Two soybean (*Glycine max* L.) GmPM proteins reduce liposome leakage during desiccation

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ABSTRACT. Immature seeds can acquire the traits of desiccation tolerance and germination after precocious maturation. This process is associated with the accumulation of non-reducing sugars and late embryogenesis abundant (LEA) proteins. LEA proteins were first identified from late developmental seeds and were proposed to be associated with desiccation tolerance. In the present study, we examined whether purified LEA fusion proteins from soybean seeds can maintain the integrity of artificial liposomes under desiccation. We also studied the relationship between the protective effects of LEA protein–sucrose glasses and desiccated membrane. GmPM6 (LEA II) but not GmPM16 (LEA IV) provided significant protection to dehydrated liposomes. However, neither GmPM6- nor GmPM16-sucrose glasses could protect these liposomes. We discuss the functions of cellular glass in mature orthodox seeds.

Keywords: Dehydration; Glass; GmPM; LEA proteins; Liposome; Soybean.

INTRODUCTION

Immature seeds of certain plants, when detached and slowly dried, acquire traits that mimic those of mature seeds, such as germination and desiccation tolerance (Rosenberg and Rinne, 1986). Thirty-five days after flowering, soybean seeds may germinate at a very slow rate and may not grow into seedlings. They are also not desiccation tolerant. If moisture is gradually withdrawn over at least 4 days, seeds become functionally similar to mature seeds in at least five respects: 1) accelerated germination rate, 2) seedling growth, 3) desiccation tolerance acquisition, 4) soluble sugars accumulation, and 5) late embryogenesis abundant (LEA) proteins accumulation. Soluble sugar levels are markedly different in axes of seeds undergoing slow drying and high relative humidity control treatment (Blackman et al., 1992). The accumulation of soluble non-reducing sugars, a characteristic of most mature seeds, appears to be important in the development of desiccation tolerance (Koster and Leopold, 1988). The hydroxyl constituents of the sugars are believed to replace the hydration

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LEA proteins are a subset of osmotic responsive proteins; their accumulation has been observed during embryo maturation in nearly all vascular plants (Dure et al., 1989; Shih et al., 2008). Hundreds of LEA proteins have been identified from plants, fungi, bacteria, and even animals (Tunnacliffe and Wise, 2007; Shih et al., 2008). Most LEA proteins are hydrophilic and remain soluble at high temperature (Dure, 1993). Five groups of LEA proteins have been identified from common amino acid sequence domains and have been proposed to contribute to desiccation tolerance in the embryo (Shih et al., 2008). The accumulation of LEA proteins is proposed to be increased in drought-stressed plants, and these proteins may play a protective role against desiccation-induced cellular damage (Dure et al., 1989; Dure, 1993). For instance, the ectopic expression of LEA proteins in model plants resulted in increased tolerance to water stress (e.g., Xu et al., 1996; Borrell et al., 2002; NDong et al., 2002). Moreover, recombinant yeast or bacteria expressing LEA proteins were reported to be less susceptible to growth inhibition of growth in media of high osmolarity (e.g. Imai et al., 1996; Swire-Clark and Marcotte, 1999; Zhang et al., 2000; Lan et al., 2005). Several in vitro experiments have involved the function of LEA proteins. Cryoprotection or desiccation protection assays showed that several LEA proteins maintained the function of stress-sensitive enzymes after freezing or dehydration (e.g., Kazuoka and Oeda, 1994; Honjoh et al., 2000; Goyal et al., 2005; Grelet et al., 2005; Nakayama et al., 2008). FTIR analysis results indicated that LEA proteins increased the glass transition temperature (T_g) of non-reducing sugars and reduced the phase transition temperature (T_m) of phospholipids in the dehydrating condition (Wolkers et al., 2001; Shih et al., 2004; 2010a; 2010c).

Using 4-day pod-dried (PD) soybean seeds at 35 days post-flowering as material, we prepared a cDNA library (Hsing et al., 1990; Hsing and Wu, 1992) and identified 41 cDNA clones designated Glycine max physiologically mature (*GmPM*). Twenty-two of them belonged to five groups of LEA genes. Molecular biology, expression profiling, or structural biology was used to reveal the functions of these proteins (e.g., Hsing et al., 1995; Lee et al., 2000; Shih et al., 2004; 2010a; 2010b; 2010c; Tsai et al., 2008). Most of the soybean LEA proteins studied accumulated in precociously or naturally matured seeds. Hydrophilic LEA proteins (groups I to IV) were members of natively unfolded proteins or intrinsically disordered proteins, and the proteins can change their conformation and interact with macromolecules, such as non-reducing sugars, phospholipids, or polypeptides.

Here, we present the results of desiccation protection assay of two hydrophilic soybean LEA proteins, GmPM6 (LEA II, 23.7 kDa, pI value 6.1) (Soulages et al., 2003; Shih et al., 2010c) and GmPM16 (LEA IV, 14.7, pI value 9.7) (Shih et al., 2004) by monitoring the integrity of liposomes after desiccation. We discuss the possible role of the LEA proteins in desiccation and dry storage tolerance.

MATERIALS AND METHODS

Bacterial strains and media

Escherichia coli strains XL1-Blue and BL21(DE3) were used as the host. XL1-Blue was used for cloning plasmids, and BL21(DE3) was used for expressing recombinant proteins and growth analysis. All cultures were grown in LBmedium in the presence of 50 μ g/ml kanamycin at 37°C.

Recombinant plasmid construction, expression, and purification of its products

Recombinant DNA techniques were performed essentially as described (Sambrook and Russell, 2001). The *NdeI-XhoI* fragment of GmPM6 or GmPM16 containing the coding region was amplified by specific forward and reverse primers (GmPM6: 5'-ggttgacg<u>CATAT</u>Ggcaacgttg-3' and 5'-catgcatg<u>CTCGAG</u>gcacgatgc-3'; GmPM16: 5'-gaagaaca<u>CATATG</u>caatcctc-3' and 5'-gacgtact<u>CTCGA-</u> <u>Gaaacacag-3'</u>) from maintenance plasmids harboring the full-length cDNA of the GmPM clones. The PCR protocol was an initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 45 sec, 55°C for 30 sec and 72°C for



Figure 1. Scheme of construction of the GmPM6 and GmPM16 fusion plasmids.

1 min, with a final extension at 72°C for 5 min. The amplified PCR products were separated and purified by 1% (w/v) agarose gel electrophoresis. Products were digested with the restriction enzymes *NdeI* and *XhoI*, then ligated into the pET28a T7 expression vector (Novagen), which had been pre-cut with the same enzymes (Figure 1). The recombinant plasmids were introduced into *E. coli* cells, and the expression of the recombinant genes was enhanced by 1 mM IPTG induction. Recombinant proteins were the manufacturer's protocol (pET system manual ver. 5.0, Novagen, Germany). Purified recombinant proteins were examined by one-dimensional 12.5% SDS-polyacrylamide gel electrophoresis, followed by Coomassie blue staining.

Liposome preparation

Myoglobin (17.2 kDa, pI value 7.1), 5(6)-carboxyfluorescein (CF), and sucrose were from Sigma (USA). 1-palmitoyl-2-oleoyl-3-sn-phosphatidylcholine (POPC, 760 kDa) was from Avanti Polar Lipids (USA). Liposomes containing CF in Tris buffer were prepared by reversephase evaporation (Szoka and Papahadjopoulos, 1978). Five micrograms of POPC was dissolved in organic solvent into which 5 mol% dicetyl phosphate or 5 mol% stearylamine was added to give an overall negative or positive charge. Neutral liposomes were made from POPC. The solvent was removed under reduced pressure at 35°C on a rotary evaporator. The dried lipids were dissolved in 1 ml of 1 mM Tris, pH 8.0, containing 150 mM CF. The tube was sonicated in a bath-type sonicator for 10 min at 30°C until a uniform emulsion formed. Liposome suspensions (multi-lamellar vesicles) were sonicated by use of a probetype sonicator. Sonication was carried out intermittently at 5°C for 5 min. After sonication, unincorporated material was removed by passing the large unilamellar vesicles through a Sephadex G-50 column equilibrated with 1 mM Tris, pH 8.0, to stabilize the LEA proteins.

Determination of particle-size distribution and zeta potential of vesicles

Mean vesicle sizes of the liposome were measured at 25°C using a Zetasizer 3000HS apparatus (Malvern Instruments, UK). Red light at 632 nm was used as the light source. Particles were measured by photon correlation spectroscopy and were in constant random thermal or Brownian motion, which caused the intensity of light scattered from the particles to form a moving speckle pattern. Large particles moved more slowly than did small particles, so their scattered light fluctuation rate was also slower. Photon correlation spectroscopy was used to measure the rate of change of these light fluctuations to determine the size distribution of the light scattering particles. The zeta potential of the liposomes was determined from electrophoretic mobility measurements at 25°C. At some distance from the surface, the "shear plane," the ions were no longer dragged along with a moving particle but remained in the bulk solution. The potential at this distance was, by definition, the zeta potential ζ . A Zetasizer and laser Doppler electrophoresis were used to measure particle movement when they were placed in an electric field. The measurement determined the charges on the particles.



Figure 2. Properties of phospholipid intermediate unilamellar vesicle. A, mean vesicle size; B, zeta potential.

Analysis of liposome membrane integrity after desiccation

Liposomes with different zeta potential and encapsulating the fluorescent dye CF were diluted into Tris buffer containing various concentrations of protein and subjected to desiccation before rehydration. Eight-microliter droplets containing liposome and sugar or protein (ratio 1:10) were dried in a stream of dry nitrogen gas for 3 hr. One milliliter of Tris buffer (1 mM) was then added to the dry liposome preparation. CF fluorescence was determined using a spectrofluorometer (excitation wavelength 490 nm, emission wavelength 515 nm). Membrane integrity after desiccation was calculated from the fluorescence of the sample relative to a non-desiccated control sample and the total CF released from the sample after the addition of Triton X-100 to 0.1%. Liposome experiments were repeated 3 times with different preparations. Data are presented as mean±S.D.

RESULTS

Particle size and zeta potential of liposomes

Figure 2A indicates that the three kinds of liposomes prepared by the reverse-phase evaporation method had similar particle size, about 120 nm. Negative and positive liposomes clearly showed negative or positive zeta potential (Figure 2B). The neutral liposome with negative zeta potential may be due to measurement under 1 mM of Tris buffer, which may also cause underestimation of the positive charge of positive vesicles.

Maintenance of liposome membrane integrity after desiccation and rehydration

Figure 3 illustrates that the liposome membrane integrity after desiccation and rehydration could be maintained at different levels while being incubated with GmPM6 or GmPM16. Structural integrity was assessed by determining the release of the encapsulated fluorescent dye CF, with total release equivalent to complete loss of integrity. Three types of liposomes desiccated alone showed loss of membrane integrity with rehydration, whereas the liposomes desiccated in the presence of GmPM6 proteins maintained up to 67%, 52%, and 72% of membrane integrity for stearylamine (positive liposome), dicetyl phosphate (negative liposome) and POPC (neutral liposome), respectively. The liposomes desiccated in the presence of GmPM16 proteins maintained up to 24%, 19%, and 18% of membrane integrity of positive, negative and neutral liposomes, respectively. Myoglobin was chosen as a control protein because of its similar molecular size to GmPM6 and pGmPM16 (see MATERIALS AND METHODS) and its having no known function in membrane protection. The protection assay indicated that percentage membrane integrity on co-incubation with myoglobin was 14%, 8%, and 7% for positive, negative and neutral liposomes, respectively. Sucrose was also chosen as a control because non-reducing sugars had been proposed to protect mem-



Figure 3. Effect of the presence of various concentrations of myoglobin, sucrose, GmPM6, GmPM6-sucrose, GmPM16, and GmPM16-sucrose glass on the structural integrity of positive, negative, and neutral liposomes after desiccation.

brane during dehydration (Hoekstra et al., 2001). Percentage membrane integrity with sucrose was 53%, 46%, and 51% for positive, negative and neutral liposomes, respectively. Hence, GmPM6 is more efficient in protecting membrane integrity during dehydration than other proteins or sucrose.

We then investigated whether the combination of sugar and LEA proteins could improve the protection of liposomes against desiccation. Three kinds of liposomes encapsulating CF containing various concentrations of LEA proteins, sucrose, and combinations of sugar and protein (in 1:1 mass ratio) were desiccated. Compared with the protective efficiency of GmPM6 alone, the efficiency of the GmPM6-sucrose matrix was significantly decreased. The membrane integrity percentage decreased to 27%, 22%, and 8% for positive, negative and neutral liposomes, respectively. By contrast, the protective efficiency of the GmPM16-sucrose matrix was slightly increased: 33%, 25%, and 22% for positive, negative and neutral liposomes, respectively. The protective efficiency of sucrose alone for liposomes was at least two-fold that of GmPM protein-sucrose matrixes.

DISCUSSION

Using liposomes as a model membrane system, Crowe et al. (1988) proposed that adjacent lipid bilayers in liposomes are held apart by the water associated with the phosphate head group. Removal and replacement of these water molecules may alter the bilayer configuration. With the loss of water molecules, hydrogen bonding to each polar head group and the lateral spacing between the phospholipids is reduced. This situation leads to increased van der Waals interactions between the hydrocarbon chains of the phospholipid molecules and thus the formation of a solid "gel" phase and an increase in the phase transition temperature (Hoekstra et al., 1989). The hydroxyl groups

of sugars may substitute for water molecules and provide the required hydrophilic interactions for membrane stabilization (Crowe et al., 1995). In the present study, GmPM6 and GmPM16 proteins were used to test the function of membrane stabilization during dehydration. Both proteins contained similar molecular mass and shared similar amino acid contents with high amounts of charged amino acid residues (Shih et al., 2008). However, they showed very different results: GmPM6 proteins strongly prevented liposome leakage after drying, whereas GmPM16 only had a weak effect (Figure 3). LEA II proteins were proposed to stabilize plasma membranes during osmotic stresses. A localization study revealed that freezing-induced WCS120, a wheat LEA II protein, accumulated near the plasma membrane but did not integrate into the lipid bilayer (Danyluk et al., 1998). However, LEA IV proteins accumulated mainly in the cytosome, and no evidence indicates that the proteins accumulate near or inside membrane (Hsing, unpublished data). Therefore, GmPM6 proteins might function as membrane stabilizers. Although the protective effect of sucrose on the liposome results in the depression of T_m , the degree of depression of T_m for various protective materials disagrees with the protective order we found. For example, sucrose contained the lowest T_m (not datable until -50°C, Hoekstra et al., 2001) to natural liposome, whereas GmPM6 and GmPM16 slightly lowered the T_m values from 56°C to about 45°C (Shih et al., 2010c; Shih and Hoekstra, unpublished data). The order of protective effect in the current study was GmPM6, sucrose, and GmPM16. No T_m value for sucrose, GmPM6, or GmPM16-positive liposome matrix could be detected until -50°C. However, the order of protective effect was still GmPM6, sucrose, and GmPM16. Thus, the protective effect of LEA proteins on phospholipids might not be caused solely by the interaction between hydroxyl groups of proteins and hydrophilic heads of phospholipids, although such interaction indeed existed.

The results of the liposomes and protein-sucrose glasses interaction (Figure 3) illustrate the different issues in glass function. Our results indicate that proteinsugar glasses have little or no protective addictivity. The protective effect of GmPM6-sucrose glasses was markedly less than that of GmPM6 or sucrose alone. However, the LEA protein-sugar glass stabilized protein structures under osmotic stress conditions. For instance, kinetic analysis of protein aggregation suggested that the glass forming from Aphelenchus avenae LEA III proteins and trehalose efficiently prevented the heat- or dehydration-induced aggregation of citrate synthase or lactate dehydrogenase, and the glass also revealed significant addictivity (Goyal et al., 2005). These dehydration-sensitive enzymes were embedded into glass, and thus the combined effects of LEA proteins and non-reducing sugars could be detected. However, in the present study, even if the liposomes were possibly embedded into glass, the interaction between liposomes and glass did not prevent the formation of a solid gel phase during dehydration. The current model suggested that nonreducing sugars could integrate into hydrophilic heads of plasma membrane, and then maintain the liquid-crystalline phase (Crowe et al., 1992; Hoekstra et al., 2001). However, at the first hour after imbibition, the seeds still contain approximately 10% leakage of cytoplasmic solutes (McKersie and Stinson, 1980). The removal of the seed coat greatly increases the leakage to more than fivefold (Duke and Kakefuda, 1981). Of note, the detectable leakage of cytosol-organelle and mitochondrial marker enzymes suggests that the plasma membrane is damaged and loses semi-permeability at seed maturation (McKersie and Stinson, 1980). The membrane leakage might not lead to cell death; the instant repair system should be critical. Numerous enzymes and transcription factors for early germination are synthesized during maturation and stored in mature seed. These proteins are desiccation-sensitive and should be protected because they have to function immediately during early imbibition. Hence, from our results, the cellular glass may function ahead of the protein or cellular structure protection, whereas the rest of the non-reducing sugars may provide protection for plasma membranes. Further analysis is ongoing.

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兩種大豆 GmPM 蛋白能在乾燥情況下降低微脂體的滲漏

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本研究係針對大豆種子的晚胚蛋白在乾燥的環境下,對人造微脂體的保護效果。同時,我們也以 由晚胚蛋白與蔗糖所組成的玻璃質體,進行微脂體保護效果的研究。結果顯示,屬於第二群晚胚蛋白的 GmPM6對於乾燥的微脂體具有明顯的保護效果,但第四群的晚胚蛋白 GmPM16 則較低。另一方面,無 論 GmPM6或 GmPM16與蔗糖所組成的玻璃質體皆無法對乾燥的微脂體產生足夠的保護能力。因此, 雖然晚胚蛋白與微脂體之間能夠產生靜電吸引力與氫鍵,但維持微脂體的完整性可能是由於其它的作用 力。此外,關於成熟的正儲型種子的細胞玻離質體的功能也加以討論

關鍵詞:脫水;玻璃質體;GmPM;晚胚蛋白;微脂體;大豆。