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Evidence for multiple group 1 late embryogenesis abundant proteins in encysted embryos of *Artemia* and their organelles

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The presence of late embryogenesis abundant (LEA) proteins in plants and animals has been linked to their ability to tolerate a variety of environmental stresses. Among animals, encysted embryos of the brine shrimp *Artemia franciscana* are among the most stress resistant eukaryotes, and for that reason it is considered to be an extremophile. The study presented here demonstrates that these embryos contain multiple group 1 LEA proteins with masses of 21, 19, 15.5 and 13 kDa. The LEA proteins first appear in diapause-destined embryos, beginning at ~4 days post-fertilization, but not in nauplii-destined embryos. After resumption of embryonic development, the LEA proteins decline slowly in the desiccation resistant encysted stages, then disappear rapidly as the embryo emerges from its shell. LEA proteins are absent in fully emerged embryos, larvae and adults. They are abundant in mitochondria of encysted embryos, but barely detectable in nuclei and absent from yolk platelets. LEA proteins were also detected in dormant embryos of six other species of *Artemia* from hypersaline environments around the world. This study enhances our knowledge of the group 1 LEA proteins in stress tolerant crustacean embryos.

Keywords: artemia/desiccation tolerance/diapause/ late embryogenesis abundant proteins/LEA proteins.

Abbreviations: HPLC, high performance liquid chromatography; HRP, horse radish peroxidase; PAGE, polyacylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

Many small eukaryotic organisms including nematodes, rotifers, tardigrades and cysts of the brine shrimp, *Artemia franciscana*, and plant seeds, among others, are able to survive desiccation, a phenomenon known as anhydrobiosis (*1*). As dehydration proceeds, these organisms become essentially ametabolic, a stage in which they can remain for years until water becomes available. There is considerable evidence that certain carbohydrates play a key role in the stabilization of cells and their components during anhydrobiosis (*1*, *2*). But carbohydrates alone are not sufficient for the survival of desiccation-tolerant organisms (*3*, *4*). For example, a family of proteins first found in plant seeds called late embryogenesis abundant (LEA) proteins are also key players in many desiccation tolerant organisms (*4–8*). We note that not all anhydrobiotic animals contain trehalose (*9–11*), and that LEA proteins are intrinsically unstructured (*12*, *13*), a feature that is likely related to their functions (*3*, *4*, *8*).

Genes coding for LEA proteins are highly conserved in plants (*14*, *15*), and they are usually expressed in response to environmental stresses such as water deprivation, thermal extremes and high salt. Since their discovery ~30-years-ago (*5–7*), LEA proteins have been found in several invertebrates including rotifers (*16*, *17*), chironomids (*18*), nematodes (*4*, *19–22*), tardigrades (*23*), crustacea including *Artemia* (*24–27*) and in a few prokaryotes including *Bacillus subtilis* (*28*). In fact, one might argue that these proteins represent a common denominator in the desiccation tolerance of organisms from all taxa.

In general, LEA proteins are abundant in organisms or tissues that are able to withstand desiccation. However, the simple plant *Arabidopsis thaliana* that is not desiccation-tolerant contains >50 LEA genes, each with the capacity to code for a LEA protein with different functional potential. Therefore LEA proteins appear to be important to overall viability apart from the matter of stress tolerance (*14*, *29*).

Since their initial description, considerable research has shown that LEA proteins are mostly unstructured, hydrophilic and heat-soluble comprising 1–4% of cellular (non-storage) proteins in plant seeds (*4*, *30*). Intrinsically unstructured proteins show reduced binding of sodium dodecylsulphate (SDS), and therefore their apparent molecular masses by SDS–PAGE are often higher than their actual masses obtained from mass spectrometry (*13*). Another unique property of LEA proteins is their solubility in dilute acids, many but not all, being soluble in dilute trichloroacetic acid (*31*).

While we are just beginning to understand the contribution of LEA proteins to stress resistance, one function seems to involve their interaction with sugars to form molecular complexes able to protect proteins against drying induced aggregation (*27*, *32*, *33*). In that regard these proteins are involved with the formation of biological glasses, highly viscous hydrogen-bonding

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complexes that are believed to function by providing a protective, inert matrix within which cellular structures are protected in the desiccated state (*34*, *35*).

LEA proteins have been organized into at least six families or groups based mainly on their structural characteristics, with groups 1, 2 and 3 considered to be the major groups (*4*, *15*). The best-studied LEA proteins are members of group 3, proteins that are characterized by a conserved 11-amino acid segment which may be repeated several times (*4*). They occur widely among the seeds of plants and desiccation-tolerant animals including *Artemia* (*25*). Proteins in group 2, often referred to as dehydrins are characterized by distinct amino acid motifs with periodic repeats of segments containing tyrosine (Y segment), serine (S segment) or lysine (K segment) (*36*). As of this writing group 2 LEA proteins have been found exclusively in plants.

Members of group 1 contain a highly conserved 20-amino acid motif which may be repeated several times in tandem within the molecule (*4*). Among the plants, group 1 LEA proteins are considered to be seed specific, and until recently they were considered to be restricted to plants and a few prokaryotes (*4*, *21*). However, a group 1 LEA protein has been identified in cysts of *A. franciscana* as a heat-stable cytoplasmic protein of 21 kDa containing eight in tandem copies of a 20-mer amino acid sequence characteristic of group 1 LEA proteins found in plants (*27*). An *in silico* analysis of an *A. franciscana* expressed sequence tag (EST) library has revealed over 60 clones that encode proteins highly similar to the 21 kDa group 1 LEA protein indicating that these cysts contain at least six distinct mRNAs with the potential to code for group 1 LEA proteins (*37*) (Table I). In terms of potential functions of LEA proteins, it has been shown that members of groups 1 and 3, when combined with trehalose, are able to reduce drying induced protein aggregation *in vitro* and, to a limited extent, heat induced protein aggregation (*27*, *38*).

The brine shrimp, *Artemia* is considered to be an extremophile animal because its larval and adult stages flourish in hypersaline waters while its encysted embryos survive expose to many environmental stresses (*39*–*42*). Fertilized eggs have the capacity to develop directly into swimming larvae within the female brood pouch (*i.e.* ovoviviparously), or develop into encysted gastrulae which enters diapause, a stage of developmental arrest (*39*, *40*, *43*–*47*). Embryos produced by these two routes appear to be identical morphologically, but they are quite different biochemically. After diapause is terminated the embryos will resume development and eventually hatch into larvae when placed in sea water under permissive conditions of temperature and aeration (*48*, *49*).

In this article, we describe the presence of multiple group 1 LEA proteins in encysted embryos of *Artemia*. Their occurrence in various fractions from embryos was investigated using antibodies specific for *Artemia* group 1 LEA proteins, and protocols were developed for their characterization. Immunoblotting was also used to determine the presence of group 1 LEA proteins during various stages of the life cycle of *Artemia,* and in cysts of seven different species. The relationship between LEA proteins, diapause and desiccation tolerance is discussed.

Table I. Deduced group 1 LEA proteins in *Artemi*a cysts and embryos from clones in the EST database compared with the 21 kDa group 1 LEA protein[a].

Accession No.	Stage	20-mer repeats	Amino acids[b]	Mass[c]	pI	GRAVY[d]
EF656614	Cysts	8	182	19,676	7.93	−1.365
ES492349[e]	Larvae	6	151	16,475	6.12	−1.432
ES492298	Cysts	5	126	13,537	7.99	−1.298
ES492279	Cysts	5	115	12,430	8.86	−1.506
ES505891	Cysts	4	102	10,878	9.39	−1.228
ES500643[f]	Cysts	3	96	10,219	8.26	−1.199
ES506730	Cysts	2	51	5,467	8.16	−1.141
ES510519	Larvae	1	156	18,161	6.53	−0.662

[a]*Artemia franciscana* EST database from Chen *et al.* (*37*) and NCBI database.
[b]Open reading frame.
[c]Daltons calculated from ORF.
[d]Grand average hydropathy.
[e]Unique C-terminus sequence.
[f]When alternate start codon (L) is used in translation an extra 20-mer motif is present.

Materials and Methods

Materials

Encysted dried embryos of *A. franciscana* were obtained from the Great Salt Lake (Sanders Brine Shrimp Company, Ogden, UT, USA) or the San Francisco Bay (Bay Brand, Hayward, CA, USA) and kept at −15°C until needed. All other dried *Artemia* cysts/species used in this study were from the Artemia Reference Centre (Ghent, Belgium). The gel filtration column, ion-exchange chromatographic media, Percoll and the ECL western blotting reagents were from Amersham Biosciences, while the C-18 reverse phase column (Jupiter, 5 μm, 300A) was from Phenomenex. The Amicon Ultracel-10 k centrifugation filters were from Millipore. The Bradford Protein Reagent, Coomassie Brilliant Blue (CBB) R-250, acrylamides and nitrocellulose membranes (0.2 μm) were from BioRad. X-ray film and developer were from Kodak. Phenylmethane-sulphonyl fluoride (PMSF) and Protease Arrest were purchased from Calbiochem, while protein molecular weight standards were from Fermentas. All other chemicals were of reagent grade or better.

Culturing of A. franciscana

Swimming larvae of *A. franciscana* (Great Salt Lake) were obtained as described earlier (*50*) and cultured to adults in 30% Instant Ocean (Aquarium Systems, Mentor, OH, USA) under aeration, and the presence of dried blue-green algae *Spirulina sp.* obtained from a local pet food store (*51*). Adult females with oocytes were copulated and oocytes and embryos staged as described earlier (*52*, *53*). Ovisacs with either diapause-destined (oviparous) embryos or with embryos developing into nauplii (ovoviparously) were dissected with a scalpel and quick frozen in liquid N_2. Encysted gastrula embryos deposited in the culture dish were collected with a micropipette and kept either in sea water at 4°C or dried (activated) in a desiccator with Drierite for several days at room temperature. Partially emerged pre-nauplius larvae (E-1stage) and fully emerged pre-nauplius larvae (E-2 stage) obtained from encysted embryos were collected from culture dishes using a micropipette (*39*).

Analysis of group 1 LEA proteins at different stages of the life cycle

Eggs and embryos (50–100) collected at different stages of development were homogenized in 50–100 μl of buffer A (100 mM NaCl, 10 mM Tris–HCl, pH 7.4) fortified with 0.5 mM PMSF and 1× Protease Arrest. The homogenates were heated immediately at 75°C for 10 min, chilled on ice and the heat-insoluble proteins were removed by centrifugation. The heat-soluble protein content was

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determined using the Bradford assay (*54*) then the heat-soluble proteins, equivalent to 50 eggs/embryos, were analysed by SDS–PAGE, CBB staining and western blotting. In a separate experiment 200 eggs/embryos from post-fertilization stages were analysed by immunoblotting only.

Preparation of Artemia *embryo proteins*

Commercial dried embryos of *Artemia* from GLS were fully hydrated and collected on a sintered glass filter, washed well with distilled water then homogenized in 25 g batches in buffer K using a motorized mortar and pestle as described earlier (*27*). The supernatant was collected by centrifugation at 12,000*g* for 20 min then heated for 10 min at 75°C. After cooling on ice for 30 min the heat-insoluble proteins were removed by centrifugation and the solution containing the heat-soluble proteins adjusted to contain 75% ammonium sulphate (w/v). After cooling for 30 min, the ammonium sulphate precipitate was collected by centrifugation and suspended in buffer A.

LEA protein antibody production

The amino acid complement of the first group 1 LEA proteins identified in encysted embryos of *Artemia* contained 182 amino acids and eight 20-mer repeats (NCBI accession number ABR67402). Analysis of the protein sequence revealed two regions with potentially good antigenicity and devoid of glycosylation sites. These included the sequence E67 to R80 (EGYQEMGQKGGQKR) near the centre of the molecule and G156 to Y169 (GGEARKQQMSRADY) near the C-terminus of the molecule. These peptides were synthesized commercially and injected together into two rabbits (ProSci Inc., Poway, CA, USA). After 2 months, both rabbits showed excellent immune responses to each peptide as determined by direct ELISA.

Purification of the LEA proteins by gel filtration, ion-exchange and reverse-phase chromatography

Initially, the heat-soluble proteins were fractionated on a column of Superose 12 (HR 10/30) using buffer A at a flow rate of 0.5 ml/min. Column fractions (0.5 ml) were analysed for protein using SDS–PAGE and CBB staining as describe earlier (*27*), and by a standard western blotting procedure using anti-LEA antibodies to determine the elution position of the group 1 LEA proteins. Column fractions containing the LEA proteins of interest were concentrated using Ultracel-10K filters, equilibrated with buffer B (25 mM NaCl, 2.5 mM Tris–HCl, pH 7.4), then applied to a Mono Q column (HR 5/5) equilibrated with buffer B. Elution of proteins from Mono Q was achieved using a linear gradient of NaCl to 500 mM in buffer B over 60 min at a flow rate of 0.5 ml/min. Column fractions (0.5 ml) were analysed for the LEA proteins by western blotting as describe below. The LEA proteins which eluted from the column at three distinct salt concentrations were concentrated individually using Ultracel-10K filters. Cation-exchange chromatography was performed using a Mono S column (HR 5/5) equilibrated with Buffer C (25 mM sodium acetate, pH 5.4). Protein was eluted from the column using a linear gradient of NaCl to 500 mM over 60 min at a flow rate of 0.5 ml/min. Column fractions (0.5 ml) were analysed by SDS–PAGE and CBB staining and western blotting for the LEA proteins. (Supplementary Fig. S1.) Chromatography on a C-18 HPLC reverse phase column (250×4.6 mm) was carried out on LEA proteins purified on Mono Q, and also on LEA-rich protein fractions from Mono S. In this case, proteins were eluted using a gradient of acetonitrile (6–80%) in 0.1% TFA at a flow rate of 1 ml/min. The solvent was removed by lyophilization of selected column fractions. After suspension of the dry white powder in a small volume of water, SDS–PAGE and western blot analyses were performed to identify the LEA proteins.

Phosphoprotein analysis

Artemia cysts were homogenized in buffer A containing 10 mM sodium fluoride, 0.1 mM sodium pyrophosphate and 0.1 mM EDTA and the group 1 LEA proteins purified as described earlier. The LEA proteins were applied to a 10% SDS–PAGE gel along with post-mitochondrial fraction proteins as controls. Following electrophoresis, the gel was fixed in 50% methanol and 10% acetic acid for 1 h, washed well with water then stained with Pro-Q Diamond phosphoprotein gel stain (Molecular Probes) according to the manufacturer's instructions. The gel was destained and washed as recommended then visualized on Molecular Imager Fx (BioRad). Subsequently, the gel was stained with CBB to detect total proteins.

Isolation of organelles

Encysted embryos of *Artemia* contain an abundance of organelles which are easy to obtain. Nuclei were isolated from fully hydrated cysts by a modification of the method of Squires and Acey using centrifugation through a 75% gradient of Percoll as described earlier (*55*). Mitochondria were obtained from a cyst homogenate prepared using buffered sucrose according to an established method (*56*). Two additional wash steps were included prior to collection of the mitochondria by centrifugation at 10,000*g* for 15 min. Yolk platelets were obtained from a homogenate of encysted embryos prepared using the same buffered sucrose solution, and purified by centrifugation through a 75% gradient of Percoll as described earlier (*57*). All purified organelles were suspended in a small volume of buffer A containing 0.5 mM PMSF then heated for 10 min at 75°C. After cooling on ice for 15–30 min, the heat-soluble proteins were collected by centrifugation and used immediately for analysis of the LEA proteins.

Gel electrophoresis and western blotting

Embryo extracts or proteins from various chromatographic procedures were heated in SDS sample buffer and analysed on a 10% polyacrylamide gel (SDS–PAGE). The proteins on the gel were stained with CBB or transferred to a nitrocellulose membrane (0.2 µm) for western blot analysis using standard procedures. The anti-group 1 LEA antibody was used at 1 : 10,000 dilution, while the goat anti-rabbit secondary antibody (HRP) was used at 1 : 2,500 dilution. The antibody positive bands were detected using the ECL reagent according to the manufacture's instructions, and the membrane was exposed to an X-ray film for 0.5–2 min.

Analysis of the group 1 LEA proteins in different species of Artemia

Artemia cysts from different geographical locations around the world, and representing different species were provided by the Artemia Reference Centre in Ghent, Belgium. These were hydrated in 7.5% Instant Ocean washed well with water then homogenized in buffer A containing 0.5 mM PMSF and 1% Protease Arrest and the heat-soluble (stable at 75°C) proteins obtained by centrifugation at 12,000*g* were analysed by SDS–PAGE, western blotting and the Bradford reagent for group 1 LEA proteins as described earlier.

Mass spectrometry

Protein solutions were prepared in HPLC water at a concentration of 75–150 ng/µl. Matrix solutions, α-cyano-4-hydroxycinnaminic acid (Sigma, 10 mg/ml) and sinapinic acid (Applied Biosystems, 10 mg/ml) were prepared fresh in 60% HPLC grade acetonitrile (ACN) with 0.1% trifluoroacetic acid (TFA) (Pierce) or 30% ACN with 0.1% TFA in HPLC H_2O, respectively. Proteins were mixed at 1 : 1 ratio with matrix solution on the target plate. Both matrix types displayed comparable yields of singly and doubly charged protein species when analysed by matrix-assisted laser desorption-ionization-time of flight (MALDI-TOF) in positive linear mode on a MALDI-TOF DE-Pro Voyager instrument (Applied Biosystems) using an accelerating voltage of 25,000 V. Protein identification was confirmed by trypsin digestion followed by MALDI-TOF analysis as descried earlier (*27*). The instrument was calibrated externally using the Sequazyme peptide mass standards kit (Applied Biosystems). MS spectra were processed with Data Explorer software (version 4.0, Applied Biosystems).

Results

Artemia franciscana *cysts contain multiple group 1 LEA proteins*

Previously, a 21 kDa heat-stable protein isolated from dormant cysts of *A. franciscana* was identified as a group 1 LEA protein, the first to be described in an animal species (*27*). The present study showed that dried and re-hydrated *A. franciscana* cysts contain at least three heat-stable proteins designated as LEA-1a

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(21 kDa), LEA-1b (19 kDa) and LEA-1c (15.5 kDa) as detected on western blots, irrespective of whether (or not) the homogenization buffer contained added protease inhibitors (Fig. 1). High speed sediments obtained by centrifugation of cyst homogenates were essentially devoid of group 1 LEA proteins (data not shown). LEA proteins soluble in homogenizing buffer at 75°C were also soluble in 1.5% TCA, but not in 3% TCA (Fig. 1).

Identification of group 1 LEA proteins in encysted A. franciscana *embryos*

As determined by western blotting, group 1 LEA proteins eluted from a gel filtration HPLC column of Superose 12 mainly in fractions 24–28 min, which also revealed an additional polypeptide of 13 kDa termed LEA-1d (Fig. 2). The contents of fractions 24–28 min were pooled, concentrated and applied to a Mono Q column resulting in partial fractionation of two major and two minor LEA proteins (Fig. 3A). The group 1 LEA proteins eluted in three distinct fractions designated as 'I', 'II' and 'III' (inset, Panel A). The LEA proteins which did not bind to Mono Q (fraction I) contained mainly LEA-1b and LEA-1c, those in fraction II contained mainly LEA-1b, while those in fraction III contained both LEA-1a and LEA-1b [please note that the Mono Q procedure is an improvement over that reported earlier (*27*)]. The contents of the column fractions containing these proteins were re-chromatographed individually on Mono Q columns and they eluted in the same position as they did originally (Fig. 3B–D). The insets in each panel show the western blot analyses of the group 1 LEA proteins present originally in fractions I, II and III. Because LEA-1b appeared in all three Mono Q fractions, and might be complexed with other group 1 LEA proteins, the Mono Q fractions (Fig. 3) were applied separately to the cation-exchange column Mono S. The fraction I LEA proteins eluted from Mono S as distinct peaks representing LEA-1b, LEA-1c. Additionally, LEA-1a and LEA-1b (from fractions II and III in Fig. 3) were partially resolved on Mono S suggesting that the group 1 LEA proteins are not tightly complexed with one another (Supplementary Fig. S1).

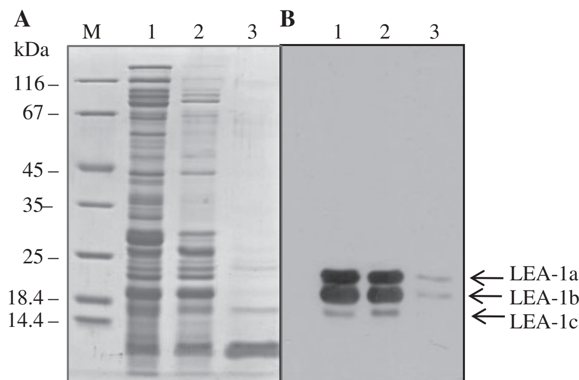

Fig. 1 Analysis of heat-stable and TCA soluble proteins in the supernatant of encysted embryos of *Artemia* by SDS–PAGE and western blotting. (A) CBB stained gel. (B) Western blot with group 1 LEA antibody. Lane M, molecular weight markers, lanes 1, heat-soluble cytosol proteins, 10 µg; lanes 2, 1.5% TCA-soluble, (and heat-soluble) cytosol proteins, 5 µg; lanes 3, 3% TCA-soluble (and heat-soluble) cytosol proteins, 2.3 µg.

Throughout our purification studies the group 1 LEA proteins were readily purified from other proteins by HPLC using reverse phase chromatography on a C-18 column, but they all eluted from the column at 31–32% acetonitrile in the elution buffer. When the Mono S purified LEA proteins were combined and applied to a C-18 reverse phase column, the elution pattern indicated identical or nearly identical hydrophobic properties as the proteins eluted in a single sharp peak (Fig. 4). Unequal amounts of the LEA proteins were applied to the C-18 column accounting for the different amounts of LEA protein detected.

As determined by computational analysis each of the LEA proteins contained a different number of the 20-mer repeats found in the group 1 LEA proteins (Table II). The 21 kDa protein has a mass of 19,612 Da by mass spectroscopy which compares favourably with the value of 19,676 Da calculated for the archived 21 kDa group 1 LEA protein (NCBI, accession number ABR67402). The nearly identical tryptic peptide maps for each of the *Artemia* LEA proteins (data not shown), and the known average mass for each 20-mer indicated that LEA-1b, LEA-1c and LEA-1d contain seven, six and five 20-mer motifs, respectively. The number of 20-mer motifs in LEA-1c, and LEA-1d have been confirmed by PCR and DNA sequencing, and their sequences entered into the NCBI database (accession numbers GU568033 and GU568034).

Group 1 LEA proteins are synthesized only in diapause-destined Artemia *embryos*

Immunoblot analyses were performed on heat-soluble proteins from various tissues of sexually mature females, including eggs at pre-fertilization stages in ovisacs with the potential to develop along either the oviparous pathway (diapause-destined showing shell gland activity) or the ovoviparous pathway (nauplius-destined without shell gland activity), both before and after fertilization. Group 1 LEA proteins were never detected in nauplius-destined embryos although LEA-1a and LEA-1b were observed in oviparously developing, diapause-destined embryos beyond 4 days post-fertilization and in cysts released from females 5–6 days post-fertilization (Fig. 5). Trace amounts of the LEA proteins were detected in diapause-destined embryos 4 days post-fertilization when 200 embryos were taken for analysis rather than 50 embryos (Fig. 5, bottom panel), so it would appear that the group 1 LEA proteins appear shortly before oviposition, 4–5 days post-fertilization. Drying of newly deposited cysts did not alter the LEA protein profile although differences were noted in the relative amounts of LEA-1a and LEA-1b in different experiments. However, LEA-1a and LEA-1b were observed to decline slowly in cyst following rehydration and incubation, then decreased abruptly once embryos emerged from their shells (E-1 stage, 14 h), resulting in complete disappearance from fully emerged embryos (E-2 stage, 18 h) and newly hatched swimming nauplii (N). In some experiments a small amount of

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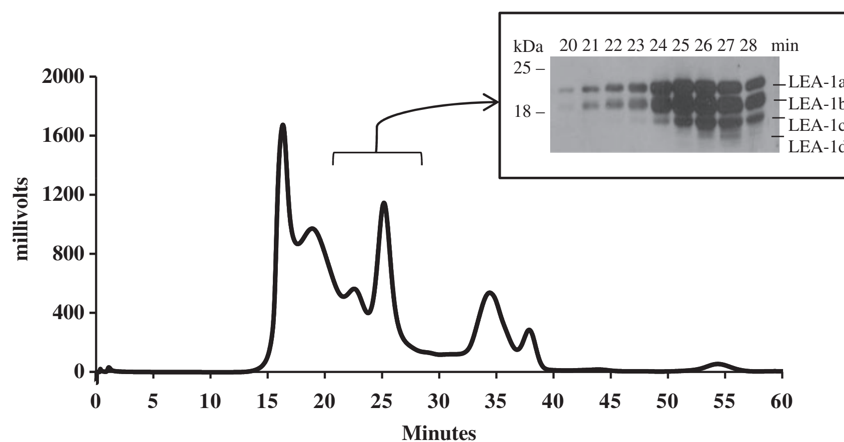

Fig. 2 Gel filtration of *Artemia* encysted embryo heat-soluble proteins on Superose 12. The heat-soluble proteins from the post-mitochondrial fraction (PMF, cytosol) were concentrated with 75% ammonium sulphate then applied to a Superose 12 column (1×30 cm) eqilibrated with 0.1 M NaCl buffered with 0.01 M Tris–HCl, pH 7.4. Column fractions were assayed by SDS–PAGE followed by western blotting with group 1 LEA antibody as shown in the insert. The LEA proteins eluted between 21 and 28 min at an elution rate of 0.5 ml/min.

LEA-1c was present in dormant cysts which also disappeared during development. A LEA protein antibody-reactive band occasionally appeared in nauplii at ~27 kDa (Fig. 5) but it has not been identified. No LEA proteins were detected in adult males or non-reproductive tissues of adult females of *Artemia* (data not shown). The data demonstrate that group 1 LEA proteins are only synthesized in diapause-destined embryos of *A. franciscana*.

Group 1 LEA proteins translocate into mitochondria of Artemia *embryos*

Heat-soluble proteins were obtained from nuclei, mitochondria and yolk platelets of encysted embryos and analysed by SDS–PAGE. As determined by western blotting, mitochondria were found to contain significant amounts of LEA-1a and LEA-1b compared with the cytosol, whereas nuclei and yolk platelets were essentially devoid of these proteins (Fig. 6). An antibody positive band of ~50 kDa appeared routinely in unheated cytosol, but it was not characterized in this study. Finally, we noted that exposure of *A. franciscana* cysts to anoxia for 8 weeks, known to cause intracellular translocations of other proteins, resulted in a little redistribution of the group 1 LEA proteins among organelles (Fig. 7). It may be noteworthy that nuclei acquired a small amount of LEA-1a in response to anoxia, while LEA-1a, LEA-1b and LEA-1c declined slightly in mitochondria.

Group 1 LEA proteins are not phosphorylated in Artemia *cysts*

Computer analysis (EXPASY) of the deduced protein from the cloned LEA-1a cDNA indicated the presence of 10 sites in the protein with a high probability (>0.938) for phosphorylation at two serine and eight tyrosine sites. Compared with the total heat-soluble proteins in the cytosol of *A. franciscana* cysts from either the Great Salt Lake (GSL) or San Francisco Bay (SFB) which contained several phosphorylated proteins, neither LEA-1a nor LEA-1b were found to be modified by phosphorylation (Fig. 8).

Group I LEA proteins in geographically dispersed Artemia *sp*

Immunostaining of western blots containing heat-stable proteins resolved in SDS polyacrylamide gels demonstrated different LEA protein profiles among seven species of *Artemia* cysts from 11 distinct geographic sites (Fig. 9). Strictly speaking, the parthenogenetic populations do not have a species designation [see editor's note in Ref. (*58*)], but we use parthenogenetica for convenience here. Of the species tested, *A. urmiana* (Iran), *A. salina* (Tunisia) and *A. salina* (Libya) contain only one LEA with a mass of ~15 kDa, similar to LEA-1c found in *A. franciscana* from the Great Salt Lake in Utah. *Artemia persimilis* from Argentina contained one LEA of ~17 kDa, while *A. tibetiana* (China) contained one LEA of ~22 kDa and another band at ~50 kDa. *Artemia parthenogenetica* strains from both Russia and Greece contained only one LEA of ~21 kDa. Only *A. franciscana* cysts produced in Vietnam from inoculations of SFB cysts, and from San Francisco Bay and the Great Salt Lake in Utah contained multiple group 1 LEA proteins. *Artemia sinica*, a bisexual strain from China, showed a considerable amount of LEA-1a despite the poor quality of its heat-soluble proteins.

Discussion

As mentioned, encysted embryos of *A. franciscana*, are arguably among the most stress-resistant of all multicellular eukaryotes (*39*, *40*, *52*, *58–60*). We believe the LEA proteins studied here are part of the adaptive repertoire providing the basis of these abilities.

We previously described a heat-stable 21 kDa protein in these embryos whose properties were consistent with the group 1 LEA proteins found in plants. We showed that the 21 kDa protein prevented drying induced protein aggregation when tested along with trehalose *in vitro* (*27*). In the present study, we used an antibody-based approach to expand our knowledge of the group 1 LEA proteins in these embryos.

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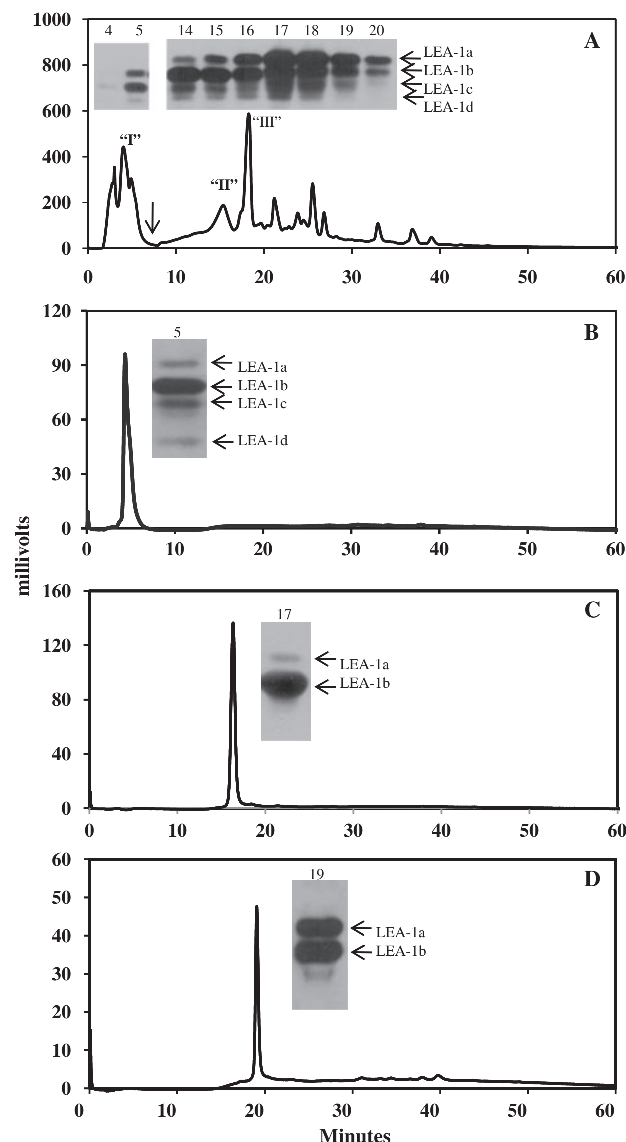

Fig. 3 HPLC of *Artemia* cyst supernatant proteins on Mono Q. (A) Column fractions 24–28 from the Superose 12 column (Fig. 2) were applied to a Mono Q column (1 × 5) cm and eluted with a NaCl (from 0.025–0.5 M) over 60 min at a flow rate of 0.5 ml/min. Selected column fractions were analysed by western blotting using the group 1 LEA antibody (see inset). (B) The LEA proteins in fraction 'I' shown in (A) were purified on a reverse phase C-18 column, then re-chromatographed on Mono Q under the same conditions used in (A). Western blot analysis using the group 1 LEA antibody of the fraction eluting at 5 min is shown in the inset. (C) The LEA proteins in fraction 'II' shown in (A) were purified on a C-18 column then re-chromatographed on Mono Q under the same conditions used in (A). Western blot analysis using the group 1 LEA antibody is shown in the inset. (D) The LEA proteins in fraction 'III' in (A) were purified on C-18 then re-chromatographed on Mono Q under the same conditions as used in (A). Western blot analysis of the main peak using the group 1 LEA antibody is shown in the inset.

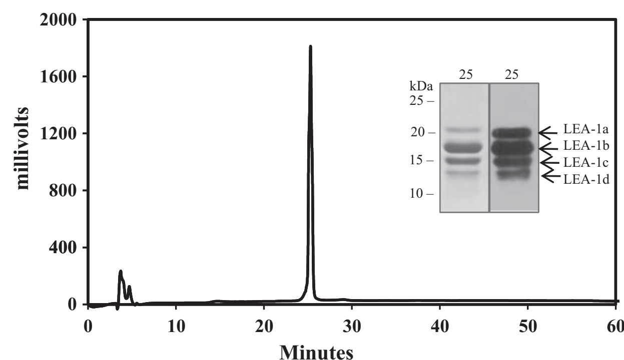

Fig. 4 Analysis of group 1 LEA proteins on a C-18 hydrophobic column. The four purified LEA proteins from the Mono Q column (LEA-1a, LEA-1b, LEA-1c, LEA-1d) were combined and chromatographed on a C-18 column using a linear gradient of acetonitrile from 6 to 80% with 0.1% TFA at a flow rate of 1 ml/min. The insert shows the CBB stained proteins from SDS–PAGE analysis (left panel) and western blot analyses using anti-group 1 LEA (right panel) of column fraction 25.

Table II. Summary of group 1 LEA proteins purified from dormant cysts of *A. franciscana* from the Great Salt Lake.

Protein	SDS–PAGE[a]	Mass spectroscopy[b]	Difference[c]	20-mer motifs[d]	Accession No.[e]
LEA-1a	21	19,612 ± 6	—	8	ABR67402
LEA-1b	19	17,448 ± 9	2164	7	–
LEA-1c	15.5	15,344 ± 9	4268	6	ADE45145
LEA-1d	13.0	13,084 ± 6	6528	5	ADE45146

[a]Mass in kilodaltons.
[b]Mass in Daltons representing the average of 4–6 measurements made on samples prepared over a period of 6 months.
[c]Difference of mass in Daltons from that in LEA-1a.
[d]Estimate for LEA-1b, LEA-1c and LEA-1d assuming an average mass of 2,150 Da for each 20-mer motif found in LEA-1a.
[e]NCBI database.

We purified several group 1 LEA proteins to homogeneity, resulting in the identification of four group 1 LEA proteins in diapause-destined embryos of *Artemia*, designated as LEA-1a, LEA-1b, LEA-1c and LEA-1d (Figs 2–4). LEA-1a and LEA-1b are the most abundant groups found in cysts from the Great Salt Lake (Utah, USA), while LEA-1c is a minor component and LEA-1d can only be detected in protein fractions enriched for the LEA proteins. Compared with known amounts of these proteins on western blots, LEA-1a and LEA-1b represent ~5% of the supernatant proteins. Based on mass spectrometry and sequencing of PCR products obtained using an *Artemia* embryo cDNA library, we determined that LEA-1a, LEA-1b, LEA-1c and LEA-1d contain eight, seven, six and five 20-mer motifs, respectively, characteristic of group 1 LEA proteins from other organisms (Table II) (*4*). Sequences for LEA-1a, LEA-1c and LEA-1d have been entered into the NCBI database (Table II). Like group 1 LEA proteins from other organisms (*4*, *31*), those from *Artemia* are thermostable, remaining soluble at temperatures up to 95°C. The LEA proteins in the heat-treated supernatant were stable to storage at 0–4°C for several weeks, while LEA proteins in untreated supernatant degraded over several days at −10°C, even in the presence of protease inhibitors. All group 1 LEA proteins studied were soluble in 1.5% TCA, but not in 3.0% TCA (or higher) as reported previously for several LEA proteins in *Arabidopsis* (*31*). LEA-1a, LEA-1b, LEA-1c and LEA-1d have identical hydrophobic

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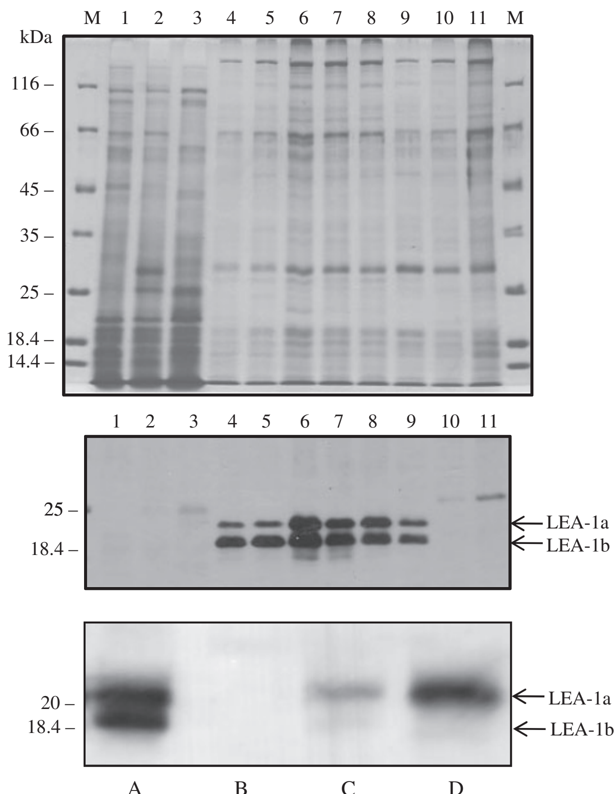

Fig. 5 Analysis of heat-soluble proteins from ovarian tissue, eggs and embryos at different stages in the life cycle of *Artemia*. Top panel, CBB stained proteins. Middle panel, immunoblot using group 1 LEA antibodies. Lane 1, three ovisacs showing shell gland activity; lane 2, three ovisacs with encysted eggs 4 days post-fertilization; lane 3, three ovisacs with nauplii-destined eggs, 6 days post-fertilization; lane 4, encysted eggs in diapause hydrated and oviposited; lane 5, encysted eggs in diapause, desiccated *in vitro*; lane 6, hydrated encysted embryos from commercial source, 0 h development; lane 7, developing encysted embryos, 5 h development; lane 8, developing encysted embryos, 8 h development; lane 9, developing encysted embryos, partially emerged (E-1 stage, 14 h); lane 10, developing, fully emerged embryos (E-2 stage, 18 h); lane 11, newly hatched nauplius larvae. Lanes 4–11 contained heat-soluble proteins from 50 embryos or larvae. M, molecular weight markers. Bottom panel, immunoblot of the heat-soluble proteins from 200 embryos at different times after fertilization. Lane A, control, hydrated commercial cysts; lane B, 2 days post-fertilization; lane C, 4 days post-fertilization, lane D, oviposited encysted embryos.

properties (Fig. 4). The predicted low grand average of hydropathy (GRAVY) value for each of these proteins (ca. −1.4, Table I), and the high degree of homology among the 20-mer motifs in these proteins (80–90%), probably accounts for their behaviour on a C-18 reverse phase column.

Using PCR methodology we confirmed the presence of mRNAs coding for LEA-1a, LEA-1c and LEA-1d in an embryo cDNA library (see NCBI database), but we have not been able to identify a cDNA coding for LEA-1b with the predicted seven 20-mer motifs. We are sure that LEA-1b is not derived from LEA-1a by proteolysis, so we are not certain about the origin of this protein, one of the two dominant LEA proteins. Perhaps two protein initiation sites are used on a single mRNA during translation, one site generating LEA-1a (21 kDa) and another giving rise to LEA-1b (19 kDa), a possibility that could be checked by N-terminal sequence data. With respect to LEA-1b, we were puzzled by the elution characteristics of this protein from Mono Q, repeatedly eluting in three distinct fractions which we designated as 'I', 'II' and 'III' (Fig. 3). Since the true mass of LEA-1b from each fraction was the same (Table II), we concluded that some LEA-1b must be complexed with other molecules, altering its ionic properties, then released upon further chromatography. This possibility, characteristic of some molecular chaperones, is currently being studied. Intrinsically unstructured proteins have reduced binding of SDS, often showing an increased apparent molecular mass on SDS–PAGE gels (*13*), and such is the case for *Artemia* LEA-1a and 1b (Table II).

Phosphorylation is an important event, especially in the control of metabolic functions. Among plants, several LEA proteins in groups 2 and 3 are phosphorylated, but no reports have appeared demonstrating phosphorylated group 1 LEA proteins despite the presence of multiple sites with high probability of occurrence (*4*, *61*, *62*). Thus, the LEA proteins identified in this study are not phosphorylated even though LEA-1a has 10 potential consensus sites for various kinases (Fig. 8).

Analysis of the *Artemia* embryos EST database indicated that it contained mRNAs with the capacity to produce group 1 LEA proteins with variable copies of the 20-mer motifs characteristic of the group 1 LEA proteins (Table I). However, despite our exhaustive search among *Artemia* embryo proteins, we have not found LEA proteins for all predictable mass sizes and characteristic 20-mer motifs based on clones in the EST database (*37*). Among the clones in the EST database with high homology to LEA-1a (21 kDa) described earlier (*27*), ∼25% of the EST entries have open reading frames coding for a group 1 LEA protein with four 20-mer motifs and a calculated mass of 10.2 kDa. We confirmed the presence of mRNA in an *Artemia* embryo cDNA library coding for an LEA protein with four 20-mer motifs; however, we have not found a group 1 protein of 10.2 kDa in any embryo fraction. Moreover, analysis of most EST clones coding for an LEA protein with four 20-mer motifs has revealed an upstream signal peptide consistent with the translocation of the putative protein to mitochondria, although we have not detected a 10.2 kDa LEA protein in mitochondria (Fig. 6). Our methodology should be able to detect a group 1 LEA protein of 10.2 kDa (if present). The question remains: why are we not able to detect a 10 kDa group 1 LEA protein when evidence suggests an abundance of its mRNA in embryos of *Artemia*? One possibility is that embryos contain a store of mRNA with the capacity to produce a 10 kDa LEA protein containing four of the 20-mer motifs when cysts undergo a stressful event. This is currently under investigation. We also noted that the EST library contains cDNAs coding for LEA proteins with two 20-mer motifs and a mass of 5.5 kDa, and confirmed its presence using PCR (see NCBI accession number GU568035), but we have not detected a group 1 LEA protein (*i.e.* LEA-1g) of this size. Overall, the smallest group 1 LEA protein

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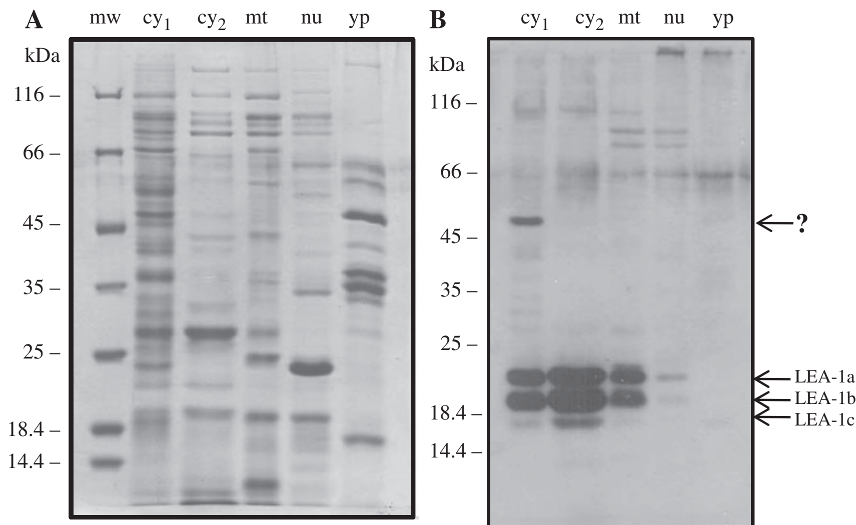

Fig. 6 SDS–PAGE and western blot analyses of heat-soluble proteins from various cell fractions of encysted embryos of *Artemia*. Five or six micrograms of heat-soluble proteins obtained from the cytosol (cy_2), mitochondria (mt), nuclei (nu) and yolk platelets (yp) were applied to duplicate lanes on the same SDS–PAGE gel along with unheated cytosol (cy_1). One-half of the gel was stained for protein with CBB (A), while proteins in the other half of the gel (B) were transferred to a NT membrane and processed with the group 1 LEA antibody.

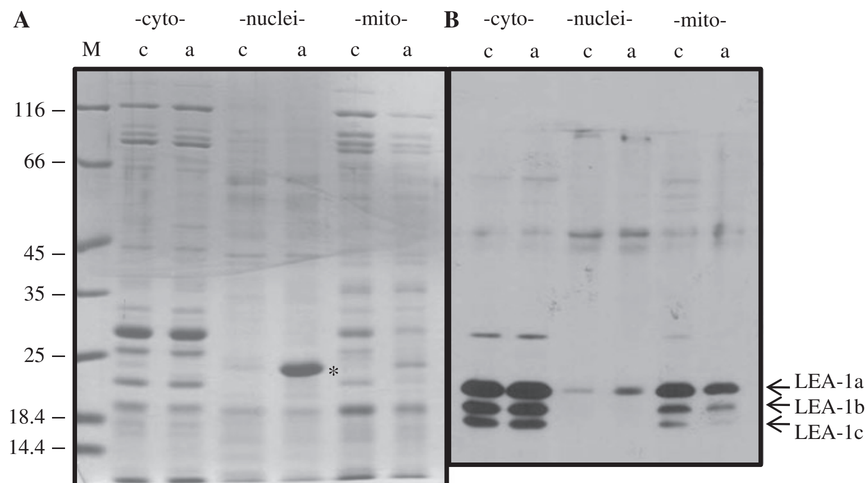

Fig. 7 Distribution of group 1 LEA proteins in anoxic embryos of *Artemia*. Nuclei, mitochondria and the post-mitochondrial (cytosol) fractions were isolated from 8 weeks anoxic embryos and control embryos as described earlier (*55*), and the heat-soluble proteins were obtained from each fraction. (A) CBB stained gel. (B) Western blot using the group 1 LEA antibody. 'c' and 'a' represent embryo fractions from control and anoxic embryos, respectively. Each lane contained 4 µg protein. Asterisk probably represents artemin which is known to translocate to the nucleus during periods of anoxia.

detected in our analysis of *Artemia* embryos is LEA-1d with five 20-mer motifs and a mass of 13 kDa.

Mitochondria in higher organisms are sensitive to desiccation, but they survive drying in plant seeds and desiccation tolerant invertebrates (*4*, *63*). In plants, LEA proteins in groups 2 and 3 have been found distributed among different organelles including nuclei, chloroplast and mitochondria, while group 1 LEA proteins have not been localized to any organelle [see review by Tunnacliffe and Wise (*4*)]. However, we detected a significant amount of LEA-1a and LEA-1b in mitochondria, a very small amount of LEA-1a in nuclei, but neither in yolk platelets (Fig. 6). LEA proteins appear to be translocated into mitochondria of diapause-destined *Artemia* embryos prior to oviposition along with AfrLEA3, a group 3 LEA protein identified recently (*25*). An mRNA coding for LEA-1a with a 35 amino acid presequence has been detected in an *Artemia* embryo cDNA library, amplified by PCR, sequenced and registered with NCBI (accession number GQ406334) (Supplementary Fig. S2). Computer analysis of the protein (MitoProt) gave a high probability (>0.933) of the protein being transported to the matrix of mitochondria. This group 1 LEA protein (Fig. 6) may serve a function similar to the LEA proteins in seed mitochondria of the pea, *Pisum sativum*, where they are thought to protect the inner mitochondrial membrane and sensitive proteins from aggregation during desiccation (*63*, *64*). However, unlike LEA-1a, the origin of LEA-1b in *Artemia* embryos is not known; nevertheless, these two proteins appear to contribute to the proteome of

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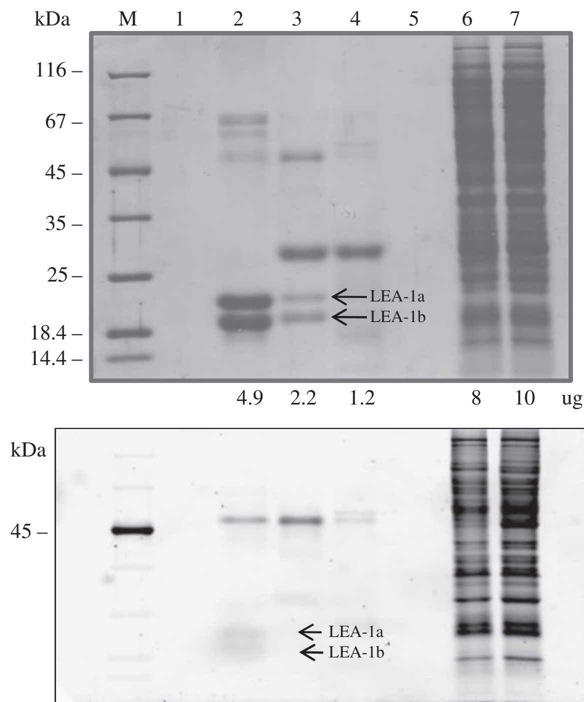

Fig. 8 Phosphate analysis of LEA proteins. Top panel, SDS–PAGE gel of various protein fractions stained with CBB. Bottom panel, Pro-Q diamond stain for phosphate of various heat-soluble protein fractions. At the left is ovalbumin which has two residues of phosphate per molecule of protein. M, molecular weight; lanes 1 and 5, blank; lanes 2–4, LEA containing fractions from C-18 column as shown in Fig. 4; lanes 6 and 7, heat-soluble proteins of *Artemia* encysted embryos from GSL and SFB cysts, respectively.

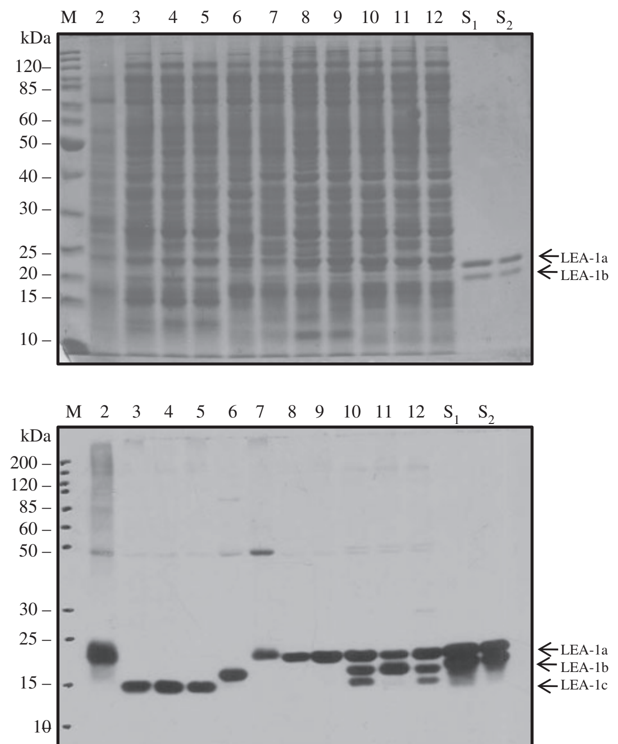

Fig. 9 SDS–PAGE and western blot analyses of different species of *Artemia* cysts for group 1 LEA proteins. Top panel, CBB stained gel. Bottom panel, western blot analysis with group 1 LEA antibodies. Lane 2 contained 10 μg of heat-soluble cytosol proteins, while lanes 3–12 contained 21 μg of heat-soluble cytosol proteins. Lane 2, *A. sinica* (China); lane 3, *A. urmiana* (Iran); lane 4, *A. salina* (Tunisia); lane 5, *A. salina* (Libya); lane 6, *A. persimilis* (Argentina); lane 7, *A. tibetiana* (China); lane 8, *A parthenogenetica* (Russia); lane 9, *A. parthenogenetica* (Greece); lane 10, *A franciscana* (Vietnam); lane 11, *A. franciscana* (GSL); lane 12, *A. franciscana* (SFB). Lanes S1 and S2 contained 0.5 and 0.25 μg purified LEA-1a and LEA-1b (from GSL cysts), respectively.

mitochondria. We note here that, in addition to the group 1 LEA proteins identified here, and AfrLEA3 mentioned above, mitochondria of encysted embryos of *Artemia* contain at least four group 3 LEA proteins to be reported on in the future.

Diapause in *Artemia* is characterized by the severe depression of metabolism and up-regulation of genes concerned with stress tolerance (*40–42*, *65*). Prior to entry into diapause, a gene is expressed that is involved with cyst shell formation, essential for entry into diapause and tolerance to water loss and other stresses (*66*). Desiccation tolerance in both plants and animals is a complex process (*3*), but when linked to embryonic diapause, as it is in *Artemia*, considerable coordination must occur to ensure survival of the embryo when development resumes.

Beginning at ~2 days post-fertilization in *Artemia* at least 17 genes are up-regulated in diapause-destined embryos, but not in nauplii-destined ones (*26*, *40*, *65*). These genes include those coding for co-transcription factor p8 (*43*, *65*), small heat shock proteins p26, (*53*), ArHsp21 (*67*) and ArHsp22 (*68*) which have protein chaperone activity, and artemin a potential RNA chaperone (*40*, *69*), as well as those coding for trehalose synthesis (*39*). The present study adds group 1 LEA proteins to that list. Newly released encysted embryos contained mainly LEA-1a but LEA-1b appeared shortly after oviposition, and both LEA proteins were present in the diapausing embryo. (Occasionally, and as shown in Fig. 5, LEA-1b is more abundant than LEA-1a in oviposited embryos for reasons which are not clear.) Following activation and resumption of development LEA proteins decreased slightly following rehydration and incubation, then disappeared in the fully emerged pre-nauplius larva (E-2 stage) (Fig. 5). Therefore, there is good correlation between stages of desiccation tolerance (encysted embryos), high concentrations of trehalose and LEA proteins, both of which might function in the formation of protective biological glasses (*27*) (and this study). Previously, we demonstrated that a 21 kDa heat-soluble protein (*i.e.* LEA-1a) together with trehalose, inhibited drying induced aggregation of citrate synthase *in vitro* (*27*). Whether the other group 1 LEA proteins function similarly remains to be determined. But our results and those of others (*24*, *25*) strongly suggest that LEA proteins and molecular chaperones noted above contribute to desiccation tolerance.

In response to anoxia, p26 translocates to the nucleus (*55*) as does a small amount of group 1 LEA-1a. However, mitochondria actually lose group 1 LEA proteins during anoxia (Fig. 7). Clearly, the group 1 LEA proteins are not required to maintain

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mitochondria under anoxia, while they may function in anoxic nuclei. The significance of these differing observations is not clear at present.

Artemia is widely distributed across five continents at ~500 sites (*70*), and there are certainly many more. *Artemia franciscana* is thought to have diverged from its sister species (*urmiana*, *tibetiana*, *sinica*) ~30-million-years ago (mya) (*71*), and emerged from its ancestral form in the Mediterranean area ~5.5 mya where both bisexual and parthenogenic individuals still exist (*72*). Parthenogenetic forms were thought to branch from bisexuals ~5 mya, in contrast to most other invertebrates where the reverse order of evolution occurs. *Artemia persimilis*, a bisexual species of the New World, is considered to be the most primitive species and most closely related to the original ancestor in the Mediterranean from which the bisexual species *A. salina*, *A. sinica*, *A. urmania*, and *A. franciscana* originated (*72*). *Artemia persimilis* uniquely has a single group 1 LEA protein of ~16 kDa compared with *A. salina* and *A. urmania*, each having one of ~15 kDa (Fig. 9). Clearly, the differences in group 1 LEA proteins among the bisexual species of *Artemia* are as genetically distinct as their alloenzyme data and DNA markers predict. Parthenogenetic species from Greece and Russia, like bisexual species from the Old World, have a single group 1 LEA protein, but of a different mass (21 versus 15 kDa).

Artemia franciscana, the species used mostly in the present study, and found naturally in hypersaline environments in North and South America (New World) is regarded as the super species in this genus. It is the only species that contains more than one group 1 LEA protein (Fig. 9). However, *A. franciscana* cysts from SFB are distinct from the GSL, but identical to those from Vietnam where it was introduced by inoculation of SFB cysts beginning in the 1980s (*40*, *72*), and inoculated sequentially since that time (N.V. Hoa, PhD dissertation, Ghent University, Belgium, 2002). Given that *A. franciscana* possesses considerable phenotypic plasticity, it was surprising that the gene(s) coding for the group 1 LEA proteins in Vietnam cysts have not changed during so many generations and despite very different environmental conditions in Vietnam, presumably reflecting the adaptive value of these proteins and their retention by selection. Two excellent reviews dealing with *Artemia* zoogeography, evolution and speciation by Gajardo *et al.* (*72*) and van Stappen (*70*) and Supplementary Table S3, provide further details about *Artemia* used in this study.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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