added at the same ratio as in the partial water loss assays. ANS was added to 5 μM final concentration in all cases. For determination of fluorescence, the same procedure described above for the partial water loss assays was used, and ANS was added immediately after the initial volume was restored with water. For heat-denaturation experiments, samples containing LDH or MDH were incubated for 40 min at 55 °C, placed on ice for 2 min and ANS added immediately after.

Protein–protein cross-linking

For cross-linking of hydrophilins to LDH or MDH we utilized Tris-bipyridylruthenium(II) di-cation ($Ru(II)bpy_3^{2+}$, Sigma) using the method described before with minor modifications (Fancy & Kodadek 1999). Briefly, after the standard partial water removal procedure (without rehydration), the protein mixture was brought to 10 μL containing 1.25 mM $Ru(II)bpy_3^{2+}$, 2.5 mM ammonium persulphate and it was flashed for 5 s through a 3 cm water-filter using a white light bulb as source (75 W). Samples were subsequently quenched with an equal volume of 2× Laemmli sample buffer containing 4% SDS and 10% β-mercaptoethanol, and resolved by SDS-PAGE. Protein bands were visualized by Western blot using anti-His tag antibody (Qiagen). To visualize LDH and MDH, enzymes were previously labelled with biotin (Boehringer) as indicated by the manufacturer, and membranes were developed with streptavidin-coupled horseradish peroxidase (Zymed, South San Francisco, CA, USA). As a reference, we also used two of the proteins that do not show protective properties in the water loss *in vitro* assays such as RNAse A and lysozyme.

All experimental data reported are means of, at least, three independent measurements with standard errors. Where no error bars are shown, the size of the symbol indicates deviations.

RESULTS

MDH and LDH activities are sensitive to controlled *in vitro* water loss

The rate of inactivation of the MDH and LDH activities during controlled water loss was determined after immedi-

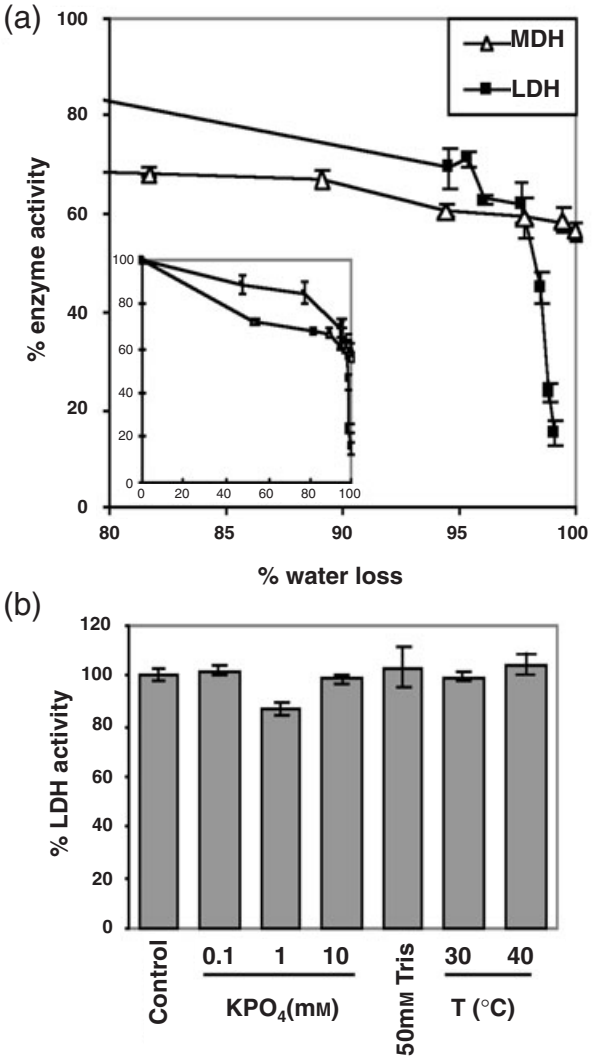

Figure 1. MDH and LDH enzyme activities decrease upon partial water loss. (a) After different degrees of partial water loss, MDH (△) and LDH (■) enzyme samples were immediately rehydrated and their remaining activity determined. The remaining activity is expressed as a percentage of control. (b) An LDH sample containing 25 mM Tris was partially dehydrated to 95% water loss at 25 °C (Control). Alternatively it contained varying initial concentrations of KPO_4 or Tris buffer or incubated at two different temperatures for 1 h as indicated.

ately restoring the samples to their initial volume. MDH and LDH activities decreased during this process and the rate of inactivation was different for each enzyme (Fig. 1a, inset). MDH was more sensitive than LDH to mild water loss treatments. MDH lost about 40% of its initial activity during the process (Fig. 1a), whereas LDH retained between 70 and 80% of its initial activity even after 99% water loss was attained (Fig. 1a). Beyond this point, LDH activity dropped to 10% whereas MDH retained about 55% of its initial activity. When both enzymes were partially dehydrated and kept at 25 °C for 3 d, their activities showed a larger decrease in the range of water loss tested (15–5% of its initial activity, data not shown).

Control experiments using different initial concentrations of the ions present in the buffer used in these assays (K^+, $Tris^-$, PO_4^-) were carried out. In the case of LDH, increasing K^+ and PO_4^- concentrations as high as 100-fold, or $Tris^-$ up to two-fold, did not affect its activity (Fig. 1b). For MDH, increasing K^+ and PO_4^- concentrations as high as 20-fold did not show any negative effects on enzyme activity (data not shown). Although temperature was carefully controlled during the assay, we confirmed that temperatures as high as 40 °C did not reduced LDH or MDH activities (Fig. 1b and data not shown). These results indicate that the decrease in enzyme activity observed during partial water loss was not due to the resulting increase in ion concentration or to eventual temperature fluctuations.

Enzyme structural changes during partial water loss

In order to correlate the loss of enzymatic activity detected during the water loss assay with possible structural modifications, we used two different approaches. Initially, protein unfolding was determined by measuring MDH aggregation in samples that had been subjected to partial water loss to various extents and rehydrated immediately. No aggregation of MDH was detected in a range of water loss (29.2 to 0.6% of remaining water, approximately –7 to –40 bars) suggesting that no extensive irreversible protein denaturation occurs under these conditions (data not shown). To detect subtle changes in conformation during partial water removal, a different, more sensitive method using ANS, a fluorescent probe, was applied. In water, ANS is essentially non-fluorescent, but its emission at 460 nm increases when bound to different hydrophobic groups (Suarez-Varela, Sanchez-Macho & Minones 1992). Thus, if the decrease in LDH or MDH activity during *in vitro* partial dehydration is the result of structural modifications, ANS would bind differently to the enzyme and change the fluorescent signal of the sample. When LDH or MDH were incubated in the presence of 5 μM ANS, low fluorescence levels were detected (no treatment, Table 1). Upon partial water removal treatment, two different stages were observed. Initially, between 90 and 98% water loss the overall ANS fluorescence was reduced by 20 and 50% for MDH and LDH, respectively (Table 1). At 99% water loss, fluorescence returned to levels similar to those of the non-treated samples, and at 99.4% water loss, fluorescence increased by nearly two-fold in LDH samples. In contrast, ANS autofluorescence did not increase over the initial value, even at 99.5% water loss. However, when MDH samples with the same water loss levels were further incubated for 3 d before being rehydrated, ANS fluorescence increased by 1.6-fold (Table 1). Changes in ANS fluorescence due to the conditions imposed by the partial dehydration were compared with those of parallel samples exposed to heat denaturation, in which a substantial unfolding of the enzymes occurs. The exposure of enzyme samples to 55 °C for 40 min

Table 1. ANS fluorescence of LDH during partial water loss

	Treatment	Fold change[a]		
		No additive[b]	+DSP16[b]	+SIP18[b]
LDH	None	1.0 ± 0.06	0.98 ± 0.10	1.0
	93.6% water loss[b]	0.5 ± 0.05	0.74 ± 0.02	1.1 ± 0.08
	95.2% water loss[b]	0.64 ± 0.1	ND	0.88 ± 0.07
	96.3% water loss[b]	0.66 ± 0.07	1.03 ± 0.14	0.92 ± 0.14
	98.8% water loss[b]	0.87 ± 0.03	ND	ND
	99.4% water loss[b]	1.80 ± 0.08	1.7 ± 0.13	ND
	40 min at 55 °C[b]	2.62 ± 0.13		
MDH	None	1.0 ± 0.06		
	96–99.1% water loss[b]	0.79 ± 0.08		
	99.3% water loss[b]	0.96 ± 0.01		
	99.5% water loss 3 d[b]	1.6 ± 0.08		
	40 min at 55 °C[b]	3.1 ± 0.12		

[a]With respect to the control sample without treatment. [b]Mean values of at least three independent measurements. ND, not determined.

resulted in a three-fold increase in ANS fluorescence for both MDH and LDH (Table 1). Accordingly, all observed changes in ANS fluorescence correlated with a decrease in both enzymatic activities (see Fig. 1a).

Different hydrophilins protect enzymatic activities during *in vitro* partial water loss

Since MDH and LDH activities are sensitive in the partial water loss assay established, we assessed whether hydrophilins play a protective role under these conditions. As reference, other proteins such as BSA, αCrystallin, RNAse A and βLactoglobulin were also tested. BSA is used as a protein stabilizer in many enzyme activity assays (Chang & Mahoney 1995) and is generally considered to be a cryoprotectant (Tamiya *et al*. 1985). αCrystallin is a molecular chaperone, which is able to prevent aggregation of heat-labile proteins (Boyle & Takemoto 1994; Horwitz 1992). RNAse A and βLactoglobulin are proteins unrelated to stress but are very stable and have molecular weight similar to the hydrophilins tested here. Using LDH as a target enzyme in this assay, we first tested two dehydrins, DSP16 and ERD10. At water loss levels up to 98% (approximately –30.24 bars), BSA recovered 75% of the enzyme activity, whereas αCrystallin, RNAse A and βLactoglobulin did not show any protective effect (Fig. 2a and data not shown). Under the same conditions, ERD10 and DSP16 showed protection levels that recovered LDH activity between 90% and 100% (Fig. 2a). In samples that had reached up to 99% water loss (approximately –50 bars) and immediately rehydrated, only DSP16 induced full protection of LDH activity. When LEA proteins from groups 3 and 4 (AtLEA76 and AtD113) were tested, protection levels similar to those of ERD10 were observed (compare Fig. 2a & b). In contrast, a group 6 LEA protein, PvLEA18, did not show any significant effect under the conditions assayed (Fig. 2b). Similar protective effects were observed when MDH was used as target enzyme (data not shown).

To further analyse the role of other hydrophilins we have extended the analysis to include those from organisms different to plants, namely one from *E. coli* (YCIG) and another from *S. cerevisiae* (Sip18). These proteins were selected since they possess the highest scores as hydrophilins as defined by Garay-Arroyo *et al*. (2000). As shown in Fig. 3, YCIG confers significant protection to LDH, up to 99% of water removal (approximately –50 bars), similar to ERD10 and DSP16. Sip18 confers protection to LDH to the same extent as BSA up to 95% of water loss (approximately –19 bars), however, between 96 and 99% of water loss (approximately –20 and –50 bars), Sip18 was a better protector (Fig. 3). When trehalose (25–100 mM), another well-known stabilizer of enzyme activities, was added in this assay, the recovered LDH activity was similar to that of Sip18 (Fig. 3). In the case of MDH, similar effects were observed (data not shown). For all hydrophilins tested, when water loss was larger than 99% (>–50 bars), LDH and MDH activities decreased dramatically in all samples and none of the proteins exerted protection.

Thus, except for PvLEA-18, we observed significant *in vitro* protection provided by hydrophilins from plant, fungi and bacteria of the activities of two enzymes.

Hydrophilins prevent structural changes in LDH upon partial water loss

Since hydrophilins showed a positive effect on the enzymatic activity recovered after partial water loss, we asked whether this effect correlated with conformational changes as measured by ANS. To this end we selected two hydrophilins from different organisms, DSP16 and Sip18. When LDH samples were partially dehydrated between 93 and 96% (approximately –18 and –30 bars) in the presence of DSP16, the fluorescence levels recovered were higher and more similar to those of the untreated samples. At 99.4% water loss (approximately –59.5 bars), no significant change in fluorescence was observed (Table 1). When Sip18 was

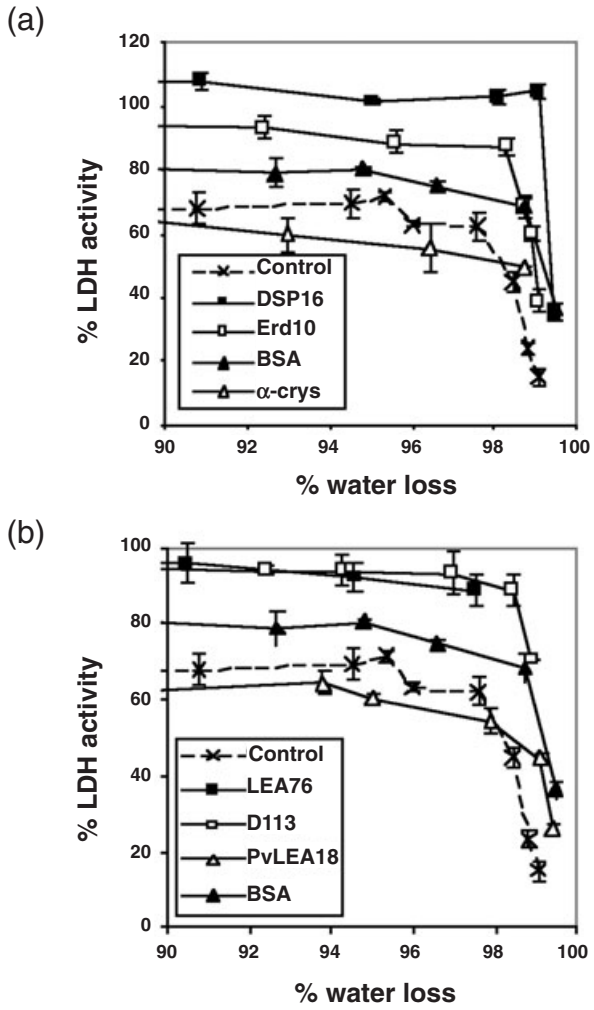

Figure 2. Effect of plant hydrophilins on LDH enzyme activity during partial water loss. LDH samples were subjected to partial water loss, immediately rehydrated and assayed for enzymatic activity. The final concentrations of LDH and proteins were at 250 nM (monomeric form) (the molar ratio of enzyme to protein was 1 : 1). In (a) a dashed line indicates enzyme activity without additives, the following symbols indicate the additives: (■) DSP16; (□) ERD10; (▲) BSA; and (△) αCrystallin. In (b) a dashed line indicates enzyme activity without additives: (■) LEA76; (□) D113; (▲) BSA; and (△) PvLEA18.

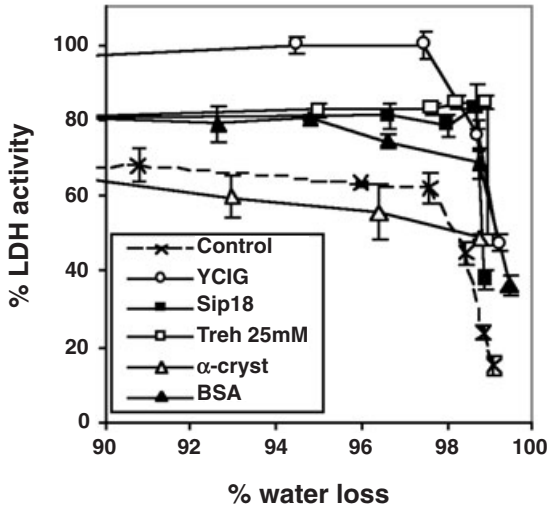

Figure 3. Effect of bacterial and yeast hydrophilins on LDH activity during dehydration. LDH samples were subjected to partial water removal, immediately rehydrated and assayed for enzymatic activity. The final concentrations of LDH and proteins were as in Fig. 2. The dashed line indicates enzyme activity without additives, the following symbols indicate the additives: (○) YCIG; (■) Sip18; (□) Trehalose 25 mM; (▲) BSA; and (●) αCrystallin.

used, similar results were obtained (Table 1). In untreated samples the presence of DSP16 or Sip18 does not increase the ANS fluorescence signal above that of LDH alone. In addition, we determined that either hydrophilin alone did not show any fluorescence before or after partial dehydration (data not shown).

Hydrophilins interact with LDH and MDH

To investigate the mechanism of enzyme protection, we asked whether a hydrophilin–enzyme interaction occurs during the process of gradual water removal. To address this question we used a ruthenium salt ($Ru(II)bpy_3^{2+}$) as a short-range cross-linking agent to detect protein–protein interactions (Fancy & Kodadek 1999). Samples containing LDH and Sip18 were subjected to partial water removal until 98% water was lost. At this point the samples were incubated in the presence of 1.25 mM ruthenium salt and flashed with an intense white light. Products of cross-linking were resolved in 12% SDS-PAGE and visualized by Western blotting using anti-His tag antibody (Fig. 4, lanes

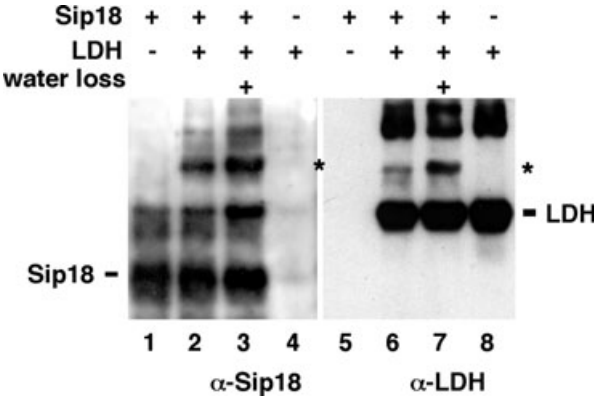

Figure 4. Hydrophilins interact with enzymes during partial water loss. Protein–protein cross-linking reactions using $Ru(II)bpy_3^{2+}$ were set with Sip18 alone (lanes 1 and 5), Sip18 and biotinylated LDH (lanes 2, 3, 6 and 7) or biotinylated LDH alone (lanes 4 and 8). LDH was incubated in the presence of Sip18 either as a concentrated mixture (lanes 2 and 6) or after 98% water loss (lanes 3 and7). Cross-linking products were resolved by 12% SDS-PAGE and visualized by Western blot with anti-His tag antibody (lanes 1–4) or with HRP-coupled streptavidin (lanes 5–8). The position of the LDH:Sip18 product is indicated by an asterisk.

1–4). Incubation of Sip18 alone under cross-linking conditions (lane 1) revealed that the fastest migrating species corresponded to Sip18, whereas the slower migrating bands are generated upon cross-linking of Sip18. Although LDH by itself does not cross-react with anti-His tag antibody (lane 4) a slower migrating species is generated when LDH and Sip18p are incubated together (lanes 2 and 3, indicated by an asterisk). The signal in this band is weaker when both proteins are incubated without prior treatment (lane 2), or absent when cross-linking is omitted (not shown), but it is enhanced upon partial water loss and cross-linking conditions (lane 3). To determine whether this band contains LDH, a Western blot was carried out to detect the previously biotinylated LDH on the same membrane. In addition to the LDH monomer and dimer forms (lane 8), a 50 kDa band was detected (lanes 6 and 7, indicated by an asterisk), that overlapped with the one detected in lanes 2 and 3. A similar cross-linking product was also detected when Sip18 and MDH were used in the assay (data not shown). These results are in agreement with a direct interaction between Sip18 and both enzymes tested under *in vitro* water-deficit conditions. We also detected a faint band corresponding to cross-link products between LDH and other proteins, which did not show any protective characteristics, such as RNAse A and lysozyme (data not shown) suggesting that a direct interaction with a hydrophilin is not necessarily sufficient to confer protection.

DISCUSSION

The aim of this study was to analyse the potential role of different water deficit-induced hydrophilins to protect or preserve enzymatic activities. Among the hydrophilins used, we included two dehydrins (group 2 LEA proteins), DSP16, a dehydrin that accumulates to high levels during dehydration in *C. plantagineum* (Piatkowski *et al*. 1990; Schneider *et al*. 1993), a plant that is able to survive water losses greater than 90% (Gaff 1971) and, ERD10, an acidic dehydrin from *A. thaliana*, responsive to freezing and dehydration conditions (Kiyosue, Yamaguchi-Shinozaki & Shinozaki 1994; Welin *et al*. 1994; Delseny *et al*. 2001; Seki *et al*. 2001). Furthermore, one representative LEA protein from each of groups 3, 4 and 6 was incorporated in this study. AtLEA76 and AtD113 correspond to groups 3 and 4 LEA proteins, respectively, from *A. thaliana*, whereas PvLEA18 is a group 6 LEA protein from *P. vulgaris*. All of them accumulate in the dry embryo and endosperm, and in vegetative tissues exposed to water deficit (Baker, Steele & Dure 1988; Lin & Thomashow 1992; Colmenero-Flores *et al*. 1999; Delseny *et al*. 2001; Seki *et al*. 2001). Finally, we also included one hydrophilin from *E. coli*, YCIG, and one from *S. cerevisiae*, Sip18, both responsive to osmotic shock (Miralles & Serrano 1995, Garay-Arroyo *et al*. 2000).

We have established an *in vitro* enzymatic test based on the progressive removal of water in the absence of other perturbing factors, such as heating or freezing. In this assay, we have used MDH and LDH, two enzymes known to be sensitive to a number of different stress conditions such as heat, lyophilization and freeze–thaw cycles (Lin & Thomashow 1992; Dong *et al*. 1995; Lee 1995; Anchordoguy & Carpenter 1996; Miller *et al*. 1998). Under the conditions tested, both enzymes gradually decrease their activity, albeit with slightly different kinetics (Fig. 1). Using ANS fluorescence assays with both MDH and LDH, we detected a clear change in the enzyme exposure of hydrophobic regions during partial water removal, correlating with the gradual loss of enzymatic activity. In a particular range of partial water loss, both enzymes exhibit a decreased level of ANS fluorescence, suggesting that hydrophobic residues are masked under these conditions. This observation is in agreement with previous reports that show that high concentration of different osmolytes shifts the equilibrium between protein states toward the most compact conformation, and induces a decrease in the volume and compressibility of protein interior by elimination of lubricant water, which may be functionally important for the active-site region (Priev *et al*. 1996; Kendrick *et al*. 1997; Nagendra, Sukumar & Vijayan 1998; Qu, Bolen & Bolen 1998; Madhavarao *et al*. 2001). As partial water loss reaches values above 99%, LDH-ANS increases its fluorescence by nearly two-fold, the same phenomenon was observed for MDH only after storage in the partially dehydrated state (Table 1). In both cases, a dramatic drop of activity correlates with this increase in ANS fluorescence (Fig. 1 & Table 1), thus indicating the occurrence of structural changes leading to exposure of hydrophobic residues, as has been observed for a number of proteins (see for example Festy *et al*. 1998; Leydier *et al*. 1998).

In addition to removal of water or to the effects of low water potentials, other factors could influence the loss of enzymatic activity during the water loss assay. One such factor could be the toxicity imposed by the transient increase in ion concentrations resulting from water loss, which at 99% water loss would increase by a 100-fold. To address this issue, we carried out experiments where $Tris^-$, K^+, PO_4^- initial concentrations were modified prior to partial dehydration of LDH. Additionally, we ruled out the possibility that minimal temperature fluctuations affect enzyme activity during the assay. These changes did not modify the profile of LDH activity loss upon partial water removal (Fig. 1b) indicating that ion toxicity and temperature are not responsible for enzyme inactivation in this assay. The amount of water available upon 99% water removal corresponds to an osmolality of 2.15 mol kg^{-1} (equivalent to approximately –55 bars), about 10-fold smaller than the one obtained after lyophilization and close to that present in some dried seeds (Hoekstra, Golovina & Buitink 2001), suggesting that the osmotic potentials attained in this *in vitro* assay are responsible for the effects observed. It is noteworthy that the available water (–10 to –30 bars) after the different degrees of water loss in this assay is within the range where desiccation sensitive organisms respond to a severe water stress (Hoekstra *et al*. 2001).

The sensitivity of MDH and LDH activities to *in vitro* partial water loss allowed us to investigate the protective effect of different hydrophilins. The overall results show

that during low water availability conditions (approximately –18 to –59 bars), most hydrophilins provide a level of protection of enzyme activities superior to that of BSA or trehalose, two well-known stabilizers. It is important to note that the trehalose concentration (25 mM) required to detect protection in this assay is 10^5-fold higher, on a molar basis, than that used for hydrophilins (0.25 μM, 1 : 1 enzyme : hydrophilin molar ratio for LDH) to obtain a similar effect, suggesting different protective mechanisms and a unique mechanism of action for hydrophilins. The protective effects of BSA, trehalose and βLactoglobulin are in agreement with their previously described cryoprotective action (Kazuoka & Oeda 1994). Only Sip18 showed a lower level of protection, comparable with that of BSA, suggesting that either it is not as active as other hydrophilins or its protection properties were not optimal under the conditions tested. In the case of PvLEA18, the results obtained suggest that, unlike other hydrophilins, it does not function as a protector of other proteins. Even though the protective characteristics of PvLEA18 could be restricted to mild water stress conditions, we cannot exclude a more complex scenario for its function, such as the need of post-translational modifications (phosphorylation or glycosylation) for optimal function, as previously suggested (Colmenero-Flores *et al*. 1999). Consistent with this last observation, the hydrophilic nature of these proteins alone would not be sufficient to confer protection, since addition of a highly hydrophilic polymer such as poly-lysine did not affect the rate of enzyme inactivation (data not shown).

In this work, we also sought to gain insight into the mechanism by which hydrophilins protect enzyme activities. As has been proposed for dehydrins (Close 1997), hydrophilins in general could stabilize cellular structures and macromolecules. Their highly hydrophilic segments could order water molecules around macromolecules thereby preventing the exposure of hydrophobic domains to the solvent. For instance, it has been shown that group 1 and 2 LEA proteins exist in equilibrium with two extended conformational states: unordered and left-handed extended helical (PII-like) structures, suggesting that a large area for water binding constitutes the basis of their functional role (Soulages *et al*. 2002; 2003). Furthermore, there is evidence indicating that a group 3 LEA protein assumes an entirely unordered conformation in solution, but upon drying, the protein adopts a largely α-helical structure (Wolkers *et al*. 2001). These results suggest that LEA proteins' structure can be strongly influenced by their immediate environment. Alternatively, hydrophilins could use their own polar residues to interact with the surface groups of other proteins, effectively replacing water (Close 1997; Hoekstra *et al*. 2001). This last possibility is more likely to occur in a more severe dehydration stage (Leydier *et al*. 1998). Here, we show that the decrease in ANS fluorescence observed at 93–97% water loss (approximately –30 to –59 bars) is partially rescued upon addition of two different hydrophilins (Table 1). These results are consistent with the hypothesis that hydrophilins prevent conformational changes of target proteins that would lead to their inactivation under water limitation. In addition, using a short-range cross-linking approach, we established that an interaction between MDH, or LDH, with Sip18 occurs under the water limitation conditions reached in this assay (Fig. 4), suggesting that direct interaction between hydrophilins and target proteins is part of the protection mechanism. However, the fact that we detected cross-link products between LDH and other proteins under these conditions (RNAse A and lysozyme, data not shown), indicates that a direct interaction with a hydrophilin is not necessarily sufficient to confer protection. These findings, together with the physicochemical properties of hydrophilins lead us to suggest that these proteins preserve enzyme activities during low water availability or low osmotic potentials by a mechanism involving both organizing water molecules and a direct protein–protein interaction which would help to prevent enzyme changes in tertiary structure.

In conclusion, this study describes a method for testing the protective effects of proteins on enzyme activity during *in vitro* partial water loss conditions. In addition, we show for the first time that hydrophilins (including LEA proteins) are able to protect enzyme activities *in vitro* in a wide range of water availability levels. The use of this assay should make possible the dissection of the protective effect of LEA proteins and other hydrophilins.

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