Journal of Biochemistry and Molecular Biology, Vol. 31, No. 2, March 1998, pp. 196–200

JOURNAL OF
Biochemistry
and
Molecular Biology

Characterization of 27K Zein as a Transmembrane Protein

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Received 9 December 1997

Zeins, maize storage proteins, are retained in the endoplasmic reticulum (ER) during the subcellular targeting process without the ER retention signal. Circumstantial data indicate that the 27K zein is an ER transmembrane protein. The potential transmembrane domain may permit the 27K zein to remain in the ER. This study investigated the potential transmembrane feature by employing alkaline extraction, proteinase K digestion, and surface biotinylation on isolated intact protein bodies. These assays consistently support the possibility of the 27K zein as a transmembrane protein. The 27K zein polypeptide was shown to be associated with alkali-stripped membranes. The polypeptide was digested by proteinase K to a smaller fragment. According to surface biotinylation, the 27K zeins was labeled to the exclusion of other classes of zeins. This study, therefore, concludes that the 27K zein has an ER transmembrane domain, which may serve as an anchor for zeins' ER retention.

Keywords: ER-resident protein, Microinjection, Seed storage protein, Zein, *Xenopus* oocyte,

Introduction

Developing maize seeds synthesize ethanol-soluble proteins called zeins on membrane-bound polyribosomes. The zein polypeptides assemble to protein bodies in the lumen of the endoplasmic reticulum (ER) unlike most seed storage proteins developing vacuolar protein bodies (Larkins and Hurkman, 1978). Zein's hydrophobic aggregation is considered as the driving force of zeins' retention in the lumen of the ER (Argos *et al.*, 1982). Recent studies suggest that zein may remain in the ER due to interaction with ER resident proteins such as BiP (Li *et al.*, 1993). The mechanism governing zeins' ER retention, however, remains to be better understood.

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Studies have suggested that the 27K zein, a member of the gamma-zeins, may have a transmembrane domain (Lending *et al.*, 1988; Lending and Larkins, 1989; Geetha *et al.*, 1991; Lopes and Larkins, 1991). The 27K zein has an apparent molecular weight of 27 kDa. The 27K zeins are primarily localized near the protein body membrane. Immunogold labeling electron microscopic studies support for 27K zein's proximity to the ER membrane (Ludevid *et al.*, 1984; Lending and Larkins, 1989; Lopes and Larkins, 1991). Fast disintegration of 27K zein, prior to other zeins, during germination also support the above observations (Torrent *et al.*, 1986; Mohammad and Esen, 1990). Indeed, the 27K zein has a potential transmembrane domain. It has a stretch of 37 hydrophobic amino acids between arginine and glutamic acid, followed by an arginine three amino acids away. An increased amount of the protein is responsible for converting the opaque phenotype to the vitreous phenotype in the quality protein maize called QPM (Geetha *et al.*, 1991; Paiva *et al.*, 1991). It is postulated that the 27K zein functions in this phenotype conversion through its cytoplasmic domain which readily forms a high-molecular-weight oligomer, thus facilitating the close packing of starch granules. These circumstantial evidences together suggest that the 27K zein may span the ER membrane and bind to the membrane to the exclusion of other zeins.

No biochemical study has yet provided direct evidences for the 27K zein as a transmembrane protein. In this study, the potential transmembrane domain of the 27K zein was characterized by alkaline extraction, *in situ* proteolytic digestion, and surface labeling by biotinylation on intact protein vesicles isolated from *Xenopus laevis* oocytes, an *in vivo* expression system, following microinjection of zein mRNAs (Hurkman *et al.*, 1981; Coleman, 1984; Wallace *et al.*, 1988).

Materials and Methods

mRNA preparation and microinjection Native zein poly (A)+ mRNA was isolated using oligo-dT cellulose from membrane-bound polysomes according to Lee *et al.* (1995). To express a

secretory protein control, chicken egg white mRNA was prepared from the oviducts of Rhode Island red hen as in zein mRNA isolation. For transmembrane control, HAenv mRNAs were prepared *in vitro* from the pSP6HAenv according to Krieg and Melton (1984). The plasmids were linearized by *Sac*I, and purified by three cycles of phenol extraction and ethanol precipitation. CAP (m7GpppG) was added in 10× molar excess of GTP which was supplemented to the concentration equivalent of CAP. The RNA transcript was purified by phenol extraction and ethanol precipitation.

For microinjection, oocytes were isolated from the ovary of an adult *Xenopus laevis* (Xenopùs I: Ann Arbor, USA). The ovary was divided into pieces of 30–50 oocytes, washed in OR2 (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 3.8 mM NaOH, 1.0 mM Na_2HPO_4, 5.0 mM HEPES, pH 7.8) and incubated in fresh OR2 at 14°C overnight until injection. Oocytes were injected with 50 μl of injection mixture containing mRNA and labeling isotope, and incubated in OR2 at 14°C for 24 h before isolation of proteins. To obtain protein vesicles, the oocyte homogenate was layered and centrifuged on a 20% sucrose cushion in phosphate buffered saline (PBS, 100 mM phosphate, 150 mM NaCl, pH 7.2) at 13,000 × *g* for 25 min.

Alkaline extraction The transmembrane feature of the 27K zein was first assayed by the alkaline extraction procedure (Gilmore and Blobel, 1985). Protein vesicles were incubated in 100 μl of 100 mM HEPES (pH 11.5) for 20 min on ice and pelleted through the 20% sucrose cushion containing 60 mM HEPES (pH 11.5). Since oocytes do not contain ethanol-soluble protein (Larkins *et al.*, 1979), zein was extracted with 70% ethanol containing 5% mercaptoethanol at 60°C and the 27K zein was immunoprecipitated by adding antisera against the 27K zein (gift of Dr. A. Esen). To assay control proteins, pellets were resuspended in the binding protein immunoprecipitation (Bip-IP) buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 mM Tris-HCl, pH 7.4) and incubated with antisera on ice for 1 h. The entire supernatant was immunoprecipitated with respective antisera in 4 volumes of Bip-IP buffer.

Proteinase K treatment of protein vesicles Oocytes were injected with the zein mRNA (25 ng/oocyte) and 0.5 mCi of [^{3}H]leucine (115 Ci/mmole). To synthesize HAenv, the ER transmembrane protein control, oocytes were injected with 10 ng of synthetic HAenv mRNA and 0.4 mCi of [^{35}S]methionine (1170 Ci/mmole). Following incubation and homogenization as described above, aliquots of the homogenates were incubated with proteinase K at 1.0 mg/ml either with 1% Triton X-100 added or omitted. Reactions were terminated by adding PMSF to a concentration of 1.0 mg/ml. Zeins were extracted with 70% ethanol containing 0 or 5% mercaptoethanol at 60°C. The ethanol-soluble fraction was collected after the insoluble portion was pelleted by centrifuging at 10,000 × *g* for 5 min. The 27K zein was immunoprecipitated by adding anti-27K zein antibody in Bip-IP buffer. Incubation was maintained in a cold chamber for 1 h before adding protein A-Sepharose (Pharmacia, Uppsala, Sweden) to 1/50 of the reaction volume. This precipitation was further maintained for 30 min on ice. The protein A-Sepharose was recovered by a brief centrifugation and washed with 1.0 ml Bip-IP buffer containing 0.5 M NaCl. The Sepharose pellets were washed four more times as follows: washes 1 and 2, 1.0 ml each with Bip-IP; washes 3 and 4, 1.0 ml each with TAB buffer (50 mM Tris-HCl, pH 7.5, 0.02% sodium azide, 2 mM benzamidine). After the final wash, the pellets were resuspended in 50 ml TAB containing 5% DTT and 2% SDS, and boiled for 3 min.

Biotinylation of exposed proteins The protein vesicles were resuspended in 0.5 ml of 0.2 M sucrose, 70 mM potassium phosphate, pH 7.0 and treated with a 2% biotin amidocaproate N-hydroxysuccimide ester (Pierce, Rockford, Illinois) in 10 μl N,N-dimethyl formamide (DMF) according to Johnson (1990). The reaction was incubated at 37°C for 90 min. Zein was immunoprecipitated by antisera against total zein or the 27K zein, respectively, in four volumes of Bip-IP and recovered by protein A-Sepharose. Samples were electrophoresed on 11.5% SDS-polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane at 14 V overnight. Biotinylated proteins were detected with avidin-labeled horse radish peroxidase in ECL (Enhanced Chemiluminescence, Amersham) reagents.

Results

This study had investigated whether the 27K zein has a transmembrane domain that permits the 27K zein to remain in the ER despite the indiscriminate bulk flow during the cellular protein targeting process. The potential transmembrane features of the 27K zein were analyzed by three categories of assays: alkaline extraction, proteinase K digestion, and surface biotinylation of protein vesicles containing zeins expressed in *Xenopus* oocytes.

Localization of the 27K zein with alkali-stripped membranes To assay whether the 27K zein spans the ER membrane, the isolated protein vesicles were subjected to alkaline extraction which distinguishes integral membrane protein from peripheral or soluble proteins. Under high pH conditions, biological membranes undergo a disruption to open sheets from which peripheral or soluble proteins are released. The alkaline extraction assay provided a strong evidence for the 27K zein's potential transmembrane domain. When protein vesicles containing zeins were subjected to an alkaline solution (pH 11.5), the 27K zein was released from the ER membrane.

Distinct patterns were evident between the two control proteins (Fig. 1). The HAenv, an ER transmembrane protein, was localized in the pellet, whereas ovalbumin, a secretory protein, was detected exclusively in the supernatant. The 27K zein was found to localize mainly with the membrane fraction. Minuscule amounts of the 27K zein were found in the supernatant fraction. If the 27K zein is a luminal protein, it should be localized primarily with the supernatant fraction. The pattern corresponded to that of HAenv which was localized in the membrane fraction. This result strongly indicates that the 27K zein is embedded in the ER membrane.

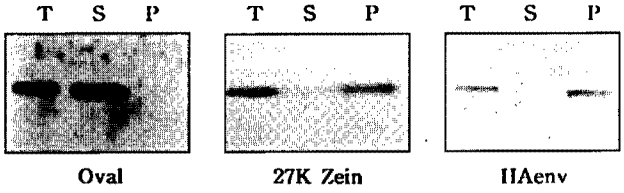

Fig. 1. Alkali extraction of membranes. Protein vesicles, containing 27K zein or control protein, were incubated in 100 mM HEPES, pH 11.5, for 20 min on ice and pelleted through a sucrose cushion containing 60 mM HEPES, pH 11.5. The 27K zein was extracted with 70% ethanol separately from pellet and supernatant fractions. Control proteins were immunoprecipitated. Protein samples were electrophoresed on 11.5% SDS-polyacrylamide gel and fluorographed. T: total oocyte homogenate; S: supernatant; P: pellet.

27K zein is trimmed *in situ* by proteinase K The potential transmembrane domain of the 27K zein was further analyzed by *in situ* proteinase K treatment on isolated intact protein vesicles, whereby the membrane-spanning domain is specifically probed. If the 27K zein is a transmembrane protein, the proteinase K digestion should produce a new band (or bands) corresponding to the portion protected within protein vesicles. Following homogenization of injected oocytes through a pipette tip, aliquots of the homogenates were incubated with proteinase K either in the absence or presence of 1% Triton X-100.

The 27K zein, associated with vesicles, was affected under proteinase K treatment (Fig. 2). Figure 2A shows the total zeins or ethanol-soluble fraction from *Xenopus* oocytes injected with zein mRNAs. The lane numbers correspond to the aliquots used in Fig. 2B. When the protein vesicles were treated with proteinase K in the presence of 1% Triton X-100, a nonionic detergent, zeins in the protein vesicles was totally degraded (lane 3). Under Triton-free treatment, however, the 27K zein was trimmed to a reduced size (lane 4, Fig. 3B). The size of the 27K zein resistant to proteinase K treatment was smaller than the untreated counterpart (lanes 1–2, Fig. 3B). The size of the 27K, resistant to the proteinase K, most likely represents the portion protected within the protein vesicles. This result indicates that the 27K zein was trimmed *in situ* by proteinase K; thus, it spans the ER membrane and protrudes out of the protein vesicles. Combined with the alkali-stripping experiment, the proteinase K digestion strongly indicates that the 27K zein polypeptides protrude on the surface of the ER membrane.

The 27K zein biotinylated *in situ* To determine whether the 27K zein spans the ER membrane, biotinylation was used to label proteins protruding out of the protein vesicles. The rationale for this experiment is that surface proteins will be biotinylated *in situ* to the exclusion of the luminal ER proteins. Following surface biotinylation, zeins were immunoprecipitated, electrophoresed on SDS-polyacrylamide gels, and transferred to nitrocellulose filter.

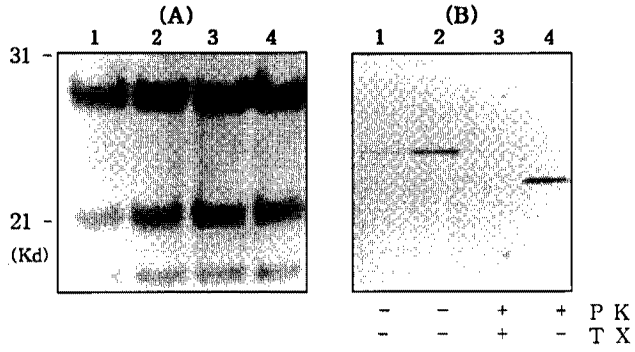

Fig. 2. Proteinase K treatment of protein vesicles from oocytes. Oocytes were injected with zein mRNA, incubated for 24 h, and homogenized in the oocyte homogenization buffer. The homogenate was divided into four aliquots. (A) Zeins were extracted from each aliquot in 70% ethanol containing 5% mercaptoethanol. (B) The aliquots as in 3A were incubated with proteinase K (PK) with 0 or 1% Triton X-100 (TX). The 27K zein was extracted using 0 (lane 1) or 5% (lane 2) mercaptoethanol following proteinase K-free incubation. The next two lanes refer to the proteinase K treatments in the presence (lane 3) or absence (lane 4) of 1% Triton X-100. The ethanol-soluble fraction was subjected to immunoprecipitation using antisera against the 27K zein. Samples were electrophoresed on a 11.5% gel and fluorographed as in Fig. 1. Each lane contains the equivalent of protein vesicles isolated from five oocytes. The sign (+/−) below the lanes denote the presence/absence of 1.0 mg/ml proteinase K (PK) or 1% Triton X-100 in the incubation mixture.

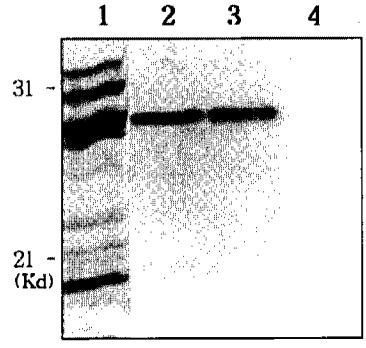

Fig. 3. Surface biotinylation of protein vesicles. Protein vesicles were biotinylated with or without Triton X-100. Biotinylated proteins were detected by avidin-labeled horseradish peroxidase following transfer to nitrocellulose filter (lane 1). Zeins were extracted in 70% ethanol containing 5% mercaptoethanol and the biotinylated fraction was detected as in lane 1 (lane 2). Ethanol-soluble fractions were immunoprecipitated by antisera against the 27K zein (lane 3) or preimmune serum (lane 4) and the biotinylated fraction was detected as in lane 1. Each lane accommodates the equivalent of five oocytes.

The biotinylated protein was detected by the avidin-conjugated horseradish peroxidase system.

The 27K zein was significantly biotinylated in isolated intact protein vesicles (Fig. 2). Zeins other than the 27K class, however, were not biotinylated. Lane 1 refers to the biotinylated protein fraction. Antisera against total zein did not precipitate any other biotinylated zein polypeptides. Only the 27K zein was shown to be biotinylated (lane 2). Immunoprecipitation with anti-27K zein verified that the biotinylated zein represented the 27K zein (lane 3). The combination of lanes 2 and 3 verified that the 27K zein spans the ER membrane and is exposed out of the ER membrane. These results are in good agreement with those obtained from the alkaline extraction and the *in situ* proteinase K experiments.

Discussion

The 27K zein has a primary structure which is typical for a transmembrane protein. It is localized primarily at the periphery of the protein body (Ludevid *et al.*, 1984; Lending *et al.*, 1988; Lending and Larkins, 1989). To be extracted from zein protein bodies, the 27K zein requires a reducing agent (e.g., 2-mercaptoethanol and dithiothreitol). Even beta-zein is readily extracted from the protein bodies without such reducing agents although it has more disulfide linkages than the 27K zein. This unusual requirement of the 27K zein is suggestive of its tighter association with the membrane of the protein bodies. Despite these circumstantial evidences, however, no study has provided a direct evidence supporting such potential transmembrane domain.

This study supports the possibility of the 27K zein as a transmembrane protein. Consistent with these circumstantial evidences, the alkali-stripping assay provides a first supporting evidence. When protein vesicles were stripped with alkali, most of the 27K zein was still associated with the alkali-stripped membrane. This distribution pattern corresponded to that of HAenv (a transmembrane protein). This was different from the secretory control, ovalbumin, which was released to the supernatant from the alkali-stripped membrane. Despite the clear result, an argument still existed that the 27K zein can remain associated with the ER membrane in the presence of alkali by unusual binding to membrane components and even by blocking agents against the alkali stripping assay. This argument could be negated considering the pair of consistent observations from proteinase K and surface biotinylation experiments.

The data from proteinase K digestion and surface biotinylation experiments supported the potential transmembrane feature of the 27K zein. The 27K zein associated with the protein vesicles was affected *in situ* by proteinase K treatment. Likewise, the 27K zein was labeled *in situ* with biotinylating agents to the exclusion of other classes of zein. These two assays indicated that the 27K zein protrudes out of the protein bodies. Therefore, it may serve as a protein body nucleating factor by virtue of its ER transmembrane feature.

The phenotype change (from opaque to glassy) of the Quality Protein Maize (QPM) endosperm strongly supports for the 27K zein's potential transmembrane feature (Geetha *et al.*, 1991; Lopes and Larkins, 1991). The QPM endosperm differs from the opaque counterpart only in the 27K zein content; the former has a higher content of the 27K zein than the latter. This phenotype conversion, therefore, has been postulated as the result of escalated interaction between the 27K zeins. Based upon the data from this study, the phenotype change can be interpreted as the direct outcome of covalent linkages between the 27K zeins across the protein body membrane, thus cementing starch granules tighter in the 27K zein-rich QPM endosperm.

The 27K zein plays an important role in protein body formation. It accumulates in the earliest endosperm protein bodies (Lending and Larkins, 1989) and in developing maize endosperm, its content determines the density and size of protein bodies (Lending *et al.*, 1988; Lending and Larkins, 1989; Lopes and Larkins, 1989). Furthermore, the 27K zein's significance in zeins' protein body assembly was also supported in *Xenopus* oocytes. Oocytes injected with mRNAs of the 27K zein and alpha-zein assembled zein polypeptides into denser vesicles. These were of equivalent density to native zein protein bodies whereas those injected singularly with alpha-zein mRNA assembled zein polypeptides into less dense vesicles (Wallace *et al.*, 1988b; Lending and Larkins, 1989). These evidences together suggest that the 27K zein may serve as a nucleating factor in zein protein body formation.

The present study indicates that 27K zein harbors a transmembrane domain which serves as a membrane-spanning anchor. The potential ER transmembrane domain may also serve as a nucleating factor in protein body formation in the lumen of the ER. Combining the three sets of data, this study concludes that the 27K zein spans the ER membrane.

Acknowledgments The author wishes to thank Dr. A. Colman for providing pSP6HAenv plasmid and antisera against hemagglutinin. His thanks also go to Dr. A. Esen for the gift of antisera against the 27K zein. The author is indebted to Dr. T. J. Anderson for revising the manuscript.

References

Argos, P., Pedersen, K., Marks, M. D., and Larkins, B. A. (1982) A structural model for maize zein proteins. *J. Biol. Chem.* **257**, 9984–9990.

Boston, R. S., Fontes, E. B. P., Shank, B. B., and Russell, L. W. (1991) Increased expression of the maize immunoglobulin binding protein homologue b-70 in three zein regulatory mutants. *Plant Cell* **3**, 497–505.

Cameron-Mills, V. and von Wettstein, D. (1980) The structure and composition of zein protein bodies. *Carlsberg Res. Commun.* **45**, 577–594.

Ceriotti, A. and Colman, A. (1988) Binding to membrane proteins within the endoplasmic reticulum cannot explain the retention of the glucose-regulated protein GRP78 in *Xenopus* oocytes. *EMBO J.* **7**, 633–638.

Chrispeels, M. J. and Raikehl, N. V. (1992) Short peptide domains target proteins to plant vacuoles. *Cell*, **68**, 613–616.

Colman, A. (1984) Translation of eukaryotic messenger RNA in *Xenopus* oocytes; in *Transcription and Translation,* Hames, B. D. and Higgins, S. J. (eds), pp. 271–302, IRL Press, Oxford.

Fontes, E B. P., Shank, B. B., Wrobel, R. L., Moose, S. P., Obrien, G.R., Wurtzel, E.T., and Boston, R.S. (1991) Characterization of an immunoglobulin binding protein homolog in the maize floury-2. *Plant Cell* **3**, 483–496.

Geetha, K. B., Lending, C. R., Lopes, M. A., Wallace, J. C., and Larkins, B. A. (1991) Opaque-2 Modifiers increase gamma-zein synthesis. *Plant Cell* **3**, 1207–1219.

Gething, M.-J., McCammon, K., and Sambrook, J. (1986) Expression of wild-type and mutant forms of influenza hemagglutinin. *Cell* **46**, 939–950.

Gilmore, R. and Blobel, G. (1985) Translocation of secretory proteins across the microsomal membrane occurs through an environment accessible to aqueous perturbants. *Cell* **42**, 497–505.

Gourdon, J. B., Lane, C. D., Woodland, H. R., and Marbiax, G. (1971) Use of frog egg and oocytes for the study of messenger RNA. *Nature* **233**, 177–182.

Hurkman, W. J., Smith, L. D., Richter, J., and Larkins, B. A. (1981) Subcellular compartmentalization of maize storage protein in *Xenopus* oocytes. *J. Cell Biol.* **89**, 292–299.

Johnson, D. J. (1990) Study of zein protein body formation. Dissertation, Dept. of Biology, Univ. of North Carolina-Chapel Hill.

Krieg, P. A. and Melton, D. A. (1984) Functional messenger RNAs are produced by SP6 *in vitro* transcription. *Nucl. Acid Res.* **12**, 7035–7056.

Larkins, B. A. and Hurkman, W. J. (1978) Synthesis and deposition of maize in protein bodies of maize endosperm. *Plant Physiol.* **62**, 256–263.

Larkins, B. A., Pedersen, K., Handa, A. K., Hurkman, W. J., and Smith, L. D. (1979) Synthesis and processing of maize storage proteins in *Xenopus laevis* oocytes. *Proc. Natl. Acad. Sci. USA* **76**, 6448–6452.

Lee, D.-H., Selester, B., and Pedersen, K. (1995) Free movement of zein in endoplasmic reticulum. *Protein Eng.* **9**, 91–95.

Lending, C. R., Kritz, A. L., Larkins, B. A., and Bracker, C. E. (1988) Structure of maize protein bodies and immunocytochemical localization of zeins. *Protoplasma* **143**, 51–62.

Lending, C. R. and Larkins, B. A. (1989) Changes in the zein composition of protein bodies during maize endosperm development. *Plant Cell* **1**, 1011–1023.

Li, X., Gillikin, J. W., Boston, R. S., and Okita, T. (1993) Rice prolamine protein body biogenesis: a BiP-mediated process. *Science* **262**, 1054–1056.

Lopes, M. A. and Larkins, B. A. (1991) Gamma-zein content is related to endosperm modification in Quality Protein Maize. *Crop Sci.* **31**, 1655–1662.

Mohammad, K. B. and Esen, A. (1990) Zein degradation in the endosperm of maize seeds during germination. *Amer. J. Bot.* **77**, 973–980.

Paiva, E., Kriz, A.L., Peixoto, M. J. V. V. D., Wallace, J. C., and Larkins, B. A. (1991) Quantitation and distribution of zein in the endosperm of maize kernels. *Cereal Chem.* **68**, 276–279

Torrent, M., Geli, M. I., and Ludevid, M. D. (1986) Storage-protein hydrolysis and protein body breakdown in germinated *Zea mays* L. seeds. *Planta* **180**, 90–95.

Wallace, J. C., Galili, G., Kawata, E. E., Cuellar, R. E., Shotwell, M. A., and Larkins, B.A. (1988a) Aggregation of lysine-containing zeins into protein bodies in *Xenopus* oocytes. *Science* **240**, 662–664.

Wallace, J. C., Galili, G., Kawata, E. E., Lending, C .R., Kriz, A. L., Bracker, C. E., and Larkins, B. A. (1988b) Location and interaction of the different types of zeins. *Biochem. Physiol. Pflanzen.* **183**, 107–114.

Wallace, J .C., Ohtani, T., Lending, C. R., Lopes, M. A., Williamson, J. D., Shaw, K. L., Gelvin, S. B., and Larkins, B. A. (1990) Factors affecting physical and structural properties of maize protein bodies. in *UCLA Symposium of Molecular and Cellular Biology*, Lamb C., and Beachy, R. (eds.), pp. 205–216, Alan R. Liss, New York.