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The intrinsically disordered protein LEA7 from *Arabidopsis thaliana* protects the isolated enzyme lactate dehydrogenase and enzymes in a soluble leaf proteome during freezing and drying

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ABSTRACT

The accumulation of Late Embryogenesis Abundant (LEA) proteins in plants is associated with tolerance against stresses such as freezing and desiccation. Two main functions have been attributed to LEA proteins: membrane stabilization and enzyme protection. We have hypothesized previously that LEA7 from *Arabidopsis thaliana* may stabilize membranes because it interacts with liposomes in the dry state. Here we show that LEA7, contrary to this expectation, did not stabilize liposomes during drying and rehydration. Instead, it partially preserved the activity of the enzyme lactate dehydrogenase (LDH) during drying and freezing. Fourier-transform infrared (FTIR) spectroscopy showed no evidence of aggregation of LDH in the dry or rehydrated state under conditions that lead to complete loss of activity. To approximate the complex influence of intracellular conditions on the protective effects of a LEA protein in a convenient in-vitro assay, we measured the activity of two Arabidopsis enzymes (glucose-6-P dehydrogenase and ADP-glucose pyrophosphorylase) in total soluble leaf protein extract (Arabidopsis soluble proteome, ASP) after drying and rehydration or freezing and thawing. LEA7 partially preserved the activity of both enzymes under these conditions, suggesting its role as an enzyme protectant in vivo. Further FTIR analyses indicated the partial reversibility of protein aggregation in the dry ASP during rehydration. Similarly, aggregation in the dry ASP was strongly reduced by LEA7. In addition, mixtures of LEA7 with sucrose or verbascose reduced aggregation more than the single additives, presumably through the effects of the protein on the H-bonding network of the sugar glasses.

1. Introduction

Anhydrobiosis or "life without water" is a phenomenon that has received much attention and although mechanisms responsible for cellular desiccation tolerance have been proposed (e.g. [1,2]), many functional aspects are still unresolved [3]. There is, however, widespread consensus that sugars and Late Embryogenesis Abundant (LEA) proteins can be major contributors to cell stability in the dry state, even in cells that naturally do not contain LEA proteins [4]. In addition, some organisms can achieve desiccation tolerance without the accumulation of sugars [5,6]. LEA proteins have been first identified in plant seeds during maturation, when the seeds attain desiccation tolerance [7], but were later also found in vegetative plant organs, in bacteria and various anhydrobiotic invertebrates [8,9].

The precise in vivo function of most LEA proteins remains unresolved, which may at least in part be due to their unstructured nature in solution, which has made functional predictions impossible. However, many of these proteins fold mainly into α-helices during drying [10]. Results from various in vitro assays suggest that some LEA proteins are involved in the stabilization of cellular constituents such as proteins and membranes, but other functions have also been proposed [8,9]. Only for the cold induced *Arabidopsis thaliana* LEA proteins COR15A and COR15B, membrane stabilization during freezing could be clearly established as their in vivo function, while enzyme stabilization could be excluded [11]. However, many in vitro investigations have shown that LEA proteins can effectively prevent inactivation of sensitive enzymes such as lactate dehydrogenase (LDH) during freezing or drying [9,12]. Under the appropriate drying conditions such enzymes aggregate, which may contribute to their inactivation. Aggregation can be prevented by many LEA proteins that are thought to function as "molecular shields" by preventing direct contact between enzyme molecules [13–15].

Abbreviations: AGPase, ADP-glucose-pyrophosphorylase; ASP, Arabidopsis soluble proteome; CF, carboxyfluoresceine; DTT, dithiothreitol; FTIR, Fourier-transform infrared; G6PDH, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase; LEA, late embryogenesis abundant; LG, β-lactoglobulin; PMSF, phenylmethylsulfonyl fluoride; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine; RNaseA, ribonuclease A.

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In addition, as an adaptive response to water loss, most organisms accumulate compatible solutes, such as sugars [16]. During drying, most sugars do not crystallize, but rather form a glass. Due to the low molecular mobility, the glassy state immobilizes macromolecules, thus providing protection e.g. to cells in dry plant seeds [17,18]. LEA proteins can be embedded in such a glassy matrix, increasing the glass transition temperature [19]. It has been proposed that H-bonding interactions between sugars and proteins may enhance the stability of cytoplasmic glasses in seeds and pollen, thereby contributing to the exceptional stability of these structures in the dry state [2,18,20].

The aim of the present study was to investigate the functional activity of the structurally disordered protein LEA7 from *A. thaliana* with respect to dehydration and freezing stress. LEA7 is located in the cytosolic compartment of plant cells [21] and increases the desiccation tolerance of transgenic yeast cells [22]. In a previous study we presented evidence that LEA7 is able to interact with lipid membranes in the fully hydrated and in the dry state [23], suggesting a function of the protein in membrane stabilization. Here, we show that LEA7 is not able to protect liposomes during dehydration, but rather has protective activity for the labile enzyme LDH and enzymes found in the total soluble proteome of Arabidopsis leaves. We present evidence from Fourier-transform infrared (FTIR) spectroscopy for interactions between LEA7 and LDH and also between LEA7 and the Arabidopsis proteome, thus decreasing the degree of protein aggregation and preservation of enzyme activity. In the presence of LEA7 the average strength of H-bonding interactions in dry sugar (sucrose and verbascose) glasses was increased, indicating that protein and sugars interact to form a more tightly packed matrix in comparison to pure sugars.

2. Materials and methods

2.1. Materials

Lactate dehydrogenase from rabbit muscle (LDH), β-lactoglobulin (LG) from bovine milk and sucrose were obtained from Sigma (St. Louis, MO), verbascose from Megazyme (Wicklow, Ireland). RNaseA from bovine pancreas (RNaseA) was from Roche (Basel, Switzerland). D_2O (99.98%) was purchased from Deutero GmbH (Kastellaun, Germany) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) was obtained from Avanti Polar Lipids (Alabaster, AL).

2.2. Expression and purification of recombinant LEA7

The *LEA7* gene (At1g52690) was cloned into the pDEST17 vector for expression with an N-terminal 6xHis-tag in *E. coli* strain BL21 Star (Invitrogen). Cloning, protein expression and purification have been described in a recent publication [23].

2.3. Liposome stability assays

POPC (10 mg) was dried from chloroform under a gentle stream of N_2 and subsequently under vacuum over night. Liposomes encapsulating 100 mM CF, 10 mM TES, 0.1 mM EDTA were formed using a handheld extruder (Avestin, Ottawa, Canada) with two layers of 100 nm pore filters [24]. External CF was removed by passing the liposomes through a Sephadex G-25 column (NAP-5, GE Healthcare) in 10 mM TES, 0.1 mM EDTA and 50 mM NaCl (TEN buffer, pH 7.4). Liposomes (10 mg/ml in TEN) were mixed with the same volume of proteins (3.33 mg/ml in TEN) and the samples were dried over silica gel in desiccators at 28 °C for 24 h in the dark. Samples were rehydrated in TEN and CF fluorescence was measured with a Fluoroskan Ascent (Labsystems, Helsinki, Finland) fluorescence microplate reader at an excitation wavelength of 444 nm and an emission wavelength of 555 nm [25,26]. While fluorescence is strongly quenched at the high concentration inside the intact liposomes, it increases when CF is released into the surrounding buffer. The total CF fluorescence (i.e. 100% leakage) was determined after lysis of the liposomes with Triton X-100 and % leakage was calculated relative to these fluorescence values [27,28].

2.4. Preparation of Arabidopsis leaf soluble proteome

Rosette leaves from *A. thaliana* were frozen in liquid nitrogen immediately after sampling and homogenized using a ball mill "Retsch MM 200" (Retsch, Haan, Germany). To 20 mg of frozen leaf powder, 1 ml of extraction buffer (50 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 1 mM ε-aminocapronic acid, 0.5 mM DTT, 1 mM PMSF, 0.1% Triton X-100, 20 μM leupeptin and 10 mg polyvinylpyrrolidone) was added. The sample was mixed thoroughly and centrifuged for 4 min at 14,000 ×*g* at 4 °C. The supernatant was passed through an NAP-5 column pre-equilibrated with extraction buffer without Triton X-100. This Arabidopsis soluble proteome (ASP) was immediately used for freezing and drying experiments.

2.5. Enzyme stability during desiccation and freezing

Samples were prepared in the wells of 96-well plates at a final volume of 4 μl in a randomized design. The effects of LEA7 and the reference proteins RNaseA and LG on LDH activity after drying and rehydration or after freeze–thaw cycles between liquid nitrogen and room temperature were tested as described recently [11]. Residual LDH activity after treatment was expressed relative to the initial activity (residual activity = (mean reaction rate of frozen or dried samples/mean reaction rate of fresh control samples) × 100).

LEA7 or reference proteins dissolved in the extraction buffer described above were mixed with ASP at a mass ratio of 5:1. The desiccation treatment was the same as for LDH and the samples were rehydrated with extraction buffer. To determine the freezing sensitivity of enzymes in the proteome, samples were frozen in liquid nitrogen for 1.5 min and then stored at −20 °C over night. Enzyme activities were determined after thawing in air at room temperature. Enzyme activity assays were performed for G6PDH (EC 1.1.1.49) and AGPase (EC 2.7.7.27) using a robot-based platform described previously [29].

2.6. FTIR spectroscopy

LEA7, reference proteins, Suc and Ver were dissolved in water. ASP was transferred into water by gel filtration chromatography as described above. ASP was mixed with all other components at the ratios indicated in the figure legends. Dry samples were prepared by spreading 50 μl on a CaF_2 window that was stored in a desiccator over silica gel at 26 °C for 24 h. Hydrated samples were dissolved in D_2O because H_2O shows an absorbance peak around 1645 cm^{-1} that overlaps with the Amide I peak of the proteins, while the D_2O peak is shifted to about 1200 cm^{-1}.

FTIR measurements were performed on a Perkin Elmer GX2000 FTIR spectrometer. Windows with dry samples were fixed in the sample holder of a vacuum chamber in the infrared beam as described before [30,31]. Samples hydrated in D_2O were analyzed between two windows separated by a 0.01 mm Teflon spacer in the same cuvette at ambient pressure [30]. Measurements were performed at 30 °C and 64 scans were accumulated. Changes in the secondary structure of proteins were estimated by analyzing the Amide I peak (1700–1600 cm^{-1}). Second derivative spectra were calculated with a five point window using Spectrum 5.0.1 software.

To analyze H-bonding interactions in the glassy state of Suc and Ver, dry samples were measured using the ATR Golden Gate device (http://www.specac.com/products/golden-gate-atr-ftir-accessory/). The position of the OH vibration peak (*ν*OH) was determined after ATR and base line correction by Spectrum 5.0.1 software. Samples (5 μl), containing sugars with or without LEA7 were applied on the ATR crystal, air dried for 20 min and then 64 scans were accumulated.

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