Evidence of Stress Proteins and a Potential Maturity Marker in Peanuts

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Stress proteins are induced in plants in response to environmental changes in temperature, oxygen, or water levels. It was hypothesized that stress proteins occur in peanut seeds during maturation and curing because these processes are known to be associated with water deficit and anaerobic metabolism in peanut seeds. To test this hypothesis, a polyclonal antibody against dehydrin, a plant stress protein, was used. Immunoblot analyses showed that a number of dehydrin-related stress proteins were detected in peanut seeds of different maturity and curing stages. Of these, only two were induced during seed curing and maturation. One (protein *a*) is potentially a peanut maturity marker because it was shown to occur only in uncured fully mature seeds. Immunoblot analyses of alcohol dehydrogenase (ADH), an enzyme known to be induced in mature peanut seeds, showed that ADH was not recognized by the antibody. This suggests that ADH is probably not related to protein *a* or dehydrin.

Keywords: *Peanut seeds; maturity marker; stress proteins; curing and maturation; dehydrin; polyclonal antibodies; immunoblot; alcohol dehydrogenase (ADH)*

INTRODUCTION

Plants undergo stresses when they are exposed to environmental changes in temperature, oxygen, or water levels. Plants respond to these changes by accumulation of abscisic acid (ABA) and genetically expressing stress-related proteins (Millar and Dennis, 1996; Sachs et al., 1996; Oliver and Bewley, 1997). A number of genes that respond to environmental changes have recently been described (Bartels et al., 1997; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997). The functions of the gene products (i.e., stress proteins) are thought to protect cells from damage, confer tolerance, and maintain homeostasis.

Of the plant stress proteins, dehydrins are the most commonly known (Artlip et al., 1997; Close, 1997; Hey et al., 1997; Ismail et al., 1997; Pelah et al., 1997; Cellier et al., 1998). Dehydrins accumulate in cells in response to the conditions of dehydration. They are members of the late embryogenesis abundant (LEA) proteins (Close, 1997; Colmenero-Flores et al., 1997; Moons et al., 1997; Zegzouti et al., 1997) that are induced during embryo maturation in the seeds. Dehydrins from different plants have similar amino acid sequences that are characterized by a conserved 15 amino acid, lysine-rich sequence near the carboxyl terminus (Close, 1997). The availability of a polyclonal antibody against the amino acid sequence has made possible the detection of dehydrin-related stress proteins in seeds.

In this study, we hypothesized that dehydrin-related proteins occur in peanut seeds during the maturing and curing stages because these stages are associated with water loss and an increase in the activities of glycolytic enzymes and alcohol dehydrogenase (ADH) (an anaerobic condition) (Chung et al., 1996, 1997). These conditions have been shown to be associated with stresses in other seeds (e.g., soybean, rice, cotton, maize) (Russell et al., 1990; Umeda and Uchimiya, 1994; Millar and Dennis, 1996; Sachs et al., 1996). Also, we assumed that ADH is a member of the LEA proteins or related to dehydrin because ADH not only is known to be induced under water loss and/or anaerobic condition (Russell et al., 1990; Umeda and Uchimiya, 1994; Millar and Dennis, 1996; Sachs et al., 1996) but also is the most abundant of the glycolytic enzymes induced in mature peanut seeds (Chung et al., 1996, 1997). The objectives of this study were (1) to determine, using a polyclonal antibody against dehydrin, if dehydrin-related proteins occur in peanut seeds during maturation and curing and (2) to determine if ADH is related to dehydrin or recognized by the antibody against dehydrin.

MATERIALS AND METHODS

Apparatus. Xcell II Mini-Cell and Blot Module were purchased from Novex (San Diego, CA). Mini Rotofor isoelectric focusing cell was purchased from Bio-Rad (Hercules, CA). Mini 2-gel device was purchased from Integrated Separation Systems (Natick, MA).

Reagents. Precast 4−20% Tris−glycine gels and SDS running buffer were purchased from Novex. Rotofor starter kit, prestained low molecular standards, Coomassie Brilliant Blue R-250, and alkaline phosphatase conjugate substrate kit were purchased from Bio-Rad Laboratories. Isoelectric focusing (IEF) standards, IEF SepraSol (2× concentrate), and IEF

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MiniPlus SepraGel (pH 3−10) were purchased from Integrated Separation Systems. Ponceau S solution was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Immobilon-P transfer membrane was purchased from Millipore Corp. (Bedford, MA). Anti-rabbit IgG alkaline phosphatase conjugate, bovine serum albumin (BSA), Tween 20, phosphate-buffered saline (PBS), nicotinamide adenine dinucleotide (NAD^+), *p*-iodonitrotetrazolium violet (INT-violet), and diaphorase (20 units) were purchased from Sigma Co. (St. Louis, MO). Anti-dehydrin polyclonal antibody was purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Chemical Co. (Rockford, IL).

Peanut Samples and Treatment. Peanut seeds (*Arachis hypogaea* L. var. Florunner) were planted at the USDA-ARS National Peanut Research Laboratory (Dawson, GA), dug 120 days after planting, and subjected to windrow drying, where samples were taken 0, 1, 2, 3, and 4 days after windrow drying, and day 4 samples were further dried to 10% moisture content with heated air. Day 0 samples refer to samples before curing. At each sample date, seeds were subjected to gentle abrasion to remove the exocarp, sorted by pod mesocarp color, hand shelled, and stored at −80 °C. Seed maturity (defined as yellow, orange, brown, and black) was based on the visual hull-scrape color method (Williams and Drexler, 1981).

Preparation of Peanut Protein Extracts. Extracts were prepared according to the method of Chung et al. (1997). Briefly, 0.7 mL of 0.02 M sodium phosphate buffer, pH 7, was added to 0.1 g of defatted meals from seeds of differing maturity and curing stages. The mixture was stirred for 1 h at 4 °C and centrifuged at 8500*g* for 10 min. The resultant supernatants were centrifuged again and then used for immunoblotting and fractionation by Rotorfor IEF cell. Protein concentration in the supernatant was determined using the BCA protein assay kit (Pierce Chemical Co).

Immunoblotting. This included gel electrophoresis, blotting or transfer of proteins to a membrane, and detection of stress proteins by antibodies against dehydrins.

Gel Electrophoresis and Blotting. Tris−glycine SDS polyacrylamide gel electrophoresis (4−20%; SDS−PAGE) or IEF (pH 3−10) gel electrophoresis and transfer to an Immobilon-P membrane were, respectively, performed according to the instructions of the manufacturers (Novex and Integrated Separation Systems). Briefly, seed extracts (5 μL each; 10 mg/mL; no reducing reagent added) from different maturity or curing stages were applied to the gel in the Xcell II Mini-Cell (for SDS gel) or Mini 2-gel device (for IEF gel). After gel running, the proteins were stained with Coomassie Brilliant Blue R-250 or transferred from the gel to the Immobilon-P membrane in the Xcell II blot module. The membrane was then stained with Ponceau S solution or incubated with 1% BSA in PBS−0.05% Tween 20 at 4 °C overnight and then washed three times (5 min each on a shaker) with the buffer before being subjected to antibody treatment.

Detection of Peanut Stress Proteins by Antibodies against Dehydrin. After blocking with BSA, the protein-bound membrane was incubated (on a shaker) for 30 min at room temperature (RT) with a preimmune rabbit serum (1:50) or a polyclonal antibody against dehydrin (1:1000) in 10 mL of PBS containing 1% BSA and 0.05% Tween 20. The membrane was then washed three times (5 min each on a shaker) with PBS−Tween 20 and incubated for 30 min at RT with an anti-rabbit IgG alkaline phosphatase conjugate (1:15000) in 15 mL of PBS containing 1% BSA and 0.05% Tween 20. The resultant membrane was again washed and incubated for 30 min at RT with 25 mL of a substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (substrate kit from Bio-Rad Laboratories). During this time, purple bands corresponding to stress proteins were seen on the membrane. After 30 min of incubation, the reaction (color development) was stopped by washing the membrane with distilled water, and the membrane was air-dried.

Fractionation of Stress Proteins and ADH by Rotofor Cell. Fractionation was based on the isoelectric point (p*I*) of the proteins and performed according to the instructions of the manufacturer (Bio-Rad Laboratories). Briefly, a mixture containing 1 mL of seed extract, 0.91 mL of ampholyte (pH 3−10), 0.91 mL of glycerol, and 15.38 mL of distilled water was applied to the Rotofor cell containing 0.1 M NaOH in the cathode chamber and 0.1 M H_3PO_4 in the anode chamber. The cell with water circulating at 4 °C was allowed to run for 3 h at 10 W. After the run, fractions (0.7 mL each) were collected and analyzed for stress proteins and ADH activity, respectively.

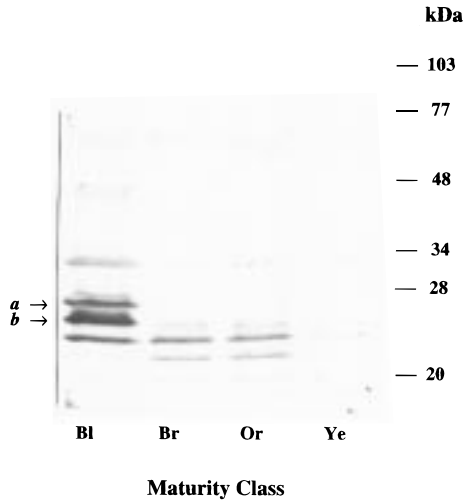

Figure 1. SDS−PAGE and immunoblot analyses of stress proteins from peanut seeds of different maturity stages. Protein extracts from seeds of different maturities were applied to SDS−PAGE, followed by transfer of proteins to a membrane and detection by polyclonal antibodies against dehydrin. Maturity stages, from immature to mature, were, respectively, yellow (Ye), orange (Or), brown (Br), and black (Bl). Stress proteins *a* and *b*, as indicated, were induced at the mature stage (black). Protein *a* was considered a peanut maturity marker.

Visualization of ADH Activity. ADH activity was visually determined according to the color method of Chung et al. (1997). Briefly, an aliquot (5 μL) from each of the Rotofor fractions was added to 0.1 M sodium bicarbonate buffer (pH 9.6, 116 μL), containing ethanol (3 μL), 4 mM INT-violet (15 μL), 14.3 μM NAD^+ (10 μL), and diaphorase (1 μL). The total volume was 150 μL. Color development was allowed to proceed for 15 min. The fraction that gave the color (red) (i.e., ADH activity) was further analyzed in immunoblots for its (ADH) reactivity with the antibody against dehydrin.

RESULTS AND DISCUSSION

In this study, the presence of stress proteins in peanut seeds at different maturity and curing stages was determined by immunoblotting with a polyclonal antibody against the conserved amino acid sequence of dehydrin.

Stress Proteins at Different Maturity Stages: Identification of a Potential Peanut Maturity Marker. Maturity (from immature to mature) was classified as yellow, orange, brown, and black (Williams and Drexler, 1981). Figure 1 shows the pattern of stress proteins at each maturity stage of uncured peanut seeds. Several proteins were detected at all maturity stages. Of these, two (proteins *a* and *b*) were distinct and occurred predominantly in the mature (black) stage. This suggests that only two stress proteins were induced during seed maturation. While protein *b* increased with maturity, protein *a* occurred solely in fully mature (black) seeds and, therefore, is potentially a maturity marker. Further analyses of the proteins at different maturity stages by IEF plus immunoblotting (Figure 2)

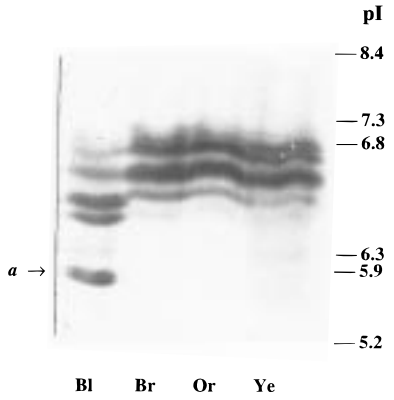

Figure 2. IEF and immunoblot analyses of stress proteins from peanut seeds of different maturity stages. Protein extracts from seeds of different maturities were applied to IEF gel (pH 3−10), followed by transfer of proteins to a membrane and detection by polyclonal antibodies against dehydrin. Bl, black (mature); Br, brown; Or, orange; Ye, yellow (immature). Stress protein *a*, as indicated, is potentially a peanut maturity marker.

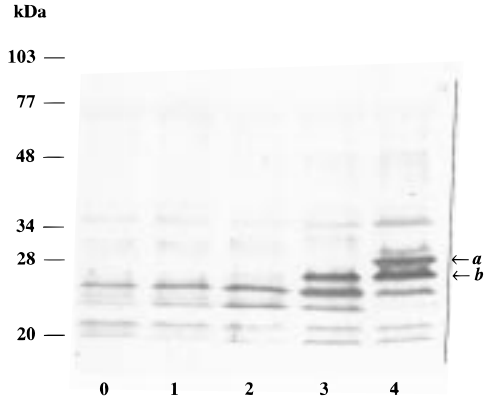

Figure 3. SDS−PAGE and immunoblot analyses of stress proteins from immature peanut seeds of different curing stages. Protein extracts from immature seeds (yellow), cured at the times indicated (day 4 included heated air-drying), were applied to SDS−PAGE, followed by transfer of proteins to a membrane and detection by polyclonal antibodies against dehydrin. Stress proteins *a* and *b*, as indicated, were induced at the final curing stage.

show a similar pattern—that is, two additional protein bands were found at the mature (black) stage. The bottom band was identified to be protein *a* after purification by electroelution and analyses with the antibody against dehydrin. Protein *a* was shown to have an isoelectric point (p*I*) of 5.9 and a molecular weight of 24 kDa (Figure 1), compared to 16−48 kDa from other plant stress proteins (Baker et al., 1995; Han and Kermode, 1996; Kader, 1996; Pelah et al., 1997). As a control, the preimmune serum did not recognize stress proteins *a* and *b* (data not shown). Also, staining with Coomassie Brilliant Blue R-250 failed to detect the difference between seeds of different maturities. This suggests that the antibody was specific for the stress proteins.

The above findings indicate that peanut seeds contain a number of stress proteins and that only two (proteins *a* and *b*) are inducible during peanut maturation. Other proteins occur at all maturity stages, indicating that they are present regardless of the physiological or environmental conditions. This is not uncommon because stress proteins have been known to occur in unstressed plants (Kader, 1996) and immature castor beans (Han and Kermode, 1996). The findings also suggest that stress proteins *a* and *b* probably are water stress-induced proteins because water stress has been known to be primarily responsible for the induction of dehydrins or dehydrin-related proteins in plants (Close, 1997; Pelah et al., 1997; Cellier et al., 1998).

In addition, the finding of stress proteins *a* and *b* supports the hypothesis that stress occurs in peanut seeds undergoing maturation. As indicated before, stress occurs because during maturation, peanut seeds undergo water loss and an increase of glycolytic enzymes and ADH (an anaerobic condition) (Chung et al., 1996, 1997). These conditions are known to be associated with stress and the induction of stress proteins (Russell et al., 1990; Umeda and Uchimiya, 1994; Millar and Dennis, 1996; Sachs et al., 1996; Bartels et al., 1997; Close, 1997; Oliver and Bewley, 1997; Pelah et al., 1997; Shinozaki and Yamagichi, 1997; Zegzouti et al., 1997; Cellier et al., 1998).

Because stress protein *a* appeared to occur solely in mature seeds (Figures 1 and 2), it was considered a potential peanut maturity marker or maturation protein. Maturation proteins have been shown to be associated with the ripening process and tolerance to water stress (Bewley and Marcus, 1990; Longhurst et al., 1990; Blackman et al., 1991, 1992). Although these proteins may be necessary for tolerance to stress, their presence alone was thought to be not sufficient to confer tolerance (Blackman et al., 1991, 1992). It was suggested that these proteins probably work in conjunction with other substances (e.g., carbohydrates) to confer tolerance. Several studies have shown that soluble sugars accumulated at high levels in plants that were exposed to stresses (Bianchi et al., 1991; Blackman et al., 1992; Crowe et al., 1997). These sugars were thought to protect protein and membrane integrity during dehydration. In peanuts, disaccharides and oligosaccharides have been shown to accumulate during seed maturation and curing (Vercellotti et al., 1995) and thereby may have a role in stress tolerance.

Stress Proteins at Different Curing Stages. Curing refers to windrow drying (for 0−4 days) and is known to be associated with a decrease in the water content in peanut seeds. Day 4 samples had ~10% water content after heated air-drying, as compared to 40% at day 0. Because of the water loss during curing, water stress was presumably created. To determine if stress proteins, especially proteins *a* and *b*, are induced due to water stress during peanut curing, immature (yellow) peanuts were used. Figure 3 shows the patterns of stress proteins in immature peanuts cured at different times (0−4 days). At the first three curing stages (i.e., days 0, 1, and 2), the patterns of stress proteins were very similar. As curing (or water loss) continued through day 3, an additional band corresponding to stress protein *b* was identified. Further curing through day 4 produced not only stress protein *b* but also stress protein *a*. This provides evidence that proteins *a* and *b* were induced during curing and that there is a correlation between water loss (or water stress) and the induction of stress proteins *a* and *b*. The differential appearance of stress proteins *b* and *a* on days 3 and 4, respectively, suggests that the induction was regulated by the extent of water loss.

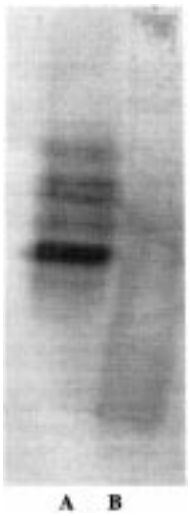

Figure 4. SDS−PAGE and immunoblot analyses of Rotorfor fractions that contained ADH activity and dehydrin-related stress proteins, respectively: (A) fraction that contained dehydrin-related stress proteins; (B) fraction that had ADH activity but no dehydrin-related stress proteins. Proteins from an extract of mature peanut seeds (black) were fractionated using the Rotorfor cell (pH 3−10). Twenty fractions were collected, and each was analyzed by immunoblot.

The above finding indicates that stress proteins *a* and *b* occurred in the yellow (immature seeds) at the final curing stages (days 3 and 4, respectively). It was observed that this also occurred in the orange/brown and black (mature seeds) (data not shown). However, unlike yellow/orange/brown, fully mature seeds (black) also contain stress proteins *a* and *b* at the early curing stages (i.e., days 0, 1, and 2) (data not shown). This was in agreement with what we found previously (see Stress Proteins at Different Maturity Stages), when stress proteins *a* and *b* were shown to be present in *noncured* (i.e., day 0) mature seeds only. It was surprising that curing did not result in induction of additional stress proteins in mature seeds. We concluded that stress proteins *a* and *b* were the only gene products induced in seeds during curing and maturation. The existence of stress protein *a* in all maturity classes after curing (4 days) indicates that stress protein *a* cannot be deemed as a maturity marker for peanut seeds that have been cured. Rather, stress protein *a* is potentially a maturity marker for uncured seeds only.

Relationship of ADH to Dehydrins. ADH is an enzyme catalyzing the conversion of acetaldehyde to ethanol in the final step of the alcohol fermentation pathway and has been known to be induced under anaerobic conditions/stress (Millar and Dennis, 1996; Sachs et al., 1996; Bray, 1997). In peanut seeds, ADH has been shown to be induced during maturation and curing (Chung et al., 1996, 1997) and, like stress protein *a*, is predominant in the mature (black) stage. This suggests that ADH may be related to dehydrin or the LEA proteins (Close, 1997; Colmenero-Flores et al., 1997; Moons et al., 1997; Zegzouti et al., 1997). If true, ADH may also be recognized by the antibody against dehydrin. To determine whether ADH is recognized by the antibody against dehydrin, fractionation of proteins from extracts of mature seeds using the Rotofor cell (based on p*I*) was carried out. Of the 20 fractions collected, 2 were identified to have ADH activity and stress proteins (including protein *a*), respectively. The fraction exhibiting ADH activity contained little protein that was recognized by the antibody against dehydrin (Figure 4). The other fraction containing stress proteins displayed little ADH activity. This suggests that ADH, despite being a stress protein, may not be related to stress protein *a* or dehydrin and that ADH and stress protein *a* (or dehydrin-related proteins) may be induced in peanuts under different stress conditions. Indeed, ADH is known to be induced under anaerobic conditions (Millar and Dennis, 1996; Sachs et al., 1996; Chung et al., 1997), whereas dehydrins or dehydrin-related proteins (e.g., protein *a*) are induced mostly under water stress (Close, 1997; Pelah et al., 1997; Cellier et al., 1998). Han and Kermode (1996) have also shown that different stresses may produce different stress proteins. For instance, one set of stress proteins has been induced by water stress/ABA, whereas another set by high salt and low temperature. In addition, two independent signal transduction pathways which affect expression of stress proteins have been suggested: that is, the ABA-independent and ABA-dependent pathways (Ingram and Bartels, 1996; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997). Water stress-inducible genes are known to respond to the ABA-dependent pathway.

Summary. Peanut seeds contain dehydrin-related stress proteins, as demonstrated in immunoblots using a polyclonal antibody against dehydrin (a plant stress protein). Among the stress proteins detected, only two (i.e., proteins *a* and *b*) were induced during seed maturation and curing. Stress protein *a* was considered a maturity marker because it was shown to occur only in uncured fully mature seeds (black). Immunoblot analysis of a Rotorfor fraction that had ADH activity showed that ADH (which is induced in mature seeds and known as an anaerobic protein) was not recognized by the antibody against dehydrin. This suggests that ADH may not be related to dehydrin and is probably induced in peanut seeds under a stress condition different from that of proteins *a* and *b*.

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