JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Preparative Procedures Markedly Influence the Appearance and Structural Integrity of Protein Storage Vacuoles in Soybean Seeds

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In legumes, vacuoles serve as the final depository for storage proteins. The protein storage vacuoles (PSVs) of soybean contain electron-transparent globoid regions in which phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate) is sequestered. This paper reports the effect of preparative procedures on the appearance and ultrastructural integrity of PSVs in soybeans. Electron microscopy examination of both developing and mature soybean seeds that were postfixed with osmium tetroxide revealed PSVs that had a homogeneous appearance with very few globoid crystals dispersed in them. Numerous electron-dense lipid bodies were readily seen in these cells. Omission of osmium tetroxide strikingly altered the appearance of PSVs and aided the visualization of the location of the globoids in the PSVs. In contrast to the osmicated tissue, lipid bodies appeared as electron-transparent spheres. The choice of dehydration reagent or staining procedure had little influence on the appearance of the PSVs. The results of this study demonstrate the profound effect of osmium tetroxide on the appearance and structural integrity of PSVs in soybean.

KEYWORDS: Electron microscopy; osmium tetroxide; soybean; phytic acid; protein storage vacuoles

INTRODUCTION

Soybeans contain about 35–40% protein on a dry weight basis. The abundant seed storage proteins of soybean, glycinin and β-conglycinin, account for about 80% of the total seed protein of soybean seed (*1, 2*). These proteins accumulate during midstage of seed development and are deposited in specialized storage organs (*3, 4*). The accumulation of storage proteins in legume seeds has been the subject of several ultrastructural investigations (*5–9*). The storage proteins that are synthesized on rough ER are transported to vacuoles as vesicles derived from Golgi apparatus (*10, 11*). In young developing seed, small protein deposits are seen at the interface of prominent vacuoles. It is suggested that as the protein deposits increase in size, the large vacuoles often fragment into small protein-filled organelles (*12*). Recent studies have established the presence of two types of vacuoles, lytic vacuoles (LVs) and protein storage vacuoles (PSVs), which can be distinguished by the presence of different tonoplast intrinsic proteins (TIPs). The membranes of LVs contain γ-TIP, whereas the PSVs reveal α-TIP and δ-TIP (*6, 13*). Ultrastructural and immunocytochemical studies have clearly established that the legume seed storage proteins are deposited in PSVs (*6*).

Phosphorus is mainly stored in seeds as phytate, a salt of phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate) (*14*).

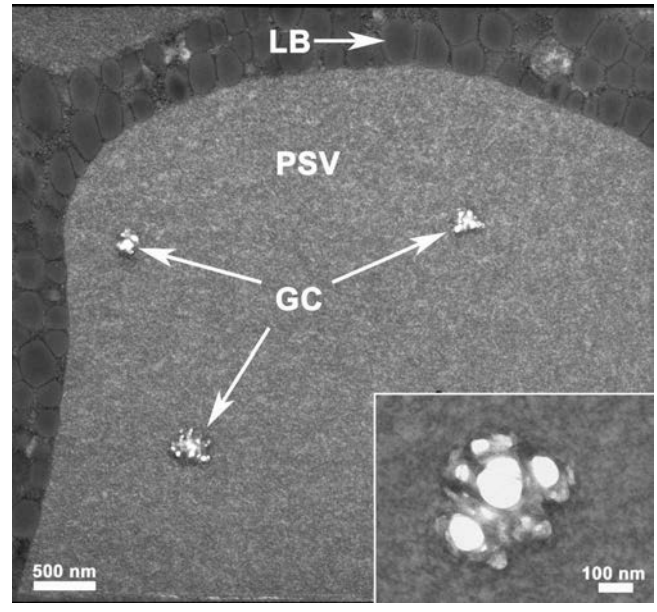

Figure 1. Transmission electron micrograph of soybean cotyledon conventionally fixed in 2.5% glutaraldehyde, postfixed in osmium tetroxide, and dehydrated in graded acetone showing globoid regions within protein storage vacuoles. A higher magnification of the globoid crystal is also shown (inset). GC, globoid crystals; PSV, protein storage vacuoles; LB, lipid bodies.

Phytate has been generally regarded as an antinutrient because it forms insoluble complexes with minerals, thereby reducing

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10.1021/jf0735228
Published on Web 04/15/2008

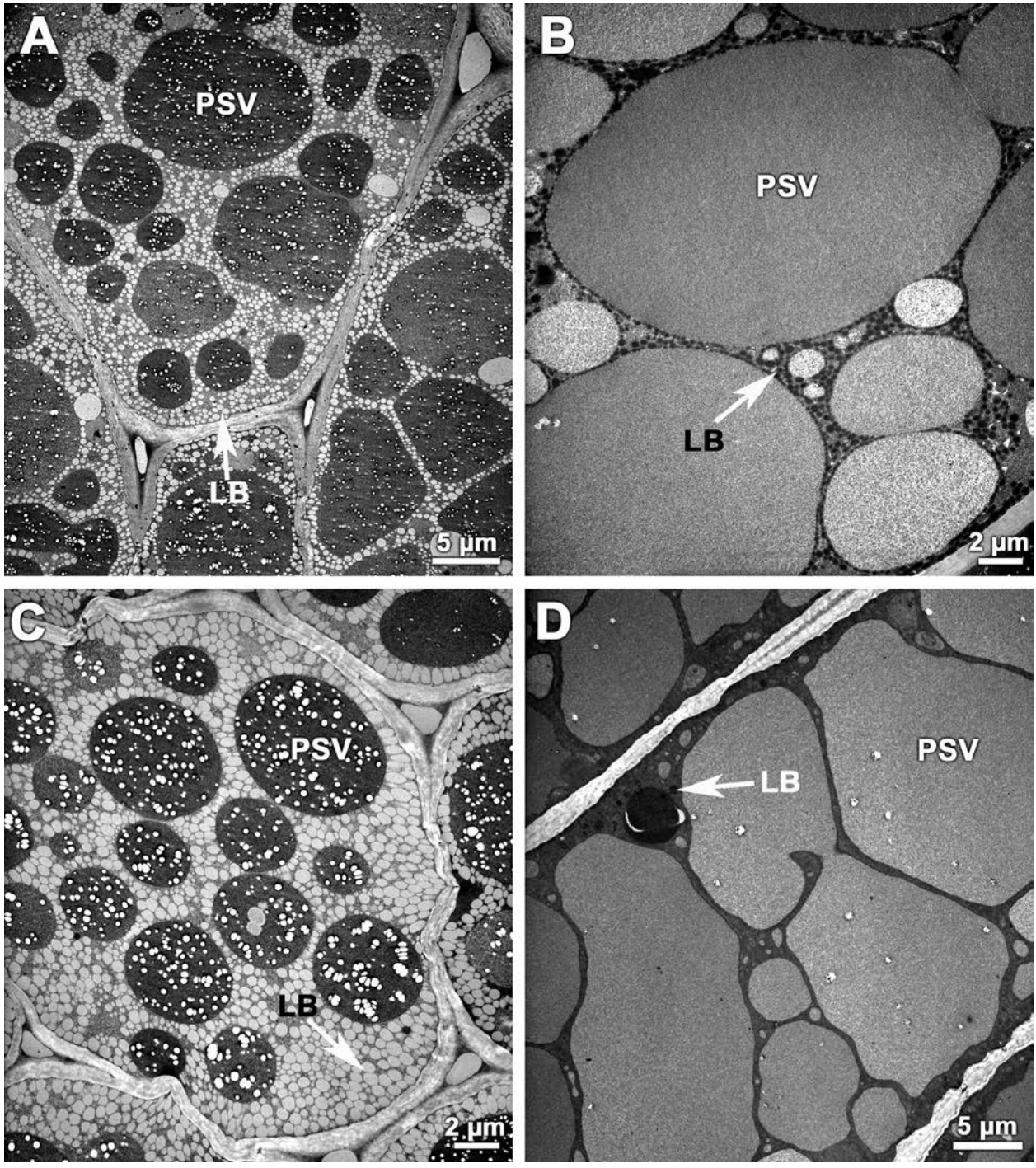

Figure 2. Effect of postfixation with osmium tetroxide and choice of dehydrating agent on the appearance of PSVs in dry soybean cotyledon. Soybean seeds were fixed in 2.5% glutaraldehyde in 50 mM phosphate buffer, pH 7.2, and subjected to dehydration with ethanol (**A**, **B**) or with acetone (**C**, **D**) and embedded in Spurr's resin. Micrographs in panels **A** and **C** are from tissue that was not postfixed with osmium tetroxide, whereas micrographs in panels **B** and **D** were obtained from cotyledon tissue that was postfixed with 1% aqueous osmium tetroxide. Note the PSVs of nonosmicated tissue show numerous globoid regions (**A**, **C**) but are absent in the osmicated tissue. PSV, protein storage vacuoles; LB, lipid bodies.

their availability. However, recent studies have shown the positive role of phytate as an antioxidant and anticolon cancer agent (*15, 16*). Additionally, phytate has been shown to affect the texture of tofu, a popular Asian food, by interacting with protein and coagulants such as calcium and magnesium salts (*17, 18*). Because phytate has a significant nutritional role, a thorough knowledge of its localization and interaction with soybean proteins is of major importance. Electron microscopy provides a convenient method to monitor the distribution of phytate within seed tissue.

In most plants, phytate is localized in globoid crystals that are found within protein bodies and PSVs (*18–22*). Variation in the distribution of globoid crystals in different regions of the cotyledon and between embryonic axis and cotyledons has been reported (*23*). Even though it has been earlier reported that soybeans do not contain globoid crystals (*24*), their presence in the seeds was conclusively demonstrated with the aid of transmission electron microscopy and energy dispersive X-ray analysis (*19, 25–27*). During the course of investigation on the distribution of globoid crystals within PSVs in soybean, we

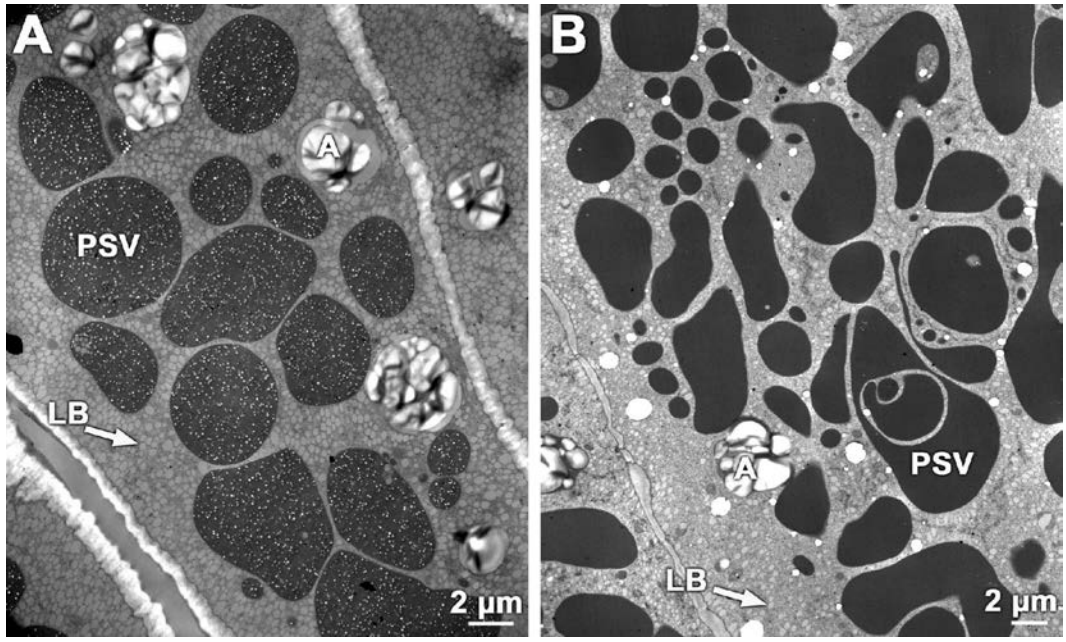

Figure 3. Effect of postfixation with osmium tetroxide on the appearance of PSVs in developing soybean cotyledon. Developing soybean seeds at R5 stage were fixed in 2.5% glutaraldehyde in 50 mM phosphate buffer, pH 7.5, subjected to dehydration with acetone, and embedded in Spurr's resin. The micrograph in panel **A** was from soybean tissue that was not postfixed with osmium tetroxide, whereas the micrograph in panel **B** was obtained from cotyledon tissue that was postfixed with 1% aqueous osmium. Note the PSVs of nonosmicated tissue show numerous globoid regions. A, amyloplast; PSV, protein storage vacuoles; LB, lipid bodies.

observed that their appearance was quite variable. An earlier study had examined the effect of pH of the fixative on the ultrastructure of soybean protein bodies (*27*). In a systematic study, Boatright and Kim (*27*) observed membrane-bound crystalloid structures within protein bodies when the soybean seeds were fixed at pH 6.4 and 5.6. When the tissue was fixed at pH 7.2, only a few membrane-bound crystalloid structures were observed, demonstrating the marked effect of the pH of the fixative on subcellular structures of soybean. In our earlier studies the presence of numerous membrane-bound crystalloid structures within PSVs was observed even when the pH of the fixative ranged from 7.0 to 7.5 (*28, 29*). This observation suggested that in addition to the fixative pH, other preparative factors could influence the structural integrity of soybean PSVs. In this study, the effects of preparative procedures on the ultrastructural integrity of PSVs were systematically investigated. The results of this study demonstrate that the inclusion or omission of osmium tetroxide as a secondary fixative has a dramatic influence on the appearance and structural integrity of PSVs in soybean.

MATERIALS AND METHODS

Soybean cultivar Williams 82 seeds were obtained from the Missouri Foundation Seed, Columbia, MO. Dry soybean seeds were imbibed in water for 5 min at room temperature and germinated for 12 h in an incubator maintained at 30 °C. Following this, the seeds were dissected into small pieces and immediately fixed in 2.5% glutaraldehyde buffered at 7.2 with 50 mM sodium phosphate. Similarly, developing seeds at R5 stage (*30*) were sliced into small cubes and fixed in the same fixative. Primary fixation was performed at room temperature for 4 h. After several washes with water, some of the seed tissues were postfixed with 1% aqueous osmium tetroxide for 1 h. Excess osmium tetroxide was removed by washing the samples three times in water. The samples were dehydrated in either a graded ethanol or acetone series. Following dehydration, the tissue was incubated in propylene oxide and infiltrated gradually in Spurr's resin. Thin sections of all embedded tissues were sectioned with a diamond knife and collected on 200 mesh copper grids. Thin sections were stained individually with Sato's triple lead citrate solution (*31*) or 5% uranyl acetate at room temperature for 10 and 18 min, respectively. Some sections were double stained with Sato's triple lead citrate solution and uranyl acetate and examined in a JEOL 1200 EX (Tokyo, Japan) transmission electron microscope at 80 kV.

RESULTS AND DISCUSSION

Effect of Postfixation with Osmium Tetroxide on the Appearance of PSVs. Soybean cotyledons that were conventionally fixed in glutaraldehyde and dehydrated in acetone (*28, 29*) contained few globoid crystals dispersed within the protein storage vacuoles (**Figure 1**). Phytate, a salt of *myo*-inositol hexaphosphoric acid, is the main component of the globoid crystals (*25, 26*). When viewed at high magnification, these globoid crystals appear to be enclosed by a membrane (**Figure 1**), which corroborates the earlier finding of Boatright and Kim (*27*). The visualization of globoid crystals within PSVs in soybean has been shown to be influenced by the preparative procedures employed for preparing seed tissue for transmission electron microscopy (*25, 27*). Therefore, the effect of postfixation with osmium tetroxide on the ultrastructure of PSVs was investigated.

Thin sections of mature soybean seeds that were fixed with or without osmium tetroxide were examined under electron microscope. The cotyledon cells contained numerous densely packed lipid bodies and prominent PSVs. In the nonosmicated tissue the lipid bodies appeared to be transparent, whereas in the osmicated tissue, the lipid bodies appeared as electron-dense dark spherical bodies (**Figure 2A**,**B**). This observation is consistent with the role of osmium tetroxide, which is known to react with unsaturated lipids resulting in the formation of hexavalent osmium, which imparts the black color (*32*). Interestingly, there was a striking difference in the appearance of PSVs between osmicated and nonosmicated tissue (**Figure 2A**,**B**). Nonosmicated tissue contained numerous globoid structures dispersed throughout the PSVs (**Figure 2A**). In contrast, very few globoid structures were seen in the osmicated tissue (**Figure 2B**). Because dehydration agents are powerful lipid solvents and could affect the structural integrity of

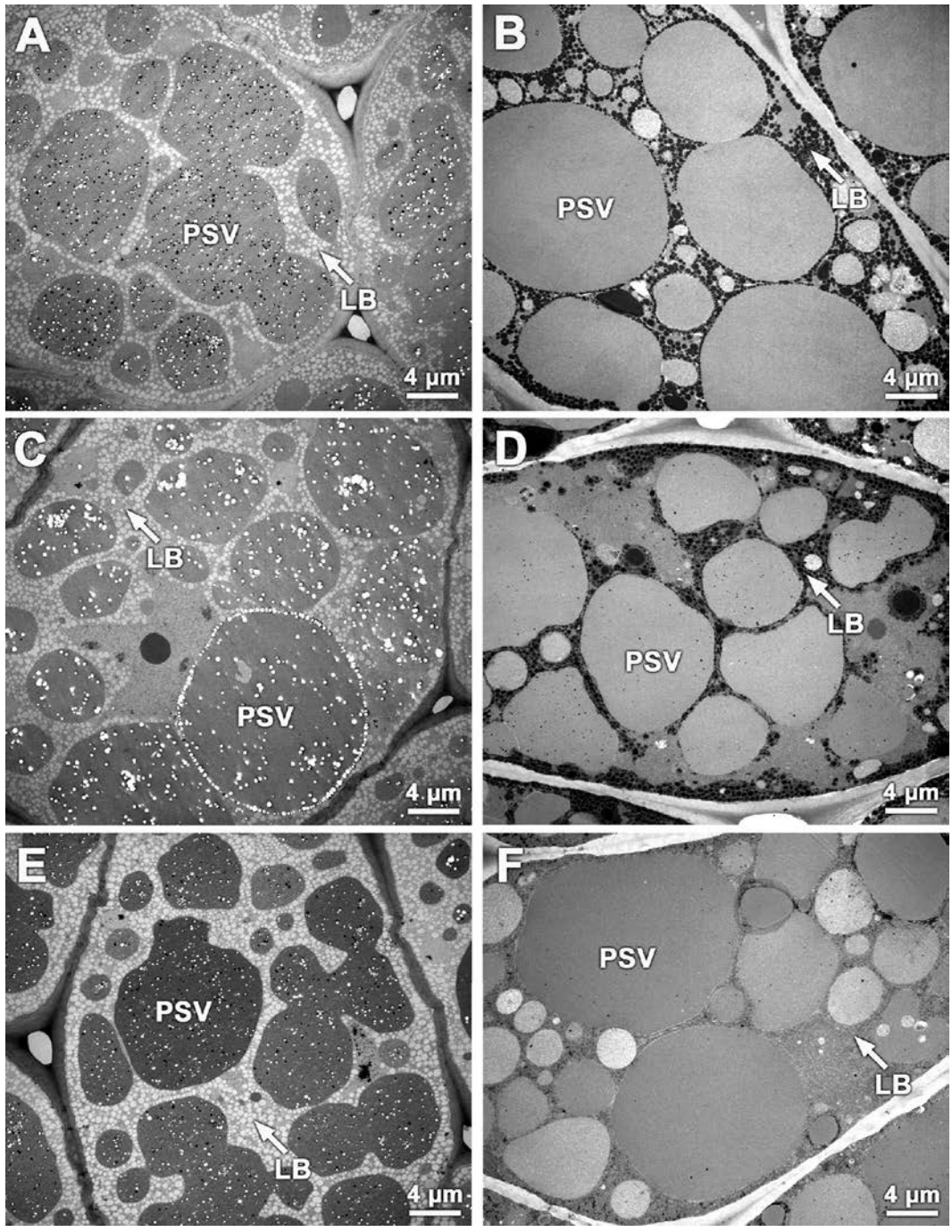

Figure 4. Effect of staining procedure on the appearance of PSVs in dry soybean cotyledon. Dry soybean seeds were fixed in 2.5% glutaraldehyde in 50 mM phosphate buffer, pH 7.5, subjected to dehydration with acetone, and embedded in Spurr's resin. Micrographs in panels **A**, **C**, and **E** are from tissue that was not postfixed with osmium tetroxide, whereas micrographs in panels **B**, **D**, and **F** were obtained from cotyledon tissue that was postfixed with 1% aqueous osmium tetroxide. Sections in panels **A** and **B** were stained with uranyl acetate; sections in panels **C** and **D** were stained with lead citrate, whereas sections in panels **E** and **F** were stained with uranyl acetate and lead citrate. PSV, protein storage vacuoles; LB, lipid bodies.

the cells, the effect of acetone and ethanol, two commonly used dehydration agents, were examined. The choice of acetone (**Figure 2A**,**B**) or ethanol (**Figure 2C**,**D**) as a dehydration reagent had little influence on the appearance of the cell contents. Irrespective of the dehydration reagent used, the omission of osmium tetroxide drastically altered the appearance of PSVs (**Figure 2A**,**C**).

Because dry soybean seeds were briefly soaked in water before processing for ultrastructural investigation, some of the observed changes in the cell contents may be related to mobilization of storage reserves initiated during the early stages of seed germination. To test this possibility, developing soybean seeds harvested at R5 stage were processed for ultrastructural analysis. Observation of developing soybean cotyledon cells revealed the presence of numerous lipid bodies and PSVs of different sizes and shapes (**Figure 3**). In addition, amyloplasts containing starch grains were also observed (**Figure 3**). As in the case of mature imbibed seeds, osmium tetroxide postfixation drastically altered the appearance of PSVs (compare panels **A** and **B** of **Figure 3**). In nonosmicated tissue numerous globoid structures that were distributed throughout the PSVs were prominently seen (**Figure 3A**), whereas in osmicated tissue the globoid structures were conspicuous by their absence (**Figure 3B**).

Effect of Staining Procedure on the Appearance of PSVs in Soybean Cotyledon. Thin sections of mature soybean seeds

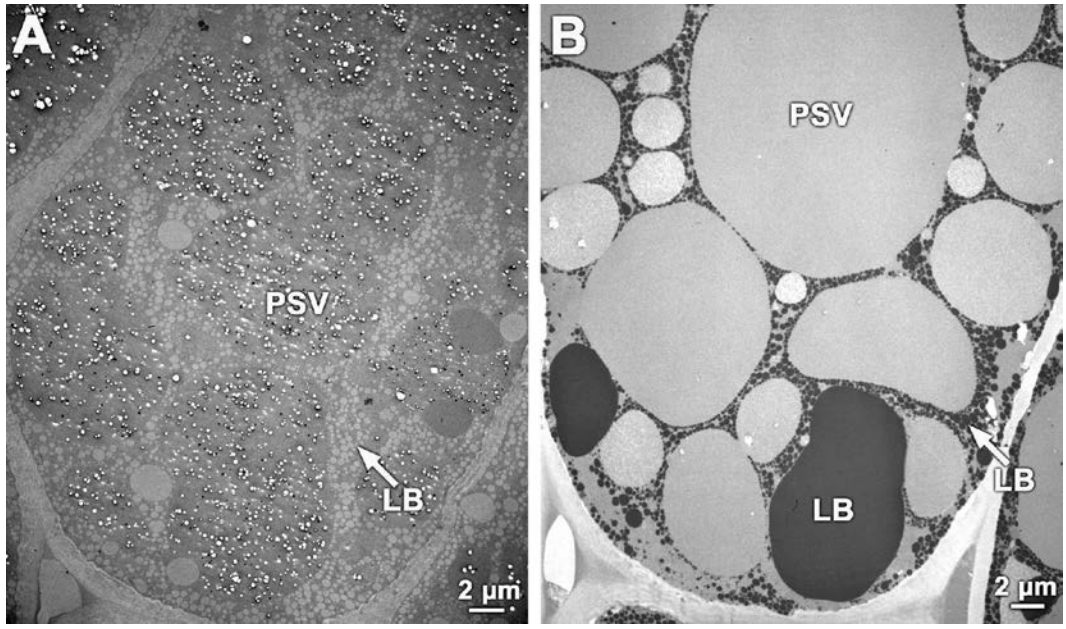

Figure 5. Globoid regions seen in nonosmicated tissue are not an artifact of the staining procedure. Dry soybean seeds were fixed in 2.5% glutaraldehyde in 50 mM phosphate buffer, pH 7.5, subjected to dehydration with acetone, and embedded in Spurr's resin. The micrograph in panel **A** is from soybean tissue that was not postfixed with osmium tetroxide, whereas the micrograph in panel **B** was obtained from cotyledon tissue that was postfixed with 2% aqueous osmium tetroxide. The micrographs were taken from unstained sections. PSV, protein storage vacuoles; LB, lipid bodies.

were individually stained with either uranyl acetate (**Figure 4A**,**B**) or lead citrate (**Figure 4C**,**D**) or double stained with uranyl acetate and lead citrate (**Figure 4E**,**F**). Electron microscopy observations revealed no obvious differences in the appearance of PSVs among the three staining procedure employed. In seeds postfixed with osmium tetroxide globoid structures were not observed in PSVs irrespective of the staining procedure (**Figure 4B**,**D**,**F**). In contrast, nonosmicated tissue contained prominent globoid structures (**Figure 4A**,**C**,**E**). Osmium tetroxide induced staining artifacts have been previously reported (*32*). Unstained thin sections of soybeans were examined under a microscope to rule out the possibility that staining artifact was responsible for the drastic differences in the appearance of PSVs (**Figure 5**). In the absence of stain, the nonosmicated tissue had lower contrast, but the presence of numerous globoid structures was clearly evident (**Figure 5A**). Surprisingly, the osmicated tissue exhibited significant contrast even in the absence of staining of the sections (**Figure 5B**). Numerous small spherical and a few large dark lipid bodies and several uniform appearing PSVs were seen (**Figure 5B**). This observation clearly indicates that the difference in the appearance of PSVs in the osmicated and nonosmicated tissue is not an artifact of the staining procedure.

Previous studies have reported variable appearance of the contents of protein bodies in pea cotyledons due to differences in preparative procedures (*33*, *34*). The appearance of protein storage vacuole contents was found to be primarily influenced by the type of buffer used as a fixative and by the osmolarity of the fixative (*35*). Similarly, Boatright and Kim (*27*) reported the effect of fixative pH on the ultrastructure of protein bodies in soybean. An examination of the micrographs of soybean cotyledon that were postfixed with osmium tetroxide from earlier publications has shown the presence of globoid crystals and globoid regions within PSVs (*19*, *25*, *26*). In these studies the researchers have employed methodology that differed in the type and concentration of chemical fixative, fixation period, osmolarity, and type of buffers of the fixatives and the duration of postfixation with osmium tetroxide. Because each of these components may affect the structural integrity, it is therefore not surprising to observe in the literature the variable appearance of PSVs in soybeans.

PSV, the primary compartment of protein storage, also functions as a site for the sequestration of mineral reserves (*20*). Within PSVs, phytate is mainly stored in the globoid regions. During seed germination, the phytate is hydrolyzed by phytases, resulting in the release of phosphoric esters of *myo*-insitol (*14*). It has been shown by energy dispersive X-ray (EDX) analysis that glutaraldehyde−OsO_4 fixation results in major elemental loss of P, Mg, and K from globoid crystals in several seeds (*36*). On the basis of this observation it was recommended to avoid the use of OsO_4 to prevent elemental loss from globoid crystals. The globoid regions seen within PSVs in the current study often lacked the electron-dense crystals. It is believed that these crystals, due to their density, are shattered during the conventional sample preparation (*25*) and consequently appear as holes within protein bodies. Postfixation of soybean seed with osmium tetroxide appears to prevent the shattering of globoid regions. The mechanism by which osmium tetroxide prevents the shattering of globoid regions is not clear. Osmium tetroxide is routinely employed as a secondary fixative in electron microscopy. It is known to react with unsaturated lipids and help their stabilization (*32*, *37*). Even though osmium tetroxide is primarily known to interact with lipids, it is also known to form cross-links with proteins (*38*, *39*). Recently, a 45 kDa protein embedded in the phytate crystals was purified (*40*). It is speculated that this protein may play an important role in phytate crystal formation. Osmium tetroxide may interact with this protein and stabilize the phytate crystals. The other possibility is that osmium may also directly interact with phytate−protein complexes present in the globoids. In the absence of postfixation with osmium tetroxide, the possible stabilization of globoid crystals may be lost, resulting in the shattering of globoids during sample preparation.

ACKNOWLEDGMENT

I thank Nathan Oehrle for critical reading of the manuscript.

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Received for review December 3, 2007. Revised manuscript received February 25, 2008. Accepted February 27, 2008. Names are necessary to report factually on available data; however, neither the University of Missouri nor the USDA guarantees or warrants the standard of product, and the use of the name by the University of Missouri or the USDA implies no approval of the product to the exclusion of others that may be suitable.

JF0735228