Effect of Electron Microscopy Fixation pH on the Ultrastructure of Soybean Protein Bodies

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Cotyledon tissues from mature soybeans were systematically prepared for transmission electron microscopy employing fixation buffer pH's of 7.2, 6.4, and 5.6. Tissue fixed at pH 7.2 showed few membrane-bound internal protein body structures. Portions of the same tissue fixed at pH 6.4 revealed numerous membrane-bound crystalloid structures and stacks of membranous sacks. Tissue prepared at pH 5.6 also contained numerous membrane-bound crystalloid structures and examples of membrane-bound globoid structures. This is the first investigation to document the effect of fixation pH on the subcellular structure of soybean protein bodies and the first to show membrane-bound crystalloid or globoid structures in soybeans.

Keywords: *Soybean; transmission electron microscopy; protein bodies; membrane; fixation pH*

INTRODUCTION

In a review of the protein bodies of seeds, Pernollet (1978) stated that "all authors are agreed about the lack of membrane bounding the crystalloid" which is an ordered, partly crystalline protein deposit and that "the boundary of the globoid is still the subject of dispute". Also, of the six investigations (referenced by Pernollet) of soybean protein bodies, none reported the presence of crystalloids or globoids. Two subsequent investigations both reported the occurrence of globoid inclusions within the protein bodies of soybeans (Lott and Buttrose, 1978; Prattley and Stanley, 1983). Neither of these investigations observed inclusions bound by a membrane. In an investigation of the composition and ultrastructure of soybean protein bodies by Boatright and Snyder (1993), numerous membrane-bound vesicles were observed in close association with internal cavities. Several stacks of membranous sacks were also observed in the internal matrix of protein bodies.

The methodology for preparing biological material for transmission electron microscopy seems to be primarily developed for mammalian tissue which has a physiological pH of 7.0−7.4 (Weakley, 1981). Investigations in the ultrastructure of soy protein body investigations have also employed a similar pH. Lott and Buttrose (1978) used a fixative pH of 7.0, and Prattley and Stanley (1983) used pH 6.8. The primary purpose of the fixation process is to maintain the physiological structure of the tissue. The pH of fixatives should remain close to the pH of the tissue because "a change in the tissue pH brought about by the fixative is bound to alter dramatically the structure and behavior of tissue proteins in solution" (Hayat, 1981). Typically, the average pH of plant cells is lower than the average pH of animal cells (Hayat, 1981) and the pH of protein bodies is lower than the pH of the surrounding cytoplasm (Ashton, 1976). This current investigation was undertaken to demonstrate the effect of varying the pH during tissue fixation on the ultrastructure of soy protein bodies.

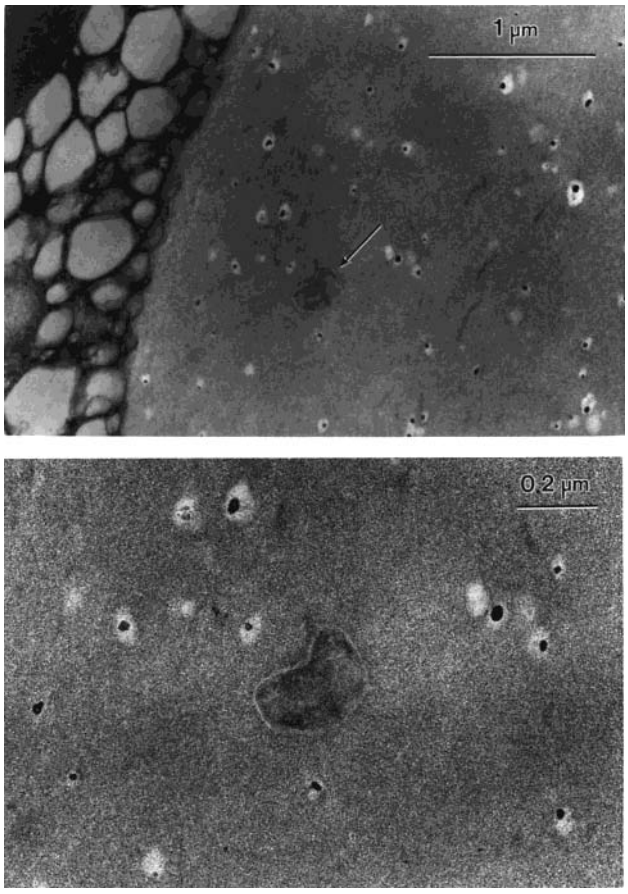

Figure 1. Transmission electron micrograph of soybean protein body fixed at pH 6.4 showing internal membrane-bound crystalloid at two different magnifications.

MATERIALS AND METHODS

Large, mature, seed-grade Forrest variety soybeans were used throughout this investigation. Cubes were cut from the

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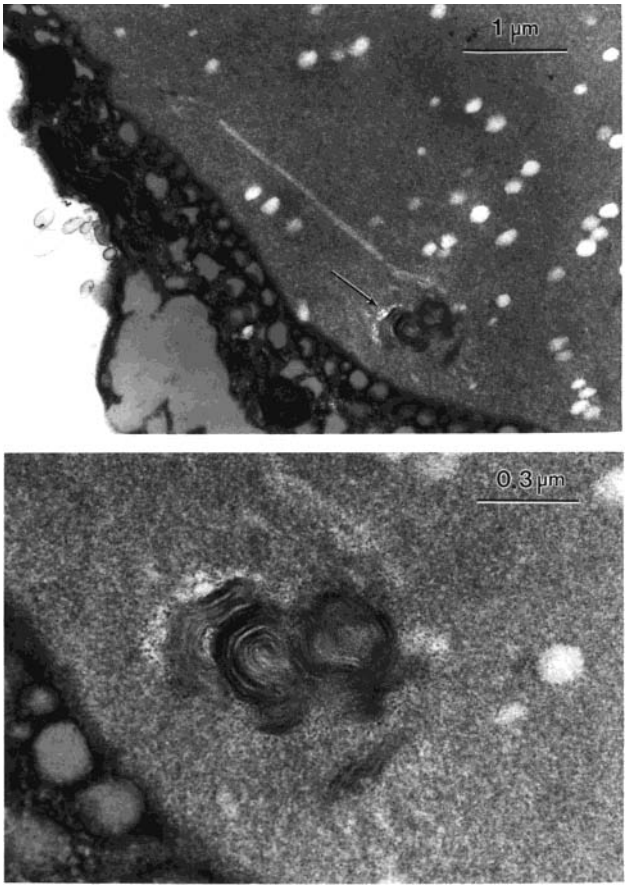

Figure 2. Transmission electron micrograph of soybean protein body fixed at pH 6.4 showing stacks of membranous material at two different magnifications.

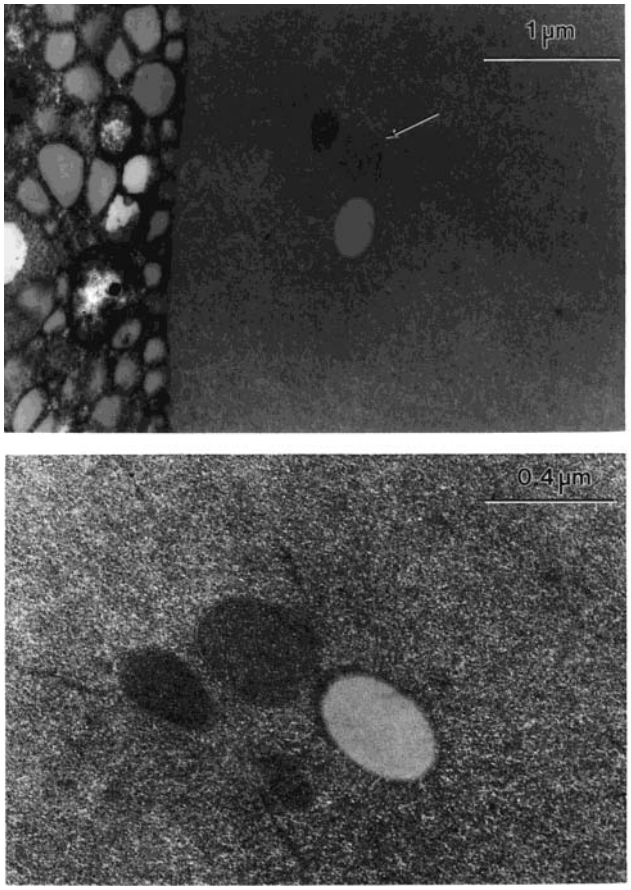

Figure 3. Transmission electron micrograph of soybean protein body fixed at pH 5.6 showing membrane-bound crystalloids at two different magnifications.

center of the cotyledons of dry seeds and subsequently cut into slices approximately 1 mm thick. The tissues were fixed for 2 h in 2% glutaraldehyde/2% paraformaldehyde at 4 °C, processed through two changes (20 min each) of 0.05 M cacodylate buffer, and post-fixed for 2 h in 1% osmium tetroxide at 24 °C. Throughout the fixation processes, all solutions were maintained at a pH of either 7.2, 6.4, or 5.6 with a 0.05 M sodium cacodylate buffer. After a water rinse, samples were prestained in 0.5% uranyl acetate overnight, dehydrated in an ethanol series followed by propylene oxide, and embedded on Spurr's epoxy resin. Silver−gold sections (ca. 800 Å) were cut with a diamond knife on a Sorvall Porter-Blum ultramicrotone. Sections were collected on 400-mesh copper grids, stained with uranyl acetate and lead citrate (Weakley, 1981), and examined with a JEOL CX electron microscope.

RESULTS AND DISCUSSION

Extensive observations of the tissues fixed at pH 7.2 revealed very few membrane-bound structures within the soybean protein bodies. However, such subcellular structures were occasionally seen (not shown) and almost always within close proximity to irregular internal vacuoles. Side-by-side evaluations were performed with portions of soybean cotyledon fixed at pH 7.2 and portions fixed at pH 6.4. The portion fixed at pH 7.2 again revealed few internal protein body structures. Within the protein bodies fixed at pH 6.4 there appeared crystalloid structures at a regular frequency. Many of these crystalloids were clearly enclosed by a bilayer membrane (Figure 1). Figure 1 also shows numerous globoid structures similar to those seen by Lott and Buttrose (1978). In addition to these crystalloid and globoid inclusions there occasionally appeared very elaborate membranous structures (Figure 2), similar to those observed previously by Boatright and Snyder (1993). The previous fixation method was accomplished with 1% osmium tetroxide at pH 5.

Additional samples prepared by lowering the fixation pH to 5.6 revealed numerous crystalloid structures with membrane boundaries clearly evident (Figure 3), similar to those seen in tissue fixed at pH 6.4. One protein body in the tissue fixed at pH 5.6 contained numerous globoid structures bound by a bilayer membrane (Figure 4). The bilayer membrane is clearly not the same type of boundary as was described by Poux (1965) surrounding the globoid of flax (*Linum usitatissimum*) and cucumber (*Cucumis sativus*). The extensive occurrence of membrane-bound structures within the soybean protein bodies of this investigation challenges previous conclusions that no such membranous structures exist, particularly surrounding the crystalloids.

Each investigation into the ultrastructure of mature soybean cotyledon tissue has used a somewhat different buffer and type and concentration of fixatives. The initial fixation scheme of Lott and Buttrose (1978) also used "dry" seeds. However, they used a 6% glutaraldehyde in 0.25 M phosphate buffer (pH 7.0) for 2 h and post-fixed for 1 h in 1% osmium tetroxide. In this current investigation we used 2% glutaraldehyde/2% paraformaldehyde at 4 °C in a 0.05 M cacodylate buffer followed with 2 h of 1% osmium tetroxide followed by a prestain with uranyl acetate. Prattley and Stanley (1982) soaked dehulled soybeans for 1 h in 0.025 M phosphate buffer (pH 6.8) at 4 °C prior to sectioning and then fixed with 2% glutaraldehyde for 12 h. This current

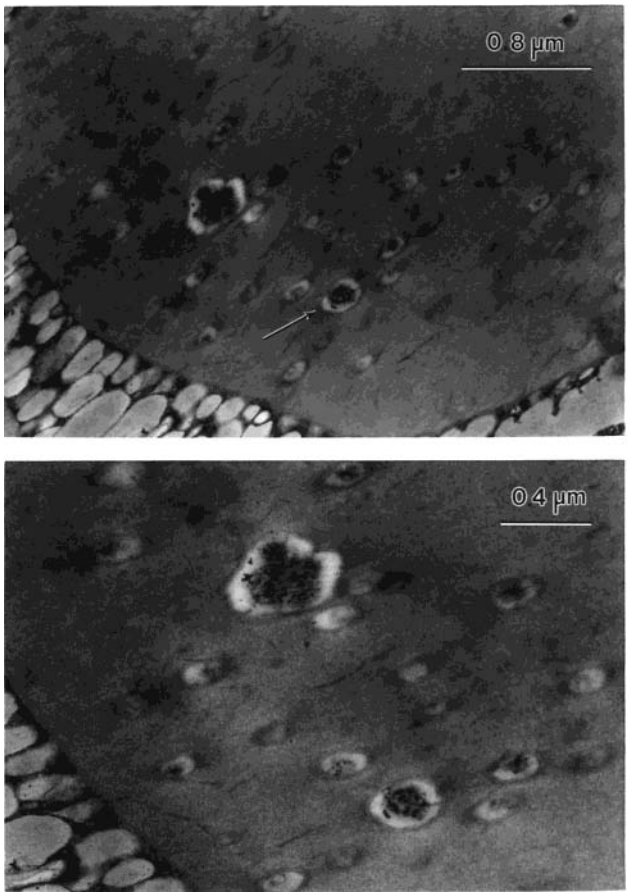

Figure 4. Transmission electron micrograph of soybean protein body fixed at pH 5.6 showing membrane-bound globoids at two different magnifications.

investigation is the first to publish the results of a systematic change in the fixative buffer. There have been many previous investigations that have reported alterations in subcellular structures resulting from variations in the conditions and types of fixatives used (Hayat, 1981). Soybean protein bodies can be isolated in an aqueous medium at pH 4.5, while at pH 7 the protein bodies rupture (Lee et al., 1983). Our results demonstrate that using chemical fixatives at pH's lower than is typically used (6.8−7.0) improves the preservation of the native subprotein body structures in soybeans. This improvement may be due to decreased mobility of the protein at the lower pH's or perhaps an inhibition of the activity of degradative enzymes at the lower pH's.

Certain degradative enzymes are associated with protein bodies prior to germination. Van Der Wilden et al. (1980) showed that protein bodies from mung beans contained *N*-acetyl-*β*-glucosaminidase, ribonuclease, acid phosphatase, phosphodiesterase, and phospholipase D. Boatright and Snyder (1993) demonstrated that phospholipase D was associated with soybean protein bodies. Many biological enzymes are associated with membranous materials (Stumpf, 1976), including *N*-acylphosphatidylethanolamine synthase in cotyledons of cotton seedlings (Chapman and Sriparameswaran, 1997), lysophosphatidate acyltransferases from soybean cotyledons (Rajasekharan and Nachiappan, 1994), and maize root phospholipase D (Brauer et al., 1991). Further study of the internal membranes of soybean protein bodies may reveal the physiological location of synthetic and degradative enzymes. Furthermore, Wang et al. (1999) observed lipoxygenase enzymes (1−3) in both the cytoplasm and protein bodies of 12-h imbibed and 4-day germinated soybean cotyledons. Seedling lipoxygenase enzymes (4−6) appeared to be primarily vacuole-localized. In a subsequent investigation (Wang et al., unpublished), auxin-induced seedling lipoxygenase enzymes appeared in highly dense proteinaceous material within the vacuoles of soybean cotyledons that were null for lipoxygenase enzymes 1−3. Compartmentalization in the mature protein bodies could contribute to prevention of premature enzymatic activity and may be one role of the bilayer membrane surrounding the crystalloid.

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