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## An abundant LEA protein in the anhydrobiotic midge, PvLEA4, acts as a molecular shield by limiting growth of aggregating protein particles



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LEA proteins are found in anhydrobiotes and are thought to be associated with the acquisition of desiccation tolerance. The sleeping chironomid *Polypedium vanderplanki*, which can survive in an almost completely desiccated state throughout the larval stage, accumulates LEA proteins in response to desiccation and high salinity conditions. However, the biochemical functions of these proteins remain unclear. Here, we report the characterization of a novel chironomid LEA protein, PvLEA4, which is the most highly accumulated LEA protein in desiccated larvae. Cytoplasmic-soluble PvLEA4 showed many typical characteristics of group 3 LEA proteins (G3LEAs), such as desiccation-inducible accumulation, high hydrophilicity, folding into  $\alpha$ -helices on drying, and the ability to reduce aggregation of dehydration-sensitive proteins. This last property of LEA proteins has been termed molecular shield function. To further investigate the molecular shield activity of PvLEA4, we introduced two distinct methods, turbidity measurement and dynamic light scattering (DLS). Turbidity measurements demonstrated that both PvLEA4, and BSA as a positive control, reduced aggregation in  $\alpha$ -casein subjected to desiccation and rehydration. However, DLS experiments showed that a small amount of BSA relative to  $\alpha$ -casein increased aggregate particle size, whereas PvLEA4 decreased particle size in a dose-dependent manner. Trehalose, which is the main hemolymph sugar in most insects but also a protectant as a chemical chaperone in the sleeping chironomid, has less effect on the limitation of aggregate formation. This analysis suggests that molecular shield proteins function by limiting the growth of protein aggregates during drying and that PvLEA4 counteracts protein aggregation in the desiccation-tolerant larvae of the sleeping chironomid.

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### 1. Introduction

Late embryogenesis abundant (LEA) proteins were first described in cotton seeds, accumulating during the late stage of development when seeds acquire desiccation tolerance (Dure et al.,

1981). Subsequently, genes encoding LEA proteins were found in the vegetative tissues of bryophytes, pteridophytes and spermatophytes, as well as in orthodox seeds (Cuming et al., 2007; Hundertmark and Hincha, 2008; Piatkowski et al., 1990). More recently, LEA proteins have also been found in anhydrobiotic animals, including certain nematodes, rotifers, tardigrades, brine shrimp embryos, collembolan species and larvae of the sleeping chironomid *Polypedium vanderplanki* (Bahrndorff et al., 2009; Browne et al., 2002; Forster et al., 2009; Hand et al., 2007; Kikawada et al., 2006; Tunnacliffe et al., 2005). Among the anhydrobiotes, the sleeping chironomid *P. vanderplanki* (Diptera) is the largest animal inhabiting the semi-arid area of the African continent (Hinton, 1951). Throughout the larval stage, it can survive in an almost completely desiccated state during a dry spell (Hinton, 1951). Additionally, *P. vanderplanki* tolerates repeated dehydration cycles and one larva was successfully revived ten times (Hinton, 1960). During a dry spell, they accumulate a large amount of

**Abbreviations:** LEA, late embryogenesis abundant; G3LEA, group 3 LEA protein; CD, circular dichroism; FTIR, Fourier transform infrared; IDP, intrinsically disordered protein; DLS, dynamic light scattering; GFP, green fluorescent protein; CHO, Chinese hamster ovary; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; LDH, L-lactate dehydrogenase.

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trehalose up to 18% of their dry mass (Watanabe et al., 2002). Trehalose, which is the main hemolymph sugar in most insects (Wyatt, 1967), functions as a chemical chaperone in the dry larvae and might protect cell components from desiccation by the replacement of water or glass formation (Crowe et al., 1992). Therefore, accumulation of trehalose might be an important event to survive drying (Sakurai et al., 2008; Watanabe et al., 2002). In addition, previously we reported that the LEA proteins PvLEA1, PvLEA2 and PvLEA3 are accumulated in response to desiccation or NaCl treatment in larvae (Kikawada et al., 2006). To our knowledge, *P. vanderplanki* is the only insect to accumulate LEA proteins. LEA protein biosynthesis is a common component of the response to drought, cold or high salinity conditions, and consequently, LEA proteins are considered to function as protectants against water-deficit stress.

LEA proteins are classified into six or more groups according to the features of their amino acid sequence (Battaglia et al., 2008; Dure, 1989; Tunnacliffe and Wise, 2007). Although most LEA proteins share the common features of high hydrophilicity and low cysteine, phenylalanine or tryptophan content, each group of LEA proteins has characteristic motifs at the amino acid sequence level (Battaglia et al., 2008; Tunnacliffe and Wise, 2007). For example, group 3 LEA proteins (G3LEAs) have several repetitions of an 11-mer motif, which is defined as a “LEA\_4 (PF02987)” in the Pfam database (Finn et al., 2010).

Computational analyses based on known structures of folded proteins predicted that G3LEAs are likely to form amphiphilic  $\alpha$ -helices that then dimerise via interactions between weakly hydrophobic regions on each chain (Dure, 1993). Intriguingly, circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy revealed that G3LEAs show a high degree of disordered conformations in aqueous solution, but adopt a largely  $\alpha$ -helical conformation upon dehydration; this process is reversible on rehydration (Goyal et al., 2003; Thalhammer et al., 2010; Tolleter et al., 2007; Wolkers et al., 2001). Therefore, G3LEAs behave as intrinsically disordered proteins (IDPs) (Tompa, 2009) in solution, but become more folded on drying.

A typical function of a G3LEA is macromolecular stabilization through so-called “molecular shield” activity (Chakrabortee et al., 2012; Wise and Tunnacliffe, 2004). LEA proteins are proposed to form a physical and/or electrostatic barrier to reduce interactions between other proteins upon drying, thus inhibiting desiccation-induced aggregation. Indeed, G3LEAs reduce the formation of protein aggregates and suppress the reduction in enzyme activity on drying (Goyal et al., 2005; Grelet et al., 2005). To investigate the anti-aggregation activity of LEA proteins, the turbidity of water stress-treated samples has been measured in many studies (Amara et al., 2012; Boucher et al., 2010; Duan and Cai, 2012; Furuki et al., 2012; Goyal et al., 2005). Turbidity increases with rising concentration of colloidal aggregates in aqueous solution and LEA proteins can reduce aggregates derived from stress-labile proteins. In fact, turbidity is affected by the particle size distribution in the colloid. For small particles, there is a marked increase in turbidity as particle size increases, but eventually turbidity declines at larger sizes (Elimelech et al., 1995). In addition, although the turbidity of one sample may indicate the same value as other samples, the specific properties (size, shape, quantity, etc.) of the particles in these suspensions will not necessarily be the same. Therefore, to improve our understanding of the anti-aggregation effect of LEA proteins, we should also focus on particle size distribution in sample suspensions. To our knowledge, there are no data on the particle size distribution in a suspension of LEA protein plus desiccation-induced protein aggregates, with the exception of one report on freeze-induced aggregates (Boucher et al., 2010).

In the present study, we characterized a novel LEA protein, PvLEA4, in the sleeping chironomid *P. vanderplanki*. Although three isolated PvLEAs (PvLEA1, PvLEA2 and PvLEA3) have some characteristics typical of classical G3LEAs, such as desiccation-inducibility, high hydrophilicity and contain several copies of an 11-mer motif, there has been no report on the protective activity of PvLEAs. PvLEA4, the novel LEA protein that we characterize in this study, appears to be the most abundantly accumulated among LEA proteins in the desiccated larvae. We studied the biochemical characteristics of PvLEA4 and also whether it shows anti-aggregation activity. For this, we used both the conventional approach of turbidity measurement, but also dynamic light scattering (DLS) to measure changes in the particle size of aggregates caused by dehydration. We report here that the sleeping chironomid LEA protein, PvLEA4, shows effective protection of desiccation-labile proteins from deactivation and aggregation caused by water stress and provide insight into the mechanism of action of molecular shield proteins.

## 2. Material and methods

### 2.1. Insect rearing

Larvae of *P. vanderplanki* were reared on a 1% (w/v) agar diet containing 2% (v/v) commercial milk under controlled light (13 h light: 11 h dark) and temperature (27 °C) conditions. Larvae of approximately 1 mg wet body weight and 7 mm in length were used for all experiments. The desiccation procedure used to induce anhydrobiosis has been described previously (Watanabe et al., 2003). Briefly, eight larvae were placed on filter paper with 0.44 ml of distilled water in a glass Petri dish (diameter 65 mm, height 20 mm), which was set in a desiccator (20 × 20 × 20 cm) with silica gel (1 kg). For rehydration, distilled water was added to dry larvae (2 mm in length) placed in a dish.

### 2.2. Proteome analysis

Larvae were homogenized in T-PER<sup>®</sup> lysis buffer (Pierce Biotechnology, Rockford, IL) with Complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). 2D gel electrophoresis, image analysis and protein sequencing were performed as described previously (Gusev et al., 2010).

### 2.3. Cloning of *PvLea4*

Based on the N-terminal amino acid sequence of the protein determined by proteome analysis, we searched the corresponding EST clones from an in-house PvEST database (Cornette et al., 2010) using Blastx. The EST clones obtained formed a single cluster containing a complete ORF. To confirm the cluster comprised full-length *PvLea4* cDNA, we performed 5'-RACE as previously reported (Kikawada et al., 2006) using the specific primers PvLEA4-5'RACE-F1 (5'-TATCAACAACCTGTATCTTTGGCTGCTG-3') and PvLEA4-5'RACE-F2 (5'-TCACGACCTTCCTCAATTTATC-3').

### 2.4. *In silico* analysis of protein sequences

Sequence identities and similarities of *PvLea4* (GenBank: AB841344) were determined using the BLAST program and the GenBank database on the NCBI web server (<http://blast.ncbi.nlm.nih.gov/>). Motif analysis was performed using the Pfam program (<http://pfam.sanger.ac.uk/>) (Finn et al., 2010). The sequence logo for 11-mer motif in PvLEA4 was designed by a web based application, WebLogo 3.3 (<http://weblogo.threeplusone.com/create.cgi>) (Crooks et al., 2004). Hydropathy plots were constructed with the Kyte and

Doolittle algorithm to analyze protein hydrophilicity (window size: 9; GENETYX-MAC ver.16, Genetyx Co., Tokyo, Japan). GRAVY index (grand average hydropathy) was calculated with the ExPASy ProtParam tool (<http://web.expasy.org/protparam/>).

### 2.5. Quantitative real-time PCR

Relative expression of *PvLea4* in wet and desiccating larvae was quantified using real-time PCR detection system CFX96 (Bio-Rad, Hercules, CA) with an SYBR<sup>®</sup> Premix Ex Taq™ kit (TaKaRa Bio, Shiga, Japan). Results were normalized using ribosomal protein L32 (*PvRpl32*, GenBank: AB244986) or elongation factor 1- $\alpha$  (*PvEfl1- $\alpha$* , GenBank: AB490338) as internal controls. The primers for the quantifications are as follows: *PvLea4*-RTP-F1, CTAGAGACCAACTTAAATCAGCTAC; *PvLea4*-RTP-R1, CTTGTCCTTTCCTTTCATAC; *PvEfl1a*-RTP-F1, AACTGACAAACCAATTGCG; *PvEfl1a*-RTP-R1, TCACCTGGTACAGCTCT; *PvRpl32*-RTP-F1, CGTCATCAGTCTGACAGATA; *PvRpl32*-RTP-R1, TTTGGGAGCATATGACGACT.

### 2.6. Western blot analysis

Proteins were extracted from larvae as described above under “Proteome analysis”. Custom anti-PvLEA4 polyclonal antibody was prepared by SIGMA-Aldrich-Japan, Tokyo, Japan. Briefly, the antibody was raised in a rabbit against a synthesized peptide corresponding to PvLEA4 sequence at positions 126–139 (TKDETLESMSKSDKN) and purified by affinity chromatography using the peptide. Anti-PvTRET1 and anti-PvTPS  $\alpha/\beta$  antibodies were prepared previously (Kikawada et al., 2007; Mitsumasu et al., 2010) were also used. Goat anti-rabbit IgG (H + L) HRP conjugated antibody (ZYMED, Life Technologies, Carlsbad, CA) was used as secondary antibody. The ECL Plus Western Blotting system (GE Healthcare Bio-Sciences, Piscataway, NJ) was used for detection according to the manufacturer's instructions. Quantification of band intensity was carried out using the image analysis software, Science Lab 2003 Multi Gauge ver.2.2 (Fujifilm, Tokyo, Japan).

### 2.7. Membrane flotation analysis

Ten desiccated larvae were rehydrated with distilled water for 30 min and smashed in “Homogenization Buffer” (10 mM HEPES-NaOH pH7.5 and 145 mM NaCl) containing a protease inhibitor cocktail (one tablet of Complete Mini, EDTA-free per 10 ml; Roche Diagnostics) using a plastic pestle, followed by homogenization using a tight-fitting Dounce homogenizer (50 strokes). The total homogenates were centrifuged at 900 g for 10 min in a micro-centrifuge to precipitate and remove the nuclei and cell debris including cuticle. The supernatants were used immediately for the following fractionation analysis, or stored at  $-80^{\circ}\text{C}$ .

Membrane flotation analysis using an iodixanol (Optiprep; Axix-Shield PoC AS, Norton, MA) density gradient was performed as described previously, with modifications (Hagiwara et al., 2003). Briefly, a working solution of 50% iodixanol (5:1 v/v mixture of OptiPrep and Homogenization Buffer) was diluted with the Homogenization Buffer to prepare a 30% iodixanol solution. Iodixanol continuous gradients were formed with 0.9 ml each of 0% and 30% iodixanol solutions in open-topped tubes by Gradient Mate (Bio-Comp Instruments Inc., Fredericton, NB, Canada) at 10 rpm for 2 min at an angle of  $78^{\circ}$ . Then, 100  $\mu\text{l}$  of the supernatant was mixed with 220  $\mu\text{l}$  of the 50% iodixanol working solution to adjust the iodixanol concentration to 35% (v/v) and was loaded into the bottom of the gradients, followed by ultracentrifugation in a Beckman TLS-55 rotor (10,000 g for 2 h at  $4^{\circ}\text{C}$ ). The gradients were fractionated into 10 fractions of 210  $\mu\text{l}$  each from the top. Fractions were stored at  $-80^{\circ}\text{C}$  or analyzed immediately by SDS-PAGE [9%

acrylamide gel (Tris–HCl pH 7.2), Tris–aspartic acid running buffer (TEFCO, Tokyo, Japan)] and Western blot analysis.

### 2.8. Subcellular localization of PvLEA4-green fluorescent protein (GFP) protein in Chinese hamster ovary (CHO) cells

A PvLEA4 expression vector, pcDNA5/FRT-PvLea4-AcGFP1, was constructed as follows. The *PvLea4* ORF lacking its stop codon was amplified using a high-fidelity DNA polymerase KOD-plus (Toyobo, Osaka, Japan) with specific primers (*PvLea4*-Bm-Kz-F1: cgggacccac-cATGGTTAAGCAAGATAACTTGGATCAA, *PvLea4*-Sm-x-R1: cccccgggACAACAATTCTTATCTTATCAGATTTTCATTGA), and inserted between the *Bam*HI and *Eco*RV sites of the pcDNA5/FRT-AcGFP1 plasmid, comprising the ORF of AcGFP1 inserted between the *Eco*RV and *Xho*I sites of pcDNA5/FRT (Life Technologies). For transient expression, 1  $\mu\text{g}$  of pcDNA5/FRT-PvLea4-AcGFP1 was transfected into CHO-K1 cells using FuGENE6 transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. Heterologous expression was allowed to proceed for 24 or 48 h. Subcellular localization of the recombinant protein was visualized using a fluorescent microscope Biozero BZ-8100 (Keyence, Osaka, Japan).

### 2.9. Expression and purification of recombinant protein PvLEA4

To synthesize recombinant PvLEA4 with an N-terminal His-tag, the *PvLea4* ORF was integrated into pET32a (+) vector (Merck, Darmstadt, Germany) replacing the sequence from the Trx-Tag to the second His-Tag. *Escherichia coli* BL21 (DE3) transformants harboring the pET32a/His-PvLEA4 vector were incubated in LB medium containing 50  $\mu\text{g}/\text{ml}$  carbenicillin, and expression of the recombinant protein was induced by addition of 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). The recombinant protein was purified according to “The handbook for high-level expression and purification of 6xHis-tagged proteins, 5th edition” (QIAGEN, Hilden, Germany), with minor modifications. Total soluble proteins in transformed *E. coli* cells were extracted by sonication in ice-cold lysis buffer (50 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM NaCl, 10 mM imidazole, pH 8.0). The crude extract was boiled for 15 min and then left on ice for 10 min, followed by centrifugation (12,000 g for 15 min at  $4^{\circ}\text{C}$ ) and the supernatant was passed over a Ni-NTA Superflow column (QIAGEN) for further purification. After washing with wash buffer (50 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM NaCl, 20 mM imidazole, pH 8.0), recombinant proteins were eluted with elution buffer (50 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM NaCl, 250 mM imidazole, pH 8.0). Eluted samples were dialyzed against MilliQ ultrapure water (Millipore, Billerica, MA) at least twice and then lyophilized. Protein profiles in the purification process were monitored by SDS-PAGE, running samples on a 9% acrylamide gel (Tris–HCl pH 7.2) with aspartic acid running buffer (TEFCO) (Supplemental Fig. 1).

### 2.10. Circular dichroism (CD) spectroscopic analysis

CD spectra were obtained with a Jasco-720 spectropolarimeter (Jasco Instruments, Tokyo, Japan). Protein solutions containing approximately 1 mg/ml of the recombinant PvLEA4 in  $\text{H}_2\text{O}$  were measured in a 0.2 mm path length cuvette. CD spectra were recorded at  $20^{\circ}\text{C}$ , or every  $10^{\circ}\text{C}$  from 20 to  $90^{\circ}\text{C}$ . The acquisition parameters were 2.0 nm bandwidth, 0.5 s response and in the 260–185 nm range.

### 2.11. Fourier transform infrared spectroscopy (FTIR) analysis

Pure recombinant PvLEA4 protein obtained as described above was dissolved in  $\text{D}_2\text{O}$  at 2 mg/ml and then lyophilized twice for deuterium exchange of the protein. Dry samples were prepared by drying 5  $\mu\text{l}$  droplets of the  $\text{D}_2\text{O}$  solution with the H-D exchanged

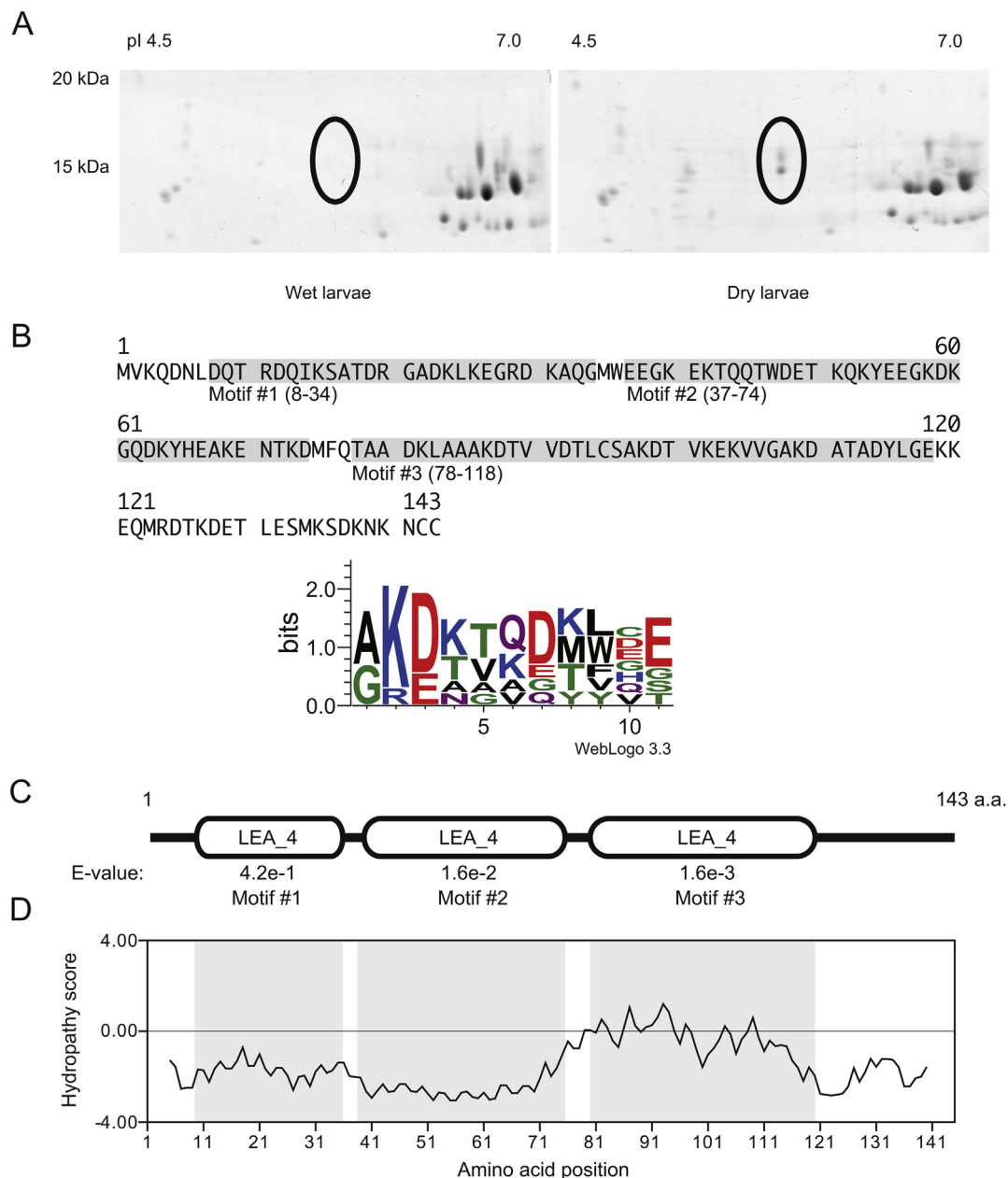
PvLEA4 protein at 10 mg/ml on a Teflon plate in a desiccator (relative humidity ca. 5%) for at least 24 h.

The dry protein films prepared above were sandwiched between two KBr plates (Jasco, Tokyo, Japan). Protein samples in D<sub>2</sub>O were poured between two circular ( $\varphi$  20 × 2 mm) CaF<sub>2</sub> plates (Jasco) with a Teflon spacer (ca. 50 mm thickness). IR spectra in the range between 4000 and 650 cm<sup>-1</sup> were recorded at room temperature, with a spectral resolution of 4 cm<sup>-1</sup> and 128 scan, on an FTIR-6100 spectrometer (Jasco) equipped with a liquid nitrogen-cooled mercury/cadmium/telluride detector and an infrared microscope (IMV-4000; Jasco). For secondary structure analysis of PvLEA4 protein, the spectral region between 1750 and 1550 cm<sup>-1</sup>, containing the amide-I absorption bands due primarily to stretch vibration of the carbonyl groups in peptide bonds on the protein

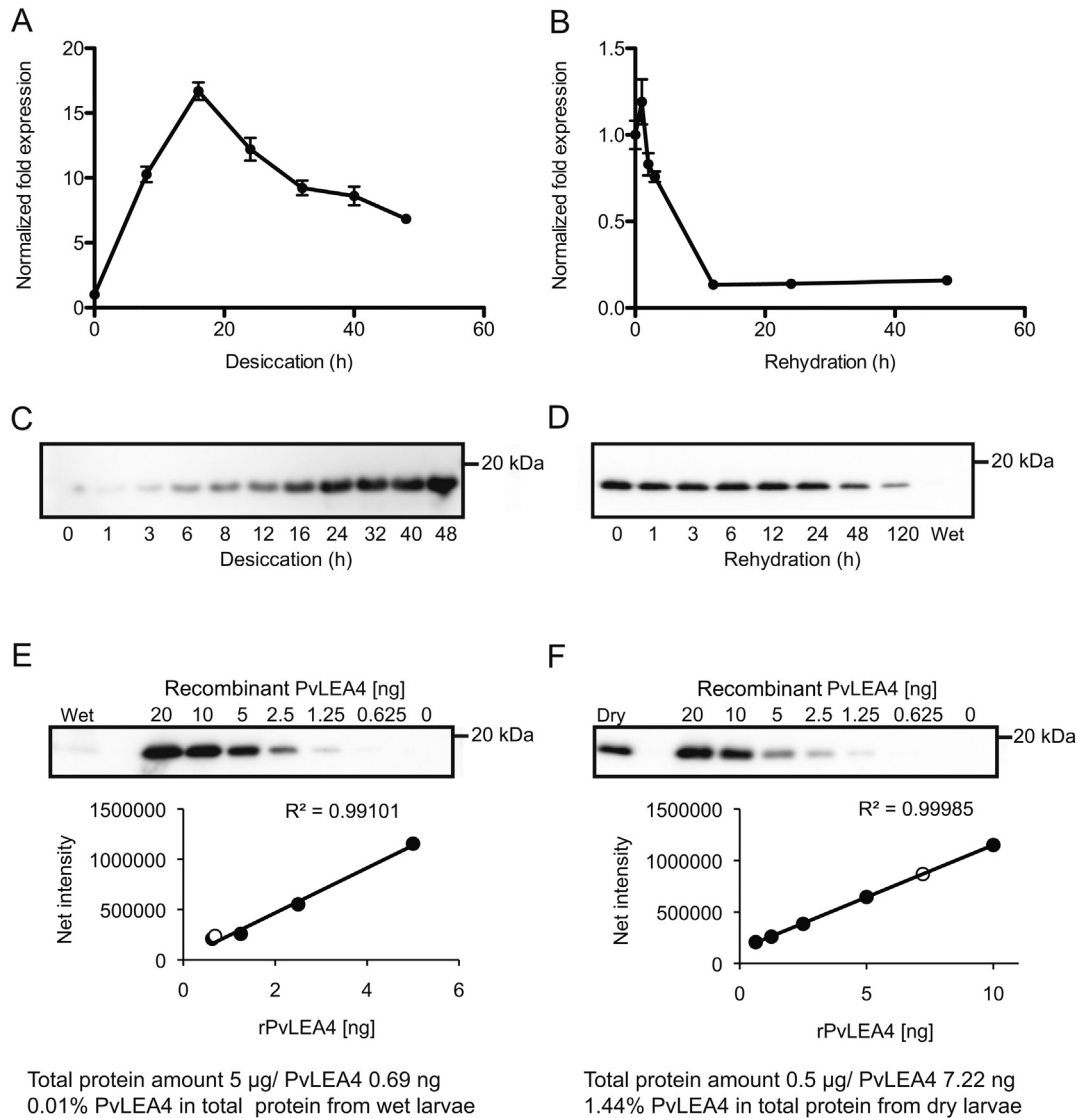
backbones (Pelton and McLean, 2000; Tamm and Tatulian, 1997), was analyzed. Secondary structure components were derived from the second derivative spectra of the amide-I bands, for which the Savitsky-Golay function (Savitzky and Golay, 1964) was used.

### 2.12. Lactate dehydrogenase (LDH) activity

Concentrated L-lactate dehydrogenase (LDH) from rabbit muscle (Roche Diagnostics) was diluted in 20 mM Tris-HCl buffer (pH 7.0) to 26 nM LDH. In a microfuge tube, 25  $\mu$ l of 26 nM LDH were mixed with 25  $\mu$ l of either recombinant PvLEA4 or BSA dissolved in 20 mM Tris-HCl buffer (pH 7.0) with or without trehalose. In this experiment, 100 mM trehalose was used, equivalent to a 7.7E+06 M ratio compared to LDH. Tubes were left in the desiccator box for 24 h and



**Fig. 1.** Biochemical features of the PvLEA4 protein. (A) 2D gel images for hydrated (left panel) and dry (right panel) larvae protein samples. PvLEA4 was particularly abundant in the dry larvae (inside oval). (B) “LEA\_4” motifs were highlighted in amino acid sequence of PvLEA4. A sequence logo was illustrated the 11-mer repeat in PvLEA4. (C) Scheme of the characteristic structure of PvLEA4. It has three “LEA\_4” motifs according to a Pfam search. (D) Hydropathy plot of the deduced PvLEA4 protein. “LEA\_4” motifs are indicated as gray area. The Kyte-Doolittle algorithm was used with a window size of 9 residues. Negative values indicate hydrophilicity.



**Fig. 2.** Desiccation-induced expression of *PvLea4* mRNA and PvLEA4 protein in *P. vanderplanki* larvae. (A, B) Temporal changes in *PvLea4* mRNA expression level were analyzed by real-time PCR. Values and bars represent means  $\pm$  SD ( $n = 3$ ). (C, D) Western blot analysis of PvLEA4 protein isolated from desiccating (C) and rehydrating (D) larvae. Equal amount of protein extracts from larvae at each drying or rehydrating treatment periods were analyzed. The position of a 20 kDa molecular mass marker is indicated. (E, F) Quantitative estimation of PvLEA4 protein isolated from hydrated (E) and dry (F) larvae. Calibration curves of recombinant PvLEA4 protein as a standard were prepared in the linear range (closed circles). PvLEA4 levels in hydrated (E; total protein loaded 5 µg) and dry (F; total protein loaded 0.5 µg) larvae were determined using each calibration curve (open circles). The position of a 20 kDa molecular mass marker is indicated.

then rehydrated in the same volume of 20 mM Tris–HCl buffer (pH 7.0). LDH activity in the buffer with or without test protein (PvLEA4 or BSA) was measured using an LDH cytotoxicity detection kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions.

### 2.13. Water-stress aggregation assay using $\alpha$ -casein

A water-stress aggregation assay using  $\alpha$ -casein was performed as described previously, with some modifications (Furuki et al., 2012). Alpha-casein from bovine milk (Sigma–Aldrich, St. Louis, MO) was dissolved in MilliQ water to 4 mg/ml. In a microfuge tube, 100 µl of 4 mg/ml  $\alpha$ -casein was mixed with 100 µl of either recombinant PvLEA4, BSA or trehalose dissolved in MilliQ. Tubes were set in a speed-vac VC-960 (TAITEC, Saitama, Japan) for desiccation by centrifugal concentration under reduced pressure for 2.5 h. Once completely desiccated, these samples were rehydrated in the same volume of MilliQ water and then the drying and rehydration cycle was repeated once. Turbidity and particle size in

the water-stress treated samples were measured using a spectrophotometer, SPECTRAMAX PLUS, (Molecular Devices, Sunnyvale, CA) and a DLS analyzer, Zetasizer nano ZS (Malvern Instruments Ltd, Worcestershire, UK), respectively.

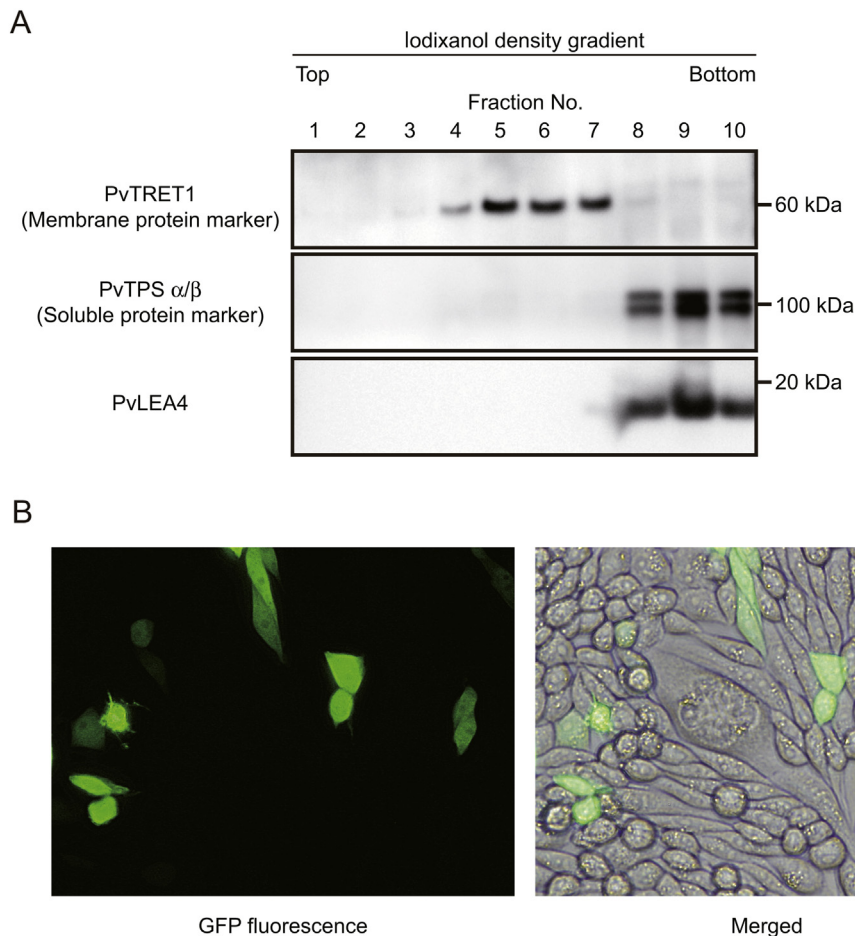
### 2.14. Statistics

Each experiment was performed with three or four replicates. Statistical relevance was determined by either one-way ANOVA and a Tukey post-hoc analysis or two-way ANOVA using Prism version 6 (GraphPad Software, La Jolla, CA).

## 3. Results

### 3.1. Identification and molecular cloning of *PvLea4*

To further investigate the molecular mechanisms underlying the extreme desiccation tolerance in *P. vanderplanki* larvae, we compared



**Fig. 3.** Subcellular localization of PvLEA4 protein. (A) Membrane flotation analysis of extracts obtained from the dry larvae. Fractions (1–10, from top to bottom of the gradient) were analyzed by SDS-PAGE and immunoblotting to detect PvTRET1 (membrane protein marker; upper panel), PvTTPS  $\alpha/\beta$  (soluble protein marker; middle panel) and PvLEA4 (lower panel). The positions of molecular mass markers (kDa) are indicated on the right. (B) GFP fluorescence images of CHO cells transiently transfected with a vector expressing a PvLEA4-GFP fusion gene.

the profiles of total protein extracted from wet and desiccated larvae using 2D gel electrophoresis. Desiccation caused a drastic alteration in the expression profile (Fig. 1A), with several new spots detected only on the 2D gel from desiccated larvae. Protein sequencing showed that the desiccation-specific spot corresponding to pI 5.50 and 15 kDa (Fig. 1A) contained the sequence VKQDNLQDT at its N-terminal end. Based on this N-terminal sequence, one cDNA cluster was identified in the *P. vanderplanki* EST database. The putative ORF of this protein encodes a polypeptide of 143 amino acid residues with a predicted molecular mass of 16.2 kDa and a pI value of 5.26. A Blastx search indicated that the putative cluster for this protein showed significant similarity to PvLEA1 protein (GenBank: BAE92616; E-value:  $4e-10$ ).

According to a Pfam search, the deduced amino acid sequence of this protein contains three “LEA\_4” motifs (PF02987) at positions 8 to 34, 37 to 74 and 78 to 118 (Fig. 1B, C). The “LEA\_4” motif consists of several repetitions of the 11-mer amino acid sequence characteristic of G3LEAs. In PvLEA4, the consensus sequence (i.e. a richer description) of the 11-mer repeat is AKDKTQDK $\Phi$ XE (Fig. 1B). Hydrophilic and charged amino acids residues, notably lysine (K), threonine (T) and glutamic acid (E), represented 47% of the total residues. Few residues of cysteine (C), tryptophan (W) or phenylalanine (F) are present in this protein, consistent with the observation that most G3LEAs possess neither C, W nor F residues. A Kyte-Doolittle hydrophathy plot predicted that this protein was predominantly hydrophilic throughout the entire amino acid sequence although the third LEA\_4 motif region

was slightly hydrophobic (Fig. 1D). The GRAVY value for this protein is  $-1.487$ . Consequently, this desiccation-specific protein was designated PvLEA4, which is the fourth LEA protein identified in the sleeping chironomid.

### 3.2. Desiccation-induced expression of *PvLea4* mRNA and PvLEA4 protein

We examined the expression profile of *PvLea4* mRNA in larvae during desiccation and rehydration using quantitative RT-PCR. Expression of *PvLea4* mRNA peaked after 16 h of desiccation treatment and gradually declined as larvae entered anhydrobiosis (Fig. 2A). *PvLea4* was apparently rapidly downregulated following rehydration (Fig. 2B). We also investigated the accumulation of PvLEA4 protein in larvae during the same regime. PvLEA4 protein was present at low levels even under unstressed conditions (0 h desiccation, Fig. 2C), but gradually accumulated upon desiccation; the protein persisted for at least 5 days after rehydration (Fig. 2C, D). In the desiccated larvae, the amount of PvLEA4 corresponded to 1.44% of total soluble proteins (Fig. 2E, F).

### 3.3. Cytosolic localization of PvLEA4

Next, we determined the localization of PvLEA4 protein in cells. The supernatants of the homogenized desiccated larvae were separated into membrane fractions and soluble fractions (Fig. 3A).

We used PvTRET1 and PvTSP  $\alpha/\beta$  as marker proteins, which are known as a membrane-bound and a soluble protein, respectively (Kikawada et al., 2007; Mitsumasu et al., 2010). Western blot analysis showed that PvLEA4 protein co-fractionates with PvTSP  $\alpha/\beta$  and is therefore a soluble protein (Fig. 3A). To determine whether PvLEA4 localizes inside or outside cells, we performed fluorescence microscope experiments using CHO cells transfected with a PvLEA4-GFP expression construct. This revealed that GFP-fused PvLEA4 localized in the cytoplasm in CHO cells (Fig. 3B), consistent with the protein being present in a soluble form in the cytoplasm.

#### 3.4. Expression and purification of the His-tagged fusion protein in recombinant *E. coli*

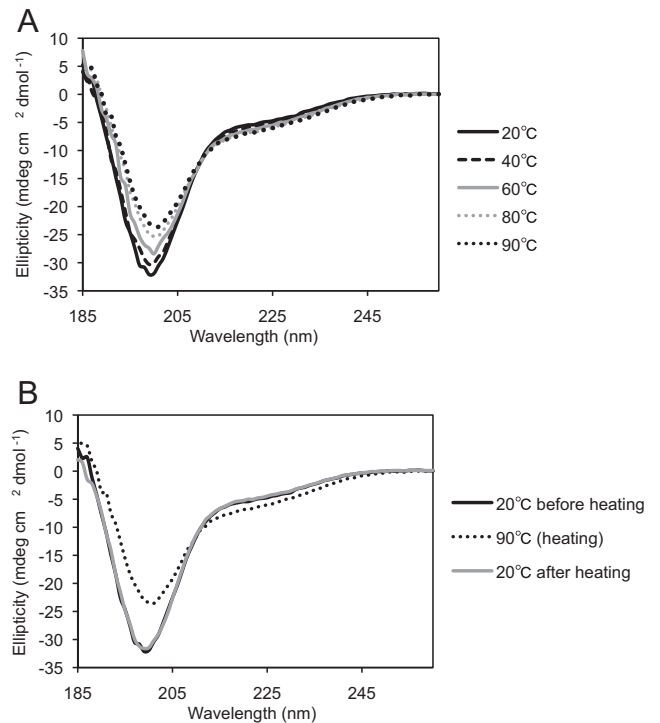
*E. coli* BL21 (DE3) transformants harboring the pET32a/His-PvLEA4 expression vector were incubated in LB medium with antibiotics. Production of His-tagged recombinant PvLEA4 was induced by 1 mM IPTG for 4 h. The supernatants of sonicated lysates from bacterial cells were boiled and His-tagged protein purified by immobilized-metal affinity chromatography. Samples of each step were examined for the presence of recombinant protein by SDS-PAGE (Supplemental Fig. 1). This recombinant protein was found in the supernatant even after boiling for 15 min. Thus, PvLEA4 protein is an entirely heat-soluble protein.

#### 3.5. Secondary structure analysis by CD under various temperature conditions

The secondary structure of PvLEA4 protein in H<sub>2</sub>O over a range of temperatures was analyzed by far-UV CD spectroscopy. The spectra of PvLEA4 protein showed a strong minimum at approximately 200 nm over the temperature range 20 °C–90 °C, which is indicative of highly disordered structures (Fig. 4A). The decrease in intensity of the 200 nm minimum with increasing temperature and the increase in intensity of the near 220 nm showed temperature-induced protein refolding. Any structural changes induced by heating were fully reversible, because the far-UV spectrum of PvLEA4 protein, after cooling from 90 °C to 20 °C, completely overlapped the spectrum of the untreated protein at 20 °C (Fig. 4B). These results revealed that hydrophilic PvLEA4 protein adopts a highly disordered conformation, but is nevertheless stable under aqueous conditions even at high temperature (90 °C). This confirms that PvLEA4 protein, like other G3LEAs, is an IDP.

#### 3.6. Secondary structure analysis by FTIR in aqueous solution and in the dry state

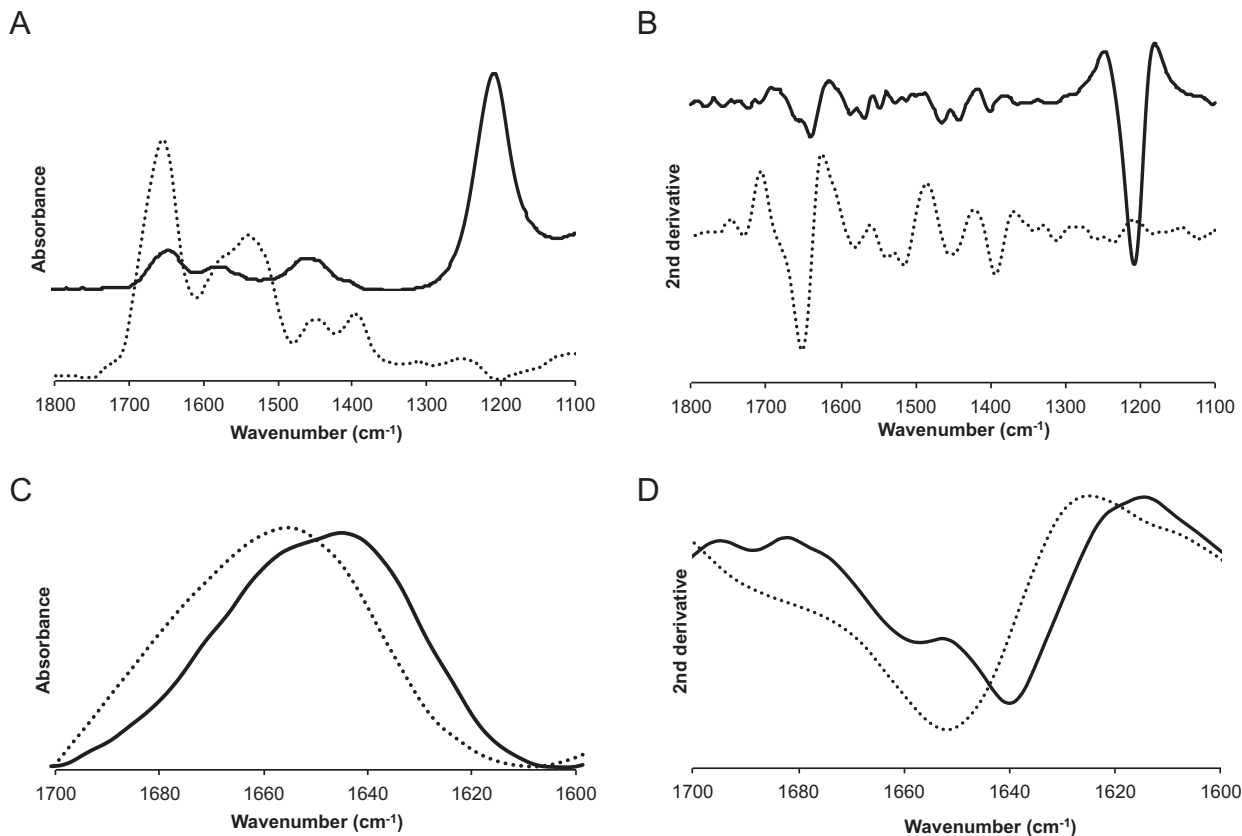
First, the FTIR spectrum of purified PvLEA4 in solution was recorded, with the protein dissolved in D<sub>2</sub>O to avoid interference of the H–O–H scissoring vibration in the range 1100–1800 cm<sup>-1</sup> (Fig. 5A). Its second-derivative spectrum was calculated to detect possible secondary structure components appearing in the amide-I band more precisely (Fig. 5B). Therefore, we focused on the spectra in the amide-I region (Fig. 5C, D). The major band with maximum absorption at 1640 cm<sup>-1</sup> was assigned to random coil structures (Pelton and McLean, 2000; Tamm and Tatulian, 1997), indicating that the protein in aqueous solution is highly disordered (Fig. 5D). In the dry state, the band at 1652 cm<sup>-1</sup> (Fig. 5C) was assigned to  $\alpha$ -helical structures (Pelton and McLean, 2000; Tamm and Tatulian, 1997) and was also clearly visible in the second-derivative spectrum of the amide-I region (Fig. 5D). This finding indicates that in the dry state the protein becomes more folded and adopts a predominantly  $\alpha$ -helical conformation.



**Fig. 4.** CD spectra of PvLEA4 solutions at various temperatures. (A) The lines represent the CD spectra of PvLEA4 at 20, 40, 60, 80 and 90 °C. (B) The two solid lines, representing PvLEA4 at 20 °C without treatment and at 20 °C after cooling from 90 °C, showed very similar spectra.

#### 3.7. Protection of LDH activity during repeated desiccation-rehydration stress

To examine whether PvLEA4 protein could protect other proteins *in vitro*, we tested the ability of the LEA protein to prevent the loss of LDH activity during repeated desiccation and rehydration. Dried LDH lost all activity, but PvLEA4 protein was able to preserve LDH activity after drying in a concentration-dependent manner (Fig. 6A). LDH activity after desiccation was 57% and 105% of the non-dried control in the presence of PvLEA4 at a molar ratio of 50:1 and 500:1 (PvLEA4/LDH), respectively (Fig. 6A), indicating that the molar ratio of 500:1 was necessary for effective dry-preservation of LDH activity. Likewise, BSA, a commonly used stabilizing agent for proteins, was also able to preserve LDH activity after drying (Fig. 6B). LDH activity after desiccation was maintained at 84% and 80% of control values in the presence of BSA at a molar ratio of 50:1 and 500:1 (BSA/LDH), respectively (Fig. 6B). Next, we examined the effect of addition of either PvLEA4 or BSA to LDH at 500:1 M ratio on the prevention of the enzyme inactivation during repeated rounds of desiccation and rehydration. LDH activity in the BSA-LDH mixture was decreased to 46% by the second round, whereas the enzyme activity in the PvLEA4-LDH mixture remained over 55% even after three drying cycles (Fig. 6C). Two-way ANOVA shows the difference in protective activity between PvLEA4 and BSA to be extremely significant ( $P < 0.0001$ , Supplemental Table 1). The results suggested that PvLEA4 protein is more effective at LDH protection under repeated dehydration conditions. We also examined the effect of trehalose when used alongside either PvLEA4 or BSA on the activity of LDH exposed to repeated desiccation-rehydration stress. The LDH activity of both PvLEA4 and BSA samples, even after four drying cycles, remained about 100% of control levels in the presence of 100 mM trehalose, although trehalose alone gave no significant effect on the preservation of LDH activity during drying



**Fig. 5.** FTIR spectroscopy of PvLEA4 in aqueous solution and in the dry state. (A) The solid line represents the FTIR spectra of PvLEA4 in D<sub>2</sub>O solution and the dashed line represents the FTIR spectra of PvLEA4 in the dry state. (C) The spectra corresponds to the Amide-I region (1700–1600 cm<sup>-1</sup>). The second-derivative spectra for the panel A and C are shown in the panel B and D, respectively.

(Fig. 6D, E, Supplemental Table 2). This demonstrates that trehalose is able to augment the protective effect of PvLEA4 and BSA on LDH activity under repeated desiccation stress.

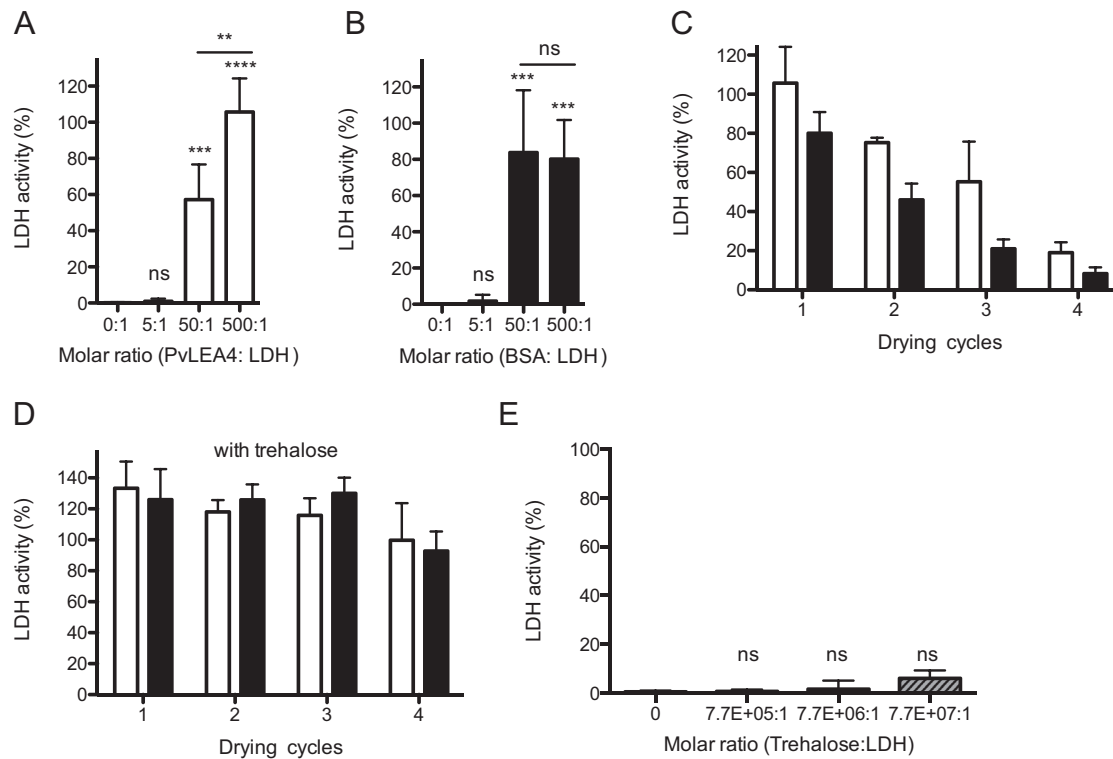
### 3.8. Anti-aggregation assay using $\alpha$ -casein under water stress conditions

LEA proteins are proposed to form a physical or electrostatic barrier to block interactions between other proteins during dehydration. In other words, LEA proteins function as molecular shields, reducing or delaying contact between other proteins, which results in inhibition of desiccation-induced aggregation (Chakrabortee et al., 2012; Wise and Tunnacliffe, 2004). To verify the role of PvLEA4 as a molecular shield, we evaluated the anti-aggregation activity of PvLEA4 using two distinct methods (i.e. turbidity and DLS), with  $\alpha$ -casein as a model aggregation-prone protein. First, we evaluated PvLEA4 anti-aggregation activity in terms of turbidity in  $\alpha$ -casein solutions. After desiccation-rehydration treatment,  $\alpha$ -casein formed irreversible aggregates and the turbidity in the solution was increased 2.5-fold (Fig. 7A–C). When either PvLEA4 or BSA was added at 0.2:1 M ratio with respect to  $\alpha$ -casein, the solutions after drying and rehydration exhibited the same degree of turbidity as before drying (Fig. 7A, B), suggesting that both PvLEA4 and BSA reduced the increase in turbidity of  $\alpha$ -casein solution due to desiccation. When trehalose was added at 80:1 M ratio with respect to  $\alpha$ -casein, the solutions after drying and rehydration exhibited the same degree of turbidity as before drying (Fig. 7C). In molar terms, PvLEA4 and BSA appeared to have almost the same inhibitory effect on the aggregation of  $\alpha$ -casein; however, the molecular weight differs greatly between PvLEA4 (16 kDa) and BSA

(67 kDa). Taking this difference into account, the data were reconfigured in terms of the mass of the added proteins and compared in the same graph as shown in Fig. 7D. This clearly indicates that a smaller mass of PvLEA4 is sufficient to reduce the turbidity of  $\alpha$ -casein solution, compared to BSA. To exert the inhibitory effect on the increasing turbidity, more than twice amount of trehalose against  $\alpha$ -casein was required (Fig. 7E).

Next, using DLS, we assessed how PvLEA4 anti-aggregation activity affected particle size distribution in  $\alpha$ -casein solutions subjected to desiccation and rehydration. The Z-average is the intensity-weighted mean hydrodynamic size of particles and a second parameter, the polydispersity index (PDI), indicates the width of the particle size distributions. Before desiccation-rehydration treatment,  $\alpha$ -casein alone solutions had polydisperse particles with the Z-average and the PDI of 95 d nm and 0.582, respectively (Fig. 8A). After the treatment resulting in the formation of aggregates in  $\alpha$ -casein alone solutions, the sample had monodisperse particles with the Z-average and the PDI of 73 d nm and 0.08 (Fig. 8B). As the molar rate of PvLEA4 to  $\alpha$ -casein increased, the Z-average decreased and the PDI increased in the PvLEA4-casein mixture after desiccation-rehydration treatment (Fig. 8C). At a 0.2:1 M ratio of PvLEA4 to  $\alpha$ -casein, a reduced Z-average (56 d nm) and low PDI (0.12) showed that smaller monodisperse particles exist in the solution after the treatment (Fig. 8C). The size distribution analysis indicated that the Z-average diameter for molecular ensemble in the PvLEA4-casein mixture was reduced clearly with the increase of the PvLEA4 amount (Fig. 8D). When 0.5:1 and 1:1 M ratios of PvLEA4:  $\alpha$ -casein mixtures underwent the treatment, the Z-average decreased to approximately 26 d nm and the PDI increased to 0.82 (Fig. 8C). The size distribution of particles in the





**Fig. 6.** PvLEA4 protein and trehalose preserve LDH activity under water stress conditions. (A, B, E) LDH activity after desiccation–rehydration treatment with PvLEA4 (A), BSA (B) and trehalose (E). (C, D) LDH activity after several cycles of desiccation and rehydration with a 500-M ratio of PvLEA4 or BSA in Tris buffer (C) or in a 100 mM trehalose solution (D). White bars, PvLEA4-LDH mixture; black bars, BSA-LDH mixture. In this experiment, 100 mM trehalose is equivalent to  $7.7E+06$  times LDH concentration by molar ratio. Each value represents mean  $\pm$  SD ( $n = 3$  or  $4$ ). \*\*\*\*,  $P < 0.0001$ ; \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; ns, not significant.

rehydrated PvLEA4-casein mixture at molar ratio (0.5:1) showed broad three peaks, which was corresponded well with that in the non-treated PvLEA4-casein mixture (Fig. 8E). These data suggest that the presence of PvLEA4 at molar ratio of over 0.5:1 effectively prevented aggregation of  $\alpha$ -casein in the rehydrated solutions. In contrast, in BSA-casein mixtures subjected to desiccation and rehydration, the Z-average initially became larger as the molar ratio of BSA to  $\alpha$ -casein increased in the range from 0:1 to 0.2:1, suggesting that BSA enhanced the association of  $\alpha$ -casein to some extent (Fig. 8F and G). At a 0.5:1 M ratio of BSA to  $\alpha$ -casein, the size distribution of particles in the rehydrated solutions showed broad three peaks (Fig. 8H). In terms of particle size distribution, an over 160-fold mole ratio of trehalose to  $\alpha$ -casein was required to confine the desiccation-induced aggregation (Fig. 8I–K). These data indicate that PvLEA4 is more effective than BSA and trehalose alone on limiting desiccation-induced aggregations.

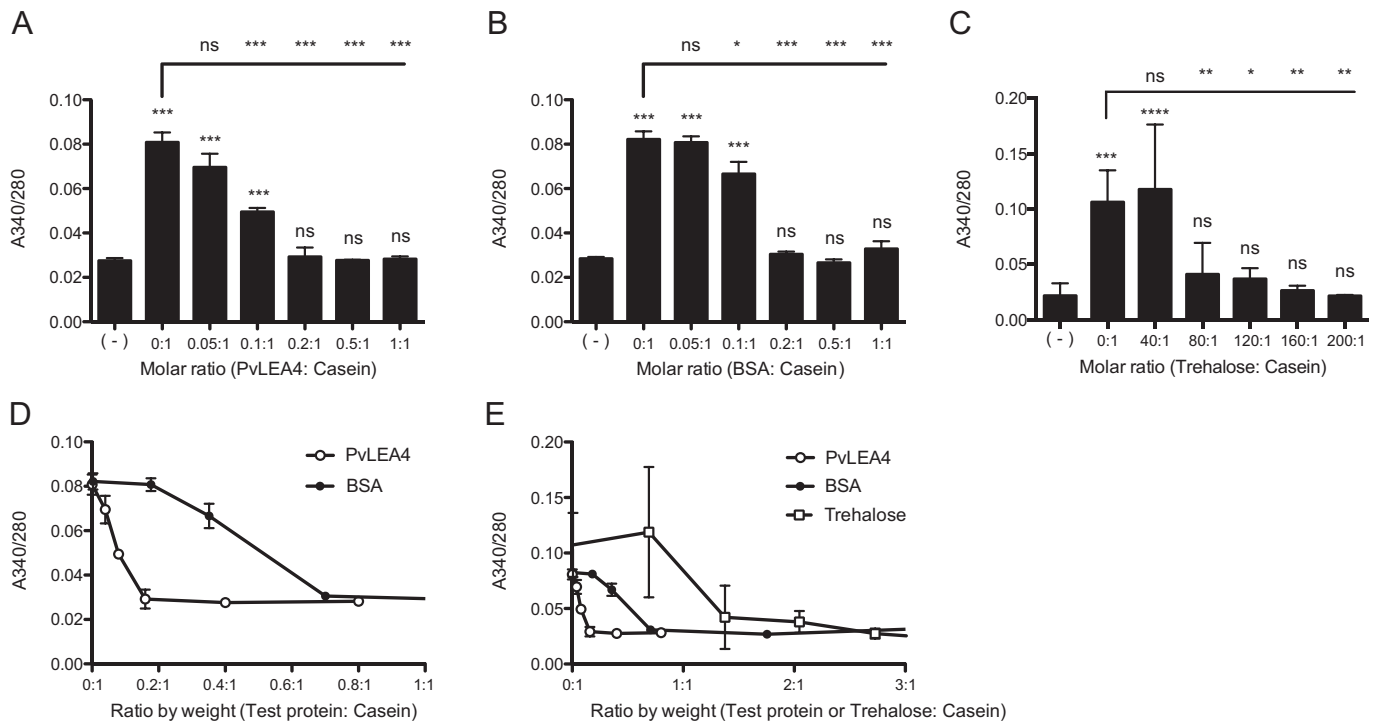
#### 4. Discussion

G3LEAs are stress-related proteins found in many organisms exhibiting desiccation tolerance. The contribution of LEA proteins to desiccation tolerance in plants, nematodes, rotifers and cysts of *Artemia* has been investigated in terms of biochemistry, physicochemistry and physiology. In insects, several LEA proteins have been found in the anhydrobiotic midge *P. vanderplanki* (Diptera); however, their biochemical and physicochemical features remain to be elucidated. Intriguingly, other dipterans, such as *Drosophila melanogaster* and *Anopheles gambiae*, have no genes encoding LEA proteins as far as we are aware. This is the first report on the functional properties of an insect LEA protein.

AvLEA1, a G3LEA from an anhydrobiotic nematode, exerts protein protection activity in a dose-dependent manner under desiccation stress conditions (Chakrabortee et al., 2007; Goyal et al.,

2005). To explain the protein aggregation reducing function of LEA proteins, Tunnacliffe and colleagues proposed the molecular shield hypothesis (Chakrabortee et al., 2012; Goyal et al., 2005; Wise and Tunnacliffe, 2004), suggesting that LEA proteins could shield aggregating proteins from irreversible interactions. Given this hypothesis, abundant shield proteins are likely to be more effective. Therefore, we focused on the most abundant LEA protein identified from proteome analysis in desiccated larvae (Fig. 1). A novel chironomid LEA protein, PvLEA4, seems to accumulate massively, so that the spot corresponding to PvLEA4 was easily confirmed in 2D gels with ordinary Coomassie staining of crude extracts of dry larvae (Fig. 1). In fact, PvLEA4 represents 1.6% of total soluble protein on 2D gels of dry larvae, according to imaging analysis software. This is consistent with results calculated from Western blot band intensities (Fig. 2F), which showed that PvLEA4 represents approximately 1.4% of total soluble protein in dry larvae. The concentrations in the larvae are very high and comparable to the abundance in plant LEA proteins in seed. For example, the D-7 LEA protein (one of G3LEAs) in mature cotton embryo comprises 2.58% of cytosolic protein (Roberts et al., 1993).

PvLEA4 has sequence similarity to PvLEA1 protein (*Polypedilum vanderplanki*; BAE92616, E-value:  $4e-10$ ), Protein LEA-1, isoform o (*Caenorhabditis elegans*, NP\_001256166, E-value:  $1e-07$ ) and its isoform proteins, AT3G53040 (*Arabidopsis thaliana*, BAH56960, E-value:  $9e-04$ ), and a group 3 LEA protein (*Oryza sativa*, AAD02421, E-value:  $9e-04$ ) as shown in Supplemental Fig. 2. Other PvLEA (PvLEA2, PvLEA3) showed no similarity with PvLEA4 although they have aligned sequences (Supplemental Fig. 3). Motif analysis using the Pfam database demonstrated that PvLEA4 has three “LEA\_4” motifs (PF02987), which comprise 11-mer repeats defined as the characteristic motif of G3LEAs (Fig. 1). Dure (1993) defined the consensus sequence for an 11-mer motif obtained from plant G3LEAs as “ $\Phi$ P $\Omega$ X $\Phi$ Ψ $\Omega$ Ψ $\Phi$ X $\Omega$ ”, where  $\Phi$ ,  $\Omega$  and  $\Psi$  represent



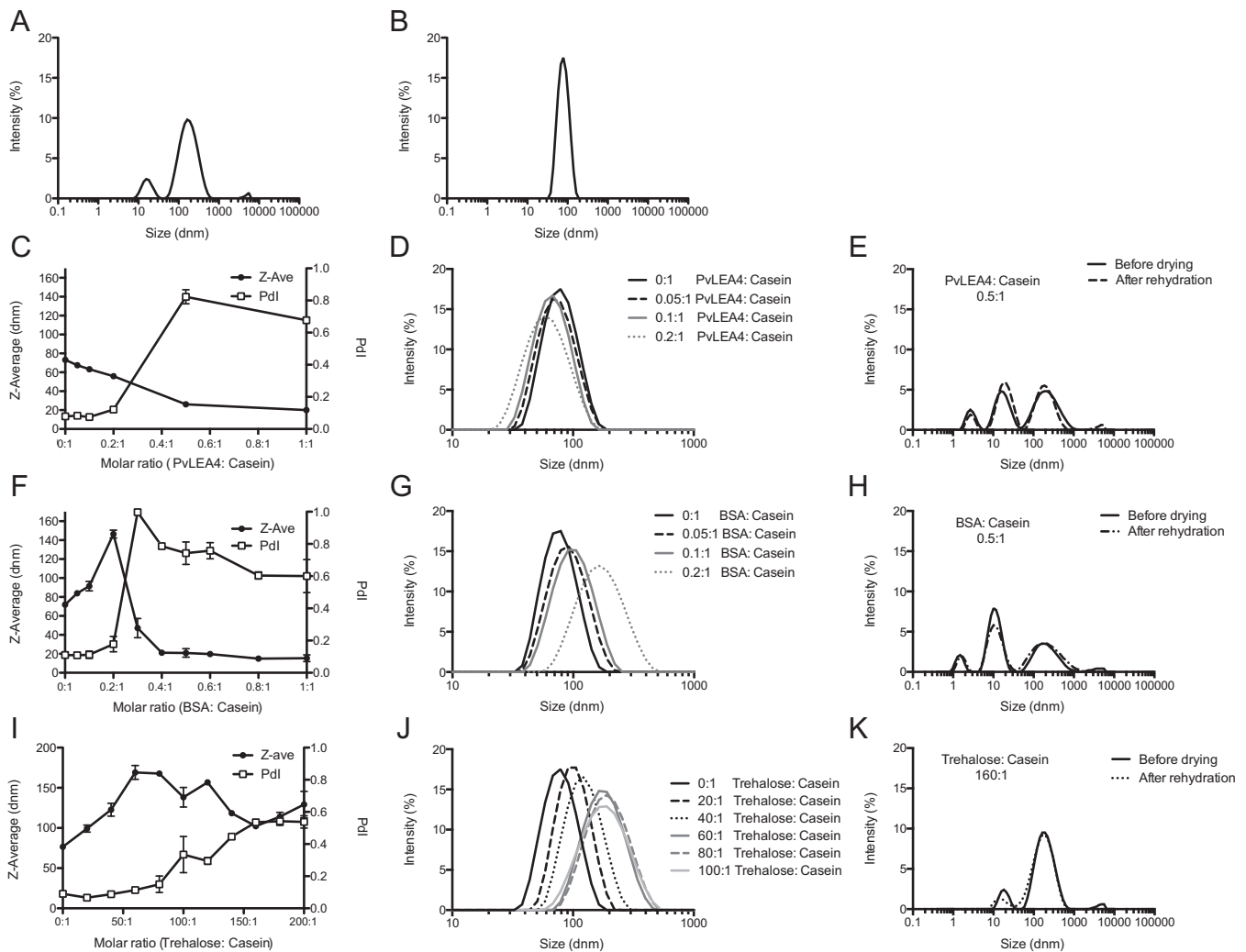
**Fig. 7.** Turbidity analysis for water-stress aggregation assay with  $\alpha$ -casein reveals differences between the anti-aggregation mechanisms of two test proteins. (A, B, C) Turbidity (apparent absorbance at 340 nm) of aqueous solutions with different molar ratios of  $\alpha$ -casein to PvLEA4 (A), BSA (B) and trehalose (C). Panel D shows the combined data of the panel A and B with x-axis values reconfigured as weight ratio. Panel E shows the combined data of the panel A, B and C with x-axis values reconfigured as weight ratio. Each value represents mean  $\pm$  SD ( $n = 3$ ). \*\*\*,  $P < 0.001$ ; \*,  $P < 0.05$ ; ns, not significant.

hydrophobic residues, negatively charged or amide residues, and positively charged residues, respectively, and X represents a nonspecifically conserved amino acid residue. He also predicted that the 11-mer motif would form an amphiphilic  $\alpha$ -helix (Dure, 1993). In the case of plant G3LEAs (e.g. LEA76, LEA D-7), the consensus sequence for the 11-mer motifs is AADXAKEKAXE (Shimizu et al., 2010), while the corresponding consensus of previously isolated chironomid LEA proteins (PvLEA1, PvLEA2, PvLEA3) is AKDXTKEKAXE (Shimizu et al., 2010). This sequence is slightly different from the plant G3LEA consensus [i.e. in position 2 lysine ( $\Psi$ ) substitutes for alanine ( $\Phi$ ), and in position 5 threonine (X) occurs instead of alanine ( $\Phi$ )], but tandem arrangements of both consensus sequences seem capable of forming an amphiphilic  $\alpha$ -helix (Supplemental Fig. 4). Indeed, FTIR analyses showed that synthesized LEA peptides containing both types of motif (i.e. the 22-mer PILEA-22 consisting of two repeats of the plant consensus, and PvLEA-22 the chironomid equivalent peptide) behaved as random coils in aqueous solution, but folded as  $\alpha$ -helices in the dry state (Shimizu et al., 2010). These results indicate that the structural characteristics of G3LEAs must be attributed to the 11-mer motifs, despite the fact that the motif sequences are not absolutely conserved. In PvLEA4, the consensus sequence of the 11-mer motif is AKDKTQDK $\Phi$ XE (Fig. 1B), which shows the same amino acid properties as the PvLEA-22 peptide. Therefore, when folded, PvLEA4 protein is likely to adopt an amphiphilic  $\alpha$ -helical conformation with both hydrophobic and hydrophilic stripes (Supplemental Fig. 5). It follows that PvLEA4 protein should have similar properties to the LEA peptides and show high affinity for water and other hydrophilic molecules or interact with ions (Furuki et al., 2012, 2011).

The function of most proteins, such as transporters, receptors and many enzymes, is closely related to their structure. However, more than 20% of proteins in higher eukaryotes are intrinsically

disordered and their functions remain to be explained (Ward et al., 2004). Many IDPs fold on binding to their biological targets (coupled folding and binding) (Dyson and Wright, 2005). In this study, the full-length PvLEA4 protein retained a random coil conformation even when heated to 90 °C, and is clearly an IDP in aqueous solution (Figs. 4 and 5). Interestingly, in common with other G3LEAs, PvLEA4 is able to undergo a conformational shift, folding into  $\alpha$ -helical structures on drying, without needing to interact with other proteins (Fig. 5). Thus, in contrast to many other proteins that denature on drying, LEA proteins become structured and exert their function as water is lost from their surroundings (Wolkers et al., 2001).

Tissue fractionation and CHO transfection experiments (Fig. 3) demonstrated that PvLEA4 is located in the cytoplasm and we therefore examined whether it was able to protect the activity of a cytoplasmic enzyme from desiccation stress, as would be experienced by the sleeping chironomid in its natural environment. Our results showed that PvLEA4 preserves the activity of LDH throughout at least four cycles of drying and rehydration; furthermore, trehalose is able to augment the protective function of PvLEA4 (Fig. 6). A similar finding has been reported for another LEA protein, AavLEA1 (Goyal et al., 2005). One possible explanation might be that LEA proteins reinforce a trehalose or other sugar-glass matrix (Wise and Tunnacliffe, 2004; Wolkers et al., 2001). In fact, the sleeping chironomid larvae produce a large amount of trehalose during desiccation and adopt a glassy state in their dried form (Sakurai et al., 2008). Furthermore, upon desiccation, synthetic LEA peptides form a glassy matrix that is reinforced by the addition of trehalose (Shimizu et al., 2010). Our data suggest that not only trehalose but also the chironomid LEA proteins might be involved in glass formation, which is considered to be one of the characteristics of desiccation tolerance in the larvae.



**Fig. 8.** DLS analysis for water-stress aggregation assay with  $\alpha$ -casein reveals differences between the anti-aggregation mechanisms of two test proteins. (A, B, D, E, G, H, J, K) Typical size distributions by intensity observed with DLS. (C, F, I) Properties of particle size distribution of rehydrated mixtures of  $\alpha$ -casein with PvLEA4 (C), BSA (F) or trehalose (I). Each value represents mean  $\pm$  SD ( $n = 3$ ). \*\*\*,  $P < 0.001$ ; \*,  $P < 0.05$ ; ns, not significant.

The protective function of LEA proteins on enzyme activity might be explained by a molecular shield mechanism (Chakrabortee et al., 2012). To examine whether PvLEA4 functions as a molecular shield, we analyzed alterations in the turbidity and particle size distribution of  $\alpha$ -casein and PvLEA4 protein mixtures undergoing cycles of desiccation and rehydration (Figs. 7 and 8). According to turbidity measurements, PvLEA4, BSA and trehalose appeared to inhibit aggregate formation of an  $\alpha$ -casein solution in a dose-dependent manner (Fig. 7). However, turbidity measurements provide little insight into the mechanisms of anti-aggregation activity. To afford more detailed information about the nature of aggregates, we also measured particle size (Z-average) and polydispersity (PDI) in  $\alpha$ -casein solutions by DLS for various molar ratios of PvLEA4, BSA and trehalose after desiccation-rehydration treatment. It was noteworthy that the anti-aggregation activity as assessed by DLS did not always correspond to that suggested by turbidity measurements. At a 0.2:1 M ratio of test proteins (PvLEA4 or BSA) to  $\alpha$ -casein, the Z-average and low PDI showed that larger size particles uniformly exist in the solution after drying and rehydration (Fig. 8C, D, F, G), although the turbidity returned to pre-desiccation levels (Fig. 7A, B). Taking into account the DLS data, a 0.5:1 M ratio of test protein (PvLEA4 or BSA) to  $\alpha$ -casein was sufficient to prevent aggregates formation.

Furthermore, the DLS technique revealed differences in the behavior of PvLEA4 and BSA in  $\alpha$ -casein solution after drying. PvLEA4 clearly decreased particle size in the test solution in a dose-dependent manner (Fig. 8C and D). On the other hand, BSA increased the particle size in the test solution at molar ratios of 0.05:1, 0.1:1 and 0.2:1 (BSA: casein), although it also decreased the particle size of aggregates at molar ratios greater than 0.2:1 (Fig. 8F and G). This suggests that BSA exerts two opposing effects under the water stress conditions imposed in our experiments: the first effect dominates at low concentrations of BSA and promotes interaction with  $\alpha$ -casein, resulting in increased aggregate size; and a second effect that predominates at high BSA concentrations, which alleviates  $\alpha$ -casein aggregation in a similar manner to PvLEA4 (Fig. 8). The molecular shield model involves a loose association of shield proteins with other polypeptides, but the model is also valid for proteins other than LEA proteins, as noted (Chakrabortee et al., 2012). In the case of a native LEA protein, such as PvLEA4, it has the ability to reduce the size of protein aggregates, even when present at low concentrations, possibly as a result of its physicochemical features. For example, on drying, LEA proteins might fold on the surface of  $\alpha$ -casein molecules, masking any hydrophobic patches that would promote aggregation. On rehydration, dispersion of  $\alpha$ -casein molecules would occur as the LEA

proteins revert to a disordered form. In addition, native PvLEA4 exerted a molecular shield effect at considerably lower concentrations than did the PvLEA-22 peptide, which was derived from another sleeping chironomid LEA protein. In the same anti-aggregation assay, a >25 M ratio of PvLEA-22 peptide to  $\alpha$ -casein was required to prevent aggregate formation during desiccation stress (Furuki et al., 2012). This suggests that the total polypeptide length or the number of G3LEA motifs in one molecule, rather than the abundance of G3LEA motifs per se in test solutions governs the anti-aggregation activity of LEA peptides or proteins, as discussed previously (Furuki et al., 2012).

The data obtained from DLS measurements clearly indicate that PvLEA4 efficiently prevents aggregation of  $\alpha$ -casein during drying by inhibiting particle agglomeration, in support of the molecular shield model. Therefore, we conclude that PvLEA4 acts as an effective molecular shield protein with a role in preventing protein aggregation.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ibmb.2013.08.004>.

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