

Immunocytolocalization of Extensin in Developing Soybean Seed Coats by Immunogold–Silver Staining and by Tissue Printing on Nitrocellulose Paper

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Abstract. In soybean seed coats the accumulation of the hydroxyproline-rich glycoprotein extensin is regulated in a developmental and tissue-specific manner. The time course of appearance of extensin during seed development was studied by Western blot analysis and by immunogold–silver localization. Using these techniques extensin was first detected at 16–18 d after anthesis, increasing during development to high levels at 24 d after anthesis. Immunogold–silver localization

of extensin in the seed coat showed marked deposition of the glycoprotein in the walls of palisade epidermal cells and hourglass cells. The immunolocalization of extensin in developing soybean seeds was also made by a new technique—tissue printing on nitrocellulose paper. It was found that extensin is primarily localized in the seed coat, hilum, and vascular elements of the seed.

EXTENSINS constitute a class of cell wall hydroxyproline-rich glycoproteins present in a wide variety of plants (Lampert, 1970). They contain the common repetitive sequence Ser-Hyp-Hyp-Hyp-Hyp, in which most of the hydroxyproline residues are glycosylated with tetra-, tri-, di-, and mono-arabinosides and many of the serine residues are galactosylated. Extensins are apparently cross-linked into the wall, and thus the level of salt-extractable glycoproteins is usually low. However, in some instances a rise above normal levels of salt-extractable glycoprotein is observed; e.g., upon wounding (Chrispeels et al., 1974; Stuart and Varner, 1980), in cells in culture (Smith et al., 1984), and during the development of specialized tissues (e.g., soybean seed coats; Cassab et al., 1985).

Lampert (1980) proposed that extensins are the major protein components of the primary cell walls and that they play a role in cell wall architecture. However, to date no reports have demonstrated that extensin is localized in all plant cell types. Previously, we have shown that in the soybean seed coat, extensin is primarily localized in the external layer of the coat, which is comprised of the epidermal palisade cells and the hourglass cells. In this system, the accumulation of hydroxyproline in the cell walls is regulated in a developmental and tissue-specific manner (Cassab et al., 1985). Here, we report the use of polyclonal antibodies specific for soybean seed coat extensin for immunocytochemical and Western blot analyses for studying the following questions: (a) when does extensin accumulation begin in developing seed coats; and (b) how is extensin distributed?

Plant hydroxyproline-rich glycoproteins or extensins are components of the extracellular matrix and can therefore be regarded as analogues of the hydroxyproline-containing col-

lagens of higher animals. Collagens are not only the major component of the animal extracellular matrix, but also influence several cell events, such as cell proliferation (Adamson, 1983), differentiation (Bunge and Bunge, 1978), migration (Bard and Hay, 1975), and specific patterns of gene expression (Lee et al., 1985). Studies of extensin localization, assembly, and interaction with other cell components should provide insights into the influence of extracellular matrices in the growth, development, and function of plant cells.

Materials and Methods

Soybean Plants

Seeds of *Glycine max* (var Provar) were obtained from plants grown in the greenhouse. The plants were maintained with natural light supplemented during the winter months with light from 1,000-W metal halide lamps (Sylvania, Seneca Falls, N.Y.; M 1000/BU-HOR) placed 1–2 m above the plants and kept on cycles of 16 h light and 8 h dark. Temperature ranged from 20° to 30°C. Developmental stage of the seed was determined as described by Meinke et al. (1981). Cell walls were prepared from fresh soybean seeds as previously reported (Cassab et al., 1985). Total cell extracts were prepared by grinding seed coats in 0.2 M CaCl₂, 2 mM Na₂S₂O₅. Seed stages examined were G (14–16 d after anthesis [daa]¹), H (16–18 daa), I (17–19 daa), J (18–20 daa), K (19–21 daa), L (20–22 daa), M (21–23 daa), and N (more than 24 daa).

Antibody Production

Soybean seed coat extensin was purified as previously described (Cassab et al., 1985). Two New Zealand White rabbits 1 and 2 were immunized by multiple-site intradermal injection of 50 µg extensin/ml of Freund's com-

1. Abbreviation used in this paper: daa, days after anthesis.

plete adjuvant (1:1, vol/vol) for the first dose, and subsequently with incomplete adjuvant after 30 d of the first injection. 1 mo after the boost, the antiserum production was monitored by ELISA.

Binding Assays

The conditions for indirect microplate ELISA were done according to Voller et al. (1976) using alkaline-phosphatase-labeled goat anti-rabbit secondary antibody. 10 ng of antigen was coated in each well of a polystyrene microtiter plate (Costar, Cambridge, MA) in 50 μ l of 50 mM NaHCO₃, pH 9.6 buffer and kept at 4°C overnight; the plate was washed four times in PBS (pH 7.4) plus Tween (0.05% Tween-20, vol/vol) pH 7.4; all the remaining protein-binding sites were blocked by addition of 200 μ l 1% BSA for 2 h at 37°C, after which the plate was washed four times with PBS-Tween. Preimmune and test sera were diluted 1:500, 1:1,000, and 1:10,000 in 200 μ l PBS-Tween, were then added to the antigen-coated wells, and were incubated at 37°C for 1 h. Antigen-coated wells were also incubated in PBS-Tween without serum as blanks. The plate was then washed four times with PBS-Tween, and 200 μ l of alkaline phosphatase-labeled goat anti-rabbit IgG (Fc) (Promega Biotec, Madison, WI) diluted 1:20,000 in PBS-Tween were added to each well. The plate was incubated at 37°C for 1 h, and then washed as before. Finally, 200 μ l of substrate solution (*p*-nitrophenyl phosphate [1 mg/ml] in 10% diethanolamine buffer (9.7%, vol/vol, diethanolamine, 0.02%, wt/vol, NaN₃, 50 μ M MgCl₂·6H₂O, pH 9.8) was added to each well and incubated at room temperature for 10 min. The reaction was stopped by the addition of 50 μ l of 3 M NaOH. The color change in each well was measured spectrophotometrically at 405 nm in an ELISA reader (model No. EL-310; Bio-Tek, Burlington, VT).

Western blot analysis was performed as described (Towbin et al., 1979). Transfer of components that were subjected to electrophoresis on a cationic neutral gel (Thomas and Hodes, 1981) to nitrocellulose paper was in the presence of 0.1 M histidine, 0.022 M MOPS (3-[*N*-morpholine] propane sulfonic acid) pH 6.8, with electrophoresis from the positive to the negative electrode at room temperature for 15 h at 0.4 A, using a Transphor Power-Lid (model No. TE 50-100VDC, 0-2A; Hoefer Scientific Instruments, San Francisco, CA). For SDS-PAGE, the transfer was done as described by King et al. (1985).

The detection of alkaline phosphatase-conjugated second antibody on protein blots was performed as described by Blake et al. (1984). Primary antibody against soybean seed coat extensin was diluted 1:15,000 and anti-rabbit IgG (Fc) alkaline phosphatase conjugate was diluted 1:20,000; and BCIP (5-bromo-4-chloroindoxyl phosphate) and NBT (nitroblue tetrazolium; Promega Biotec, Madison, WI) were used as substrate to detect the

precipitated indoxyl group. Protein blots were stained with india ink as reported by Hancock and Tsang (1983).

Chemical Analyses

Deglycosylation of purified extensin was done via anhydrous hydrogen fluoride (HF) solvolysis (Van Holst and Varner, 1984). Protein content was determined by Bradford (1976), and hydroxyproline quantitated using the method of Drozd et al. (1976).

Immunogold-Silver Staining and Light Microscopic Immunocytochemistry

Soybean seed coats were fixed for 2 h at 22°C and 14 h at 4°C in 4% paraformaldehyde in 25 mM K-phosphate (pH 7.2), 0.5 M sucrose buffer. After five changes using the same buffer, tissues were dehydrated through 10, 30, 50, 70, and 90% ethanol, and three changes of 100% ethanol (30 min per step). The sections were infiltrated for 1 h with 1:1 LR White acrylic resin, 100% ethanol, then in pure LR White for 24 h. Final embedding was done in pre-dry OO gelatin capsules, followed by polymerization in a vacuum oven at 60°C for 36 h. Sections 1–2- μ m thick were cut using glass knives and mounted on glass slides.

Immunogold-silver staining was performed as described by Springall et al. (1984). Primary antibody against extensin was diluted 1:500, and the immunogold reagent 1:100 (Auro Probe LM; Janssen Life Sciences Products, Piscataway, NJ), and incubated with sections for 2 h at room temperature. Treated sections were observed and photomicrographed with a Zeiss Axiomat light microscope in the inverted configuration. The objective used was an Axiomat lens LD-Planapochromate 25 \times /0.60 for bright field and Nomarski-DIC (differential interference contrast) optics. All photomicrographs were recorded on Kodak Technical Pan 2415 film at 18 Din. The following controls were included: (a) the omission of primary antibody or its dilution until a point that no positive reaction was observed; (b) normal rabbit serum; (c) omission of the immunogold reagent to ensure that there was no reaction of the silver developer other than with colloidal gold particles. All these controls confirmed that labeling was dependent on the presence of specific antibody.

Tissue Printing on Nitrocellulose Paper

The nitrocellulose paper was soaked in 0.2 M CaCl₂ for 30 min, and dried on paper towels. Fresh cut tissue was washed in distilled H₂O for 3 s, dried on kimwipes, and blotted onto the nitrocellulose paper for 30 s. The tissue

Table I. Antibody Characterization

Serum	Antigen [‡]	OD _{405nm}	Cross-reactivity %
R1*	Seed coat extensin	2.25	100
	Deglycosylated seed coat extensin	1.44	64
	Carrot root extensin	0.27	12
	Deglycosylated carrot root extensin	0.13	6
	CaCl ₂ Extract seed coat	1.35	60
	Seed coat extensin + HCHO	1.62	72
	Arabinogalactan protein	0.04	1.8
	Arabinogalactan	0.05	2.2
	Potato lectin	0.05	2.2
	P-R1	Seed coat extensin	0.03
R2	Seed coat extensin	2.1	100
	Deglycosylated seed coat extensin	1.72	82
	Carrot root extensin	0.97	46
	Deglycosylated carrot root extensin	0.36	17
	CaCl ₂ Extract seed coat	1.79	85
	Seed coat extensin + HCHO	1.0	47
	Arabinogalactan protein	0.06	2.8
	Arabinogalactan	0.08	3.8
	Potato lectin	0.09	4.2
P-R2	Seed coat extensin	0.01	0.5

* All the serums are diluted 1:500.

[‡] Antigen concentration is in all cases of 10 ng. Values are the means of three different experiments.

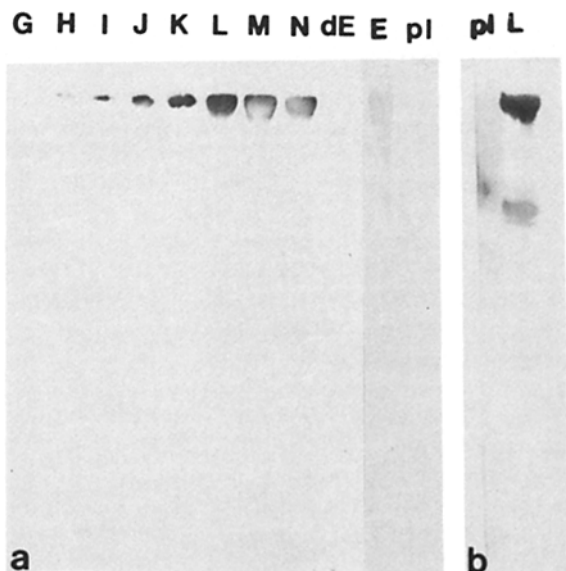


Figure 1. Western blot analysis of several soybean seed coat developmental stages with extensin antibody. (a) The lanes (from left to right) contain cell extracts from stages G (14–16 daa), H (16–18 daa), I (17–19 daa), J (18–20 daa), K (19–21 daa), L (20–22 daa), M (21–23 daa), and N (>24 daa), in which 2 μ g of total protein was loaded in each lane. dE represents 0.1 μ g of deglycosylated soybean seed coat extensin; E represents 0.5 μ g of purified soybean seed coat extensin; pI represents 15 μ g of high isoelectric point standards. Proteins were resolved by cationic neutral gel electrophoresis, and later transferred to nitrocellulose. Immune complexes were detected with alkaline phosphatase-conjugated anti-rabbit IgG antibodies. (b) India ink staining pattern of a stage L seed coat cell extract on nitrocellulose.

print was immediately dried with warm air and treated for detection of alkaline-conjugated second antibody as described above.

Results

Antibody Screening

4 wk after boosting two rabbits with 50 μ g of antigen, a strong immunogenic response was positive as determined by quantitative ELISA. Strong reaction with purified soybean seed coat extensin was obtained with sera from both rabbits at a dilution of 1:10,000. Values given in Table I were obtained with a dilution of 1:500. No cross-reactivity was obtained when primary antibody was omitted from the reaction or when preimmune serum was used. The serum cross-reacts with a salt extract from cell walls of soybean seed coat, deglycosylated soybean extensin, and glycosylated and deglycosylated carrot root extensin. Cross-reactivity of both antibodies 1 and 2 against deglycosylated soybean seed coat extensin was very high, which is unusual for proteins with high levels of O-linked glycosylation (Table I). It is possible that the protein was not completely deglycosylated, and as little as 4–5% of the original carbohydrate might account for a major part of the cross reactivity. Antibody 2 reacts more strongly against carrot extensin than antibody 1. Cross-reactivity of the serum with other hydroxyproline-rich glycoproteins such as arabinogalactan proteins and potato lectin, as well as against a polysaccharide like larch arabinogalactan

was also examined. Cross-reactivity of both serums against arabinogalactan protein and arabinogalactan was very low; similar low values were obtained for potato lectin (Table I).

Since the primary objective of this study was immunocytochemical localization of the extensin in developing soybean seed coats, the effect of fixatives on extensin by including formaldehyde in the ELISA was examined. This analysis indicates that chemical fixation reduced the antibody reactivity with both sera. In the presence of formaldehyde, cross-reactivity of antibody 1 with extensin was decreased \sim 30%, and with antibody 2 \sim 60% (Table I). Antibody 1 was used for the studies presented here.

Western Blot Analysis

To study the pattern of extensin accumulation during soybean seed coat development, protein extracts from different seed stages were prepared and subjected to gel electrophoresis for protein blot analysis. Cationic neutral gel electrophoresis was selected over SDS-PAGE because of the basic properties of extensin. Soybean seed coat extensin contains \sim 11% lysine and little aspartate and glutamate (Cassab et al., 1985). In this gel, extensin runs as a sharp band (Fig. 1) but no molecular mass can be assigned using this system (Thomas and Hodes, 1981). However, on a 10% SDS-polyacrylamide gel, soybean seed coat extensin runs as a broad band with an apparent molecular mass of \sim 180 kD (data not shown).

During soybean seed development, there is an increase in extensin from stage H throughout N in cell extracts of seed coats as determined by cationic neutral gel Western blot analysis (Fig. 1 a). No reactivity with the high isoelectric point standards run in this gel system as markers is observed. Also, india ink stain of the nitrocellulose filter after protein blotting indicates (Fig. 1 b) that a cell extract from stage L contains at least two major polypeptides; and, only extensin is labeled with the antibody.

The accumulation of extensin observed by Western blot analysis correlates with the chemical analysis of increases in hydroxyproline levels in cell wall extracts (Table II). There is an \sim 10-fold increase in the amount of extractable hydroxyproline in cell wall preparations of soybean seed coats from G to M stage, which agrees with the increase of total hydroxyproline seen in entire and dissected seed coats during seed development (Cassab et al., 1985). In stage G hydroxyproline represents 3% of the total cell wall protein and it goes to 30% at stage M. The highest amount of salt-extractable

Table II. Accumulation of Salt-extractable Hydroxyproline during Soybean Seed Coat Development

Seed stage	μ g Hydroxyproline/ μ g protein*
G	0.03
H	0.04
I	0.05
J	0.07
K	0.1
L	0.2
M	0.3
N	0.21

* Values represent the amount of extractable hydroxyproline per microgram of total protein, and they are the means of two different experiments.

extensin is seen in seeds at L and M stages. Nonetheless, at stage N the level of hydroxyproline from the cell walls decreased (Table II, Fig. 1). Once the seed matures (stage O) or desiccates, no extensin can be extracted, which suggests that it might get insolubilized in the wall matrix.

Immunogold–Silver Localization of Extensin in Developing Soybean Seed Coats

We have previously reported that hydroxyproline is primarily distributed in the external layer of the seed coat, and the ratio of hydroxyproline to dry weight is greater than in any other part of the seed. The external layer consists of two cell types, palisade and hourglass cells (Cassab et al., 1985), two microscopic characters that distinguish the Leguminous testa from other seed plants (Corner, 1951) (Fig. 2).

To assign a possible function of a protein, it is necessary to know in what type of cell it is present, and its cellular location. Preliminary efforts to separate the palisade cells from the hourglass cells by mechanical means and to discern where extensin is localized were unsuccessful, therefore, specific antibodies to soybean seed coat extensin were used for immunocytolocalization. In young seeds (stage B), measurable extensin has not accumulated in the cell walls of the different cell layers (Fig. 3 a). At this developmental stage, the hourglass cells have not yet differentiated, and the palisade cells have not matured. Fig. 3 c shows that in seeds at stage K, extensin is distributed primarily in the cell walls of the palisade and hourglass cells. There is much less staining in the parenchymatous tissue of the internal layer of the seed coat.

When seeds at stage L are stained with extensin antiserum, cell walls of the palisade cells showed stronger gold–silver deposition than seeds at stage K (Fig. 4 a). In addition, cell walls of the hourglass cells stain more strongly than the earlier seed stage K, particularly at the upper part of the cells. In the parenchyma cells there is some staining which appears not to increase during seed development in agreement with chemical determination of hydroxyproline reported previously (Cassab et al., 1985). The palisade is the major cell type in the seed coat that contains extensin in its cell walls as well as in its cytoplasm in vesicle-like structures. These later aggregates are also positive to Amido black and periodic acid-Schiff staining for proteins and glycoproteins or carbohydrates (data not shown).

In mature green seeds (stage N) the cell walls of the palisade cells react strongly with extensin antibody. The cell lumen, which is very narrow, also contains aggregates that stained with the extensin antiserum (Fig. 4 b). Cell walls of the hourglass cells are also intensely labeled at the upper part of the wall. A higher magnification micrograph of the immunolabeled palisade and hourglass cells at stage N is presented in Fig. 4 c. At this developmental stage extensin is primarily concentrated in the palisade cell walls close to the cuticle, and in the inner wall of the hourglass cells. In the control seed coat section at stage N is shown (Fig. 4 d), no gold–silver deposition is seen. In Fig. 4 e, part of the immunogold–silver stained hilum region of a seed coat at stage N is shown. In the palisade–counterpalisade cell walls, labeling is heavier than in the palisade cells not in the hilum region. In addition, cell walls of the funicle cells are stained. A control hilum region treated with normal rabbit serum at

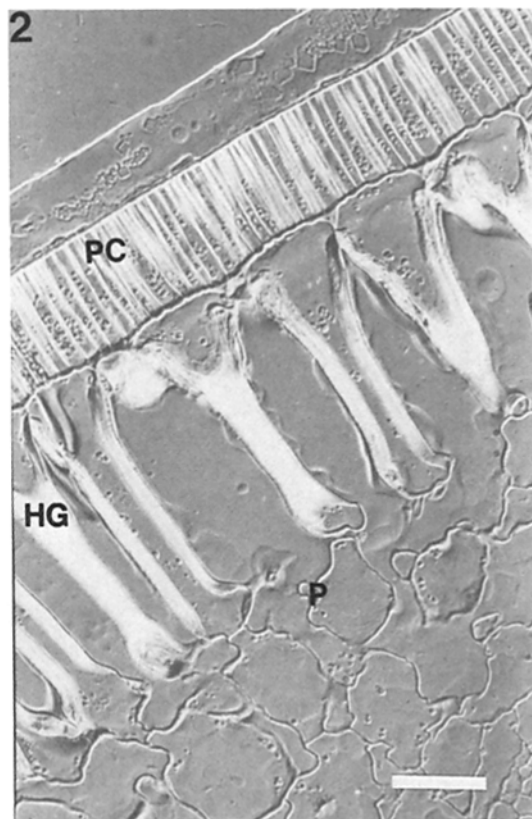


Figure 2. Soybean seed coat visualized by Nomarski microscopy. PC, palisade cells; HG, hourglass cells; P, parenchyma. Bar, 50 μ m.

stage N is in Fig. 4 f; no label can be detected in the walls of any cell.

Tissue Prints on Nitrocellulose Paper

A tissue-printing technique was developed to screen simply and quickly different plant tissues as well as plant species for the presence of extensin by using antibodies raised against soybean seed coat extensin. The tissue prints using developing soybean seeds are shown in Fig. 5 a. A direct examination of the tissue-print under the dissection microscope shows that in many regions of the print the outline of individual cells can be seen. The external layer of the testa, including the hilum, reacts strongly with the antibody. In cotyledons, the distribution and intensity of antibody staining is the same from one seed to another, and is very reproducible. This distribution seems to follow the vascular supply of the seed (Corner, 1951). The vascular system of the plant is made up of xylem, the water-conducting tissue, and phloem, the food-conducting tissue. Structurally, xylem and phloem are a complex tissue, for it consists of several different types of cells. Therefore, immunolocalization of extensin in thin sections of cotyledons will be necessary to establish the precise cellular location. There is apparently no endogenous alkaline phosphatase activity in soybean seeds since, in the absence of primary antibody, no tissue print was seen with the colored substrate. Also, no print was obtained with preimmune serum. Tissue prints can also be stained with india ink, so the total protein distribution pattern of the soybean seed can be seen (Fig. 5 b).

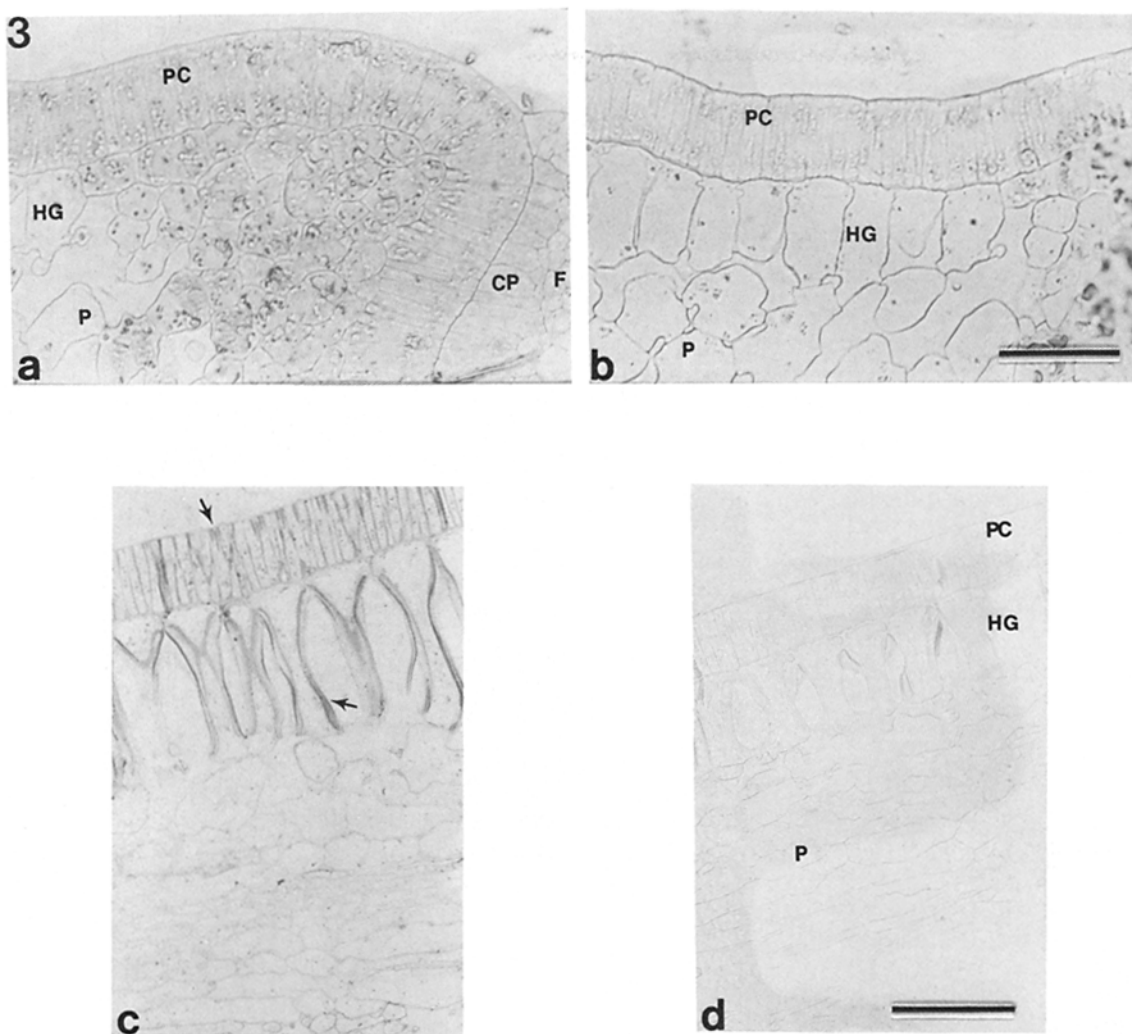


Figure 3. Immunogold-silver staining of extensin in developing soybean seed coats, at stage B and K. Seed coats at stage B (1–6 daa) were treated with (a) extensin antibody and with (b) preimmune serum. No antibody stain was observed in either case. Seed coats at stage K (19–21 daa) were stained with (c) extensin antibody and with (d) preimmune serum. The antibody stains palisade and hourglass cells (arrows in c). PC, palisade cells; HG, hourglass cells; P, parenchyma; CP, counterpalisade; F, funicle. Bars: (b) 50 μm ; (d) 100 μm .

Discussion

In the present study, the accumulation of the hydroxyproline-rich glycoprotein extensin from the soybean seed coat was studied by Western blot analysis. With this procedure extensin is first detected in cell extracts at seed stage H and increases steadily until stages M and N. This shows that marked accumulation of extensin in the seed coats occurs within a 5-d developmental period (Fig. 1, and Table II).

Immunocytochemical localization of extensin indicates that it is mainly concentrated in cell walls of the palisade and hourglass cells. The distribution of extensin among the different cells of the seed coat changes during seed development, as demonstrated by immunogold-silver staining. Extensin is not detected at early seed stages (Fig. 3 a), however some hydroxyproline extracted from cell walls can be measured at the G stage (Table II). Seeds at stage K start accumulating extensin, primarily in the cell walls of the palisade cells, but also in the cytoplasm and cell walls of the hourglass cells, as well as in the parenchyma cells. The hour-

glass cells at stage K begin a marked differentiation, a process that has been clearly described by Harris (1984). During this process, the cell walls in the region of the cell equator become heavily thickened preventing further expansion, while the ends of the cells retain their thin walls and continue to expand. At stage L, extensin is even more concentrated in the palisade cell walls as compared to stage K. There is also more label seen in the cell walls of the hourglass cells, which at this stage are fully differentiated. Finally, extensin is heavily concentrated, in the cell walls of both palisade and hourglass cells of mature green seeds, especially in the hilum region in the counter-palisade and palisade cells (Fig. 4 e). Extensin antibody labeled vesicle-like structures in the cytoplasm of palisade cells. These structures may play a special role in the synthesis and secretion of extensin. The biosynthetic pathway of extensin has been studied in wounded carrot root discs (Sadava and Chrispeels, 1978), but the subcellular localization of some posttranslational reactions is still controversial. Synthesis of the peptide, hydroxylation of

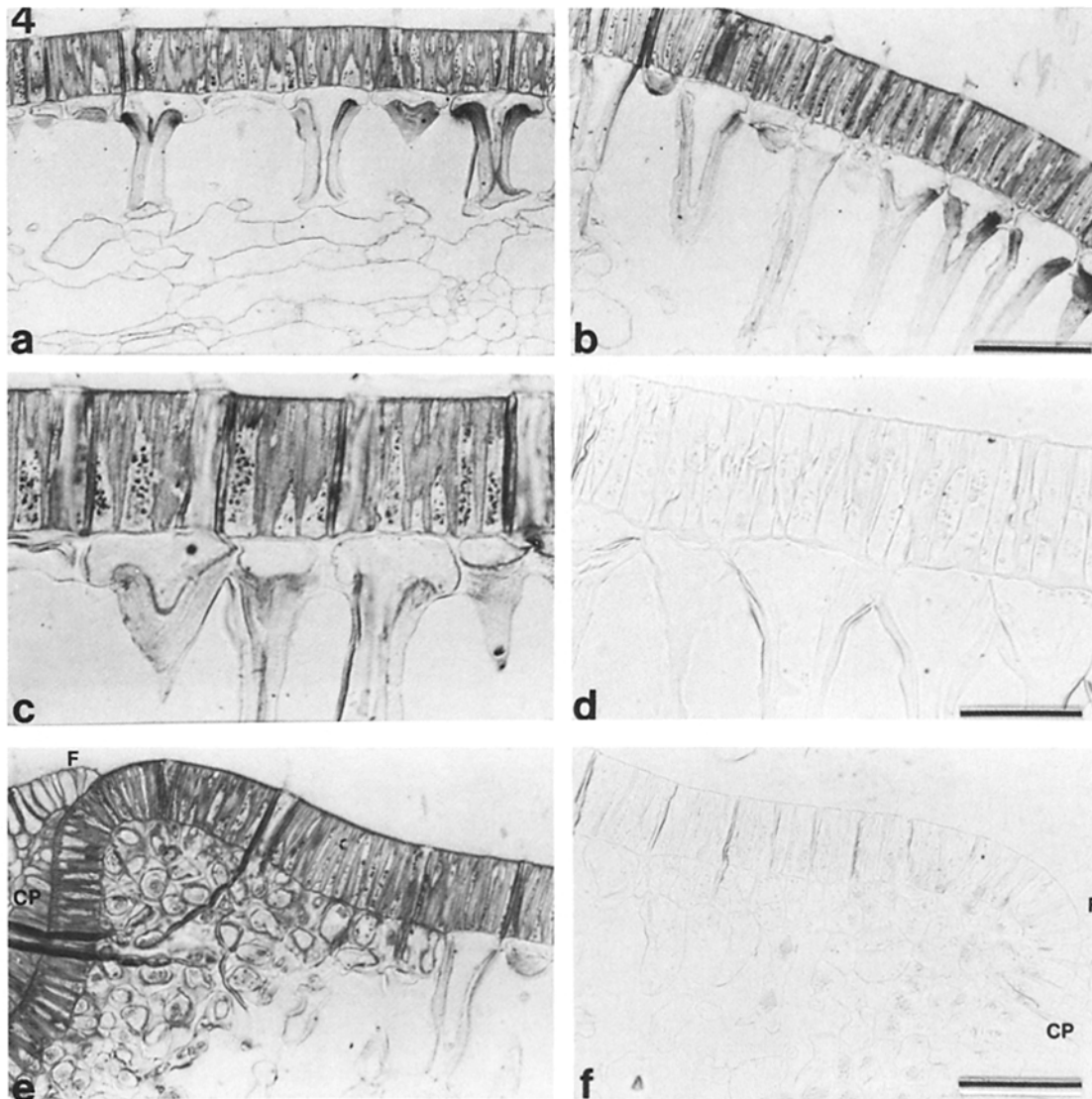


Figure 4. Immunogold-silver detection of extensin in developing soybean seed coats, at stage L (20–22 daa) and N (>24 daa). Extensin antibody stains intensely the palisade and hourglass cell walls of soybean seed coats at stage L (a) and N (b). In c, a higher magnification of a stained seed coat at stage N is shown, whereas d displays a seed coat treated with the preimmune serum. In e, a hilum at stage N stained with extensin antibody is shown, and in f, a hilum treated with the preimmune serum. CP, counter-palisade; F, funicle. Bars: (b and f) 100 μ m; (d) 50 μ m.

the proline residues, and glycosylation seems to occur sequentially in the endoplasmic reticulum and in the Golgi apparatus. Finally, the glycoprotein is secreted into the cell wall. However, the biosynthetic pathway of extensin in soybean seed coats has not been studied. Future experiments with immunoelectron microscopy should greatly improve the resolution of the antibody in the labeled aggregates of palisade cells. Moreover, these electron microscopy studies may resolve the differential distribution of extensin observed in the walls of the hourglass cells.

Overall, it can be inferred from extensin's absence in seed coats at early developmental stages that the glycoprotein may not play a role in the early differentiation of the palisade and hourglass cells. However, extensin may play a role in the maturation process of these cells since, in the hourglass cells the glycoprotein starts accumulating once the cells expand and separate.

Corner (1951) proposed that the palisade represents the obvious mechanical and protective part of the Leguminous seed; to a lesser extent the parenchyma may act as a cushion between the palisade and the embryo. On the other hand, the hourglass layer may be involved in the aeration of the seed (Corner, 1951; Harris, 1984) and the cells may be acting as columns to restrain the effect of compression from the growth of the embryo. The fact that during soybean seed development extensin is localized and accumulated in the palisade and hourglass cells just before the drying and shrinking of the seed coat starts makes extensin a good candidate for a structural protein involved in the mechanical and protective function of the seed. Interestingly, the Leguminous seed normally has a specific size, set by the differentiation of the palisade at a specific stage of development of the fruit and the seed (Corner, 1951). In some cases, however, the seed enlarges and fills the seed cavity of the pod to become over-

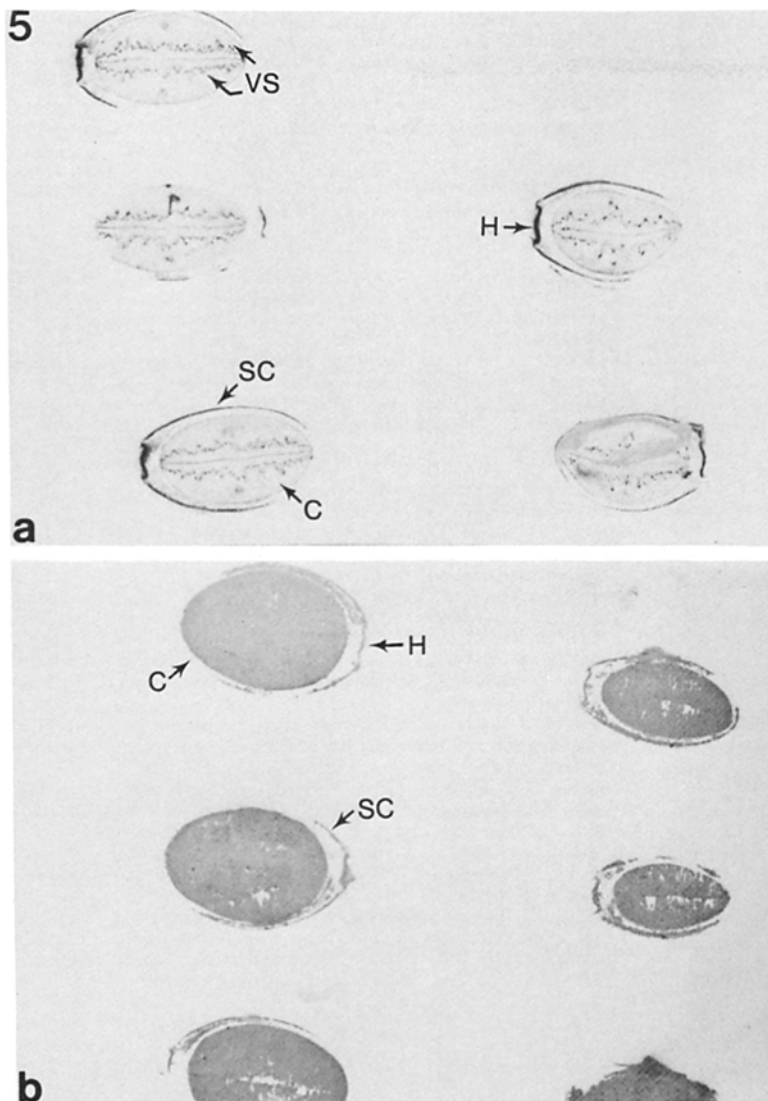


Figure 5. Developing soybean seed prints on nitrocellulose paper. (a) The prints were reacted with polyclonal antibodies, and detected with alkaline phosphatase-conjugated anti-rabbit IgG antibodies. (b) The prints were stained with india ink. C, cotyledon; H, hilum; SC, seed coat; VS, vascular supply of the seed.

grown seeds, as they are referred to in several genera of the Legumes. Their main character is that the testa remains immature, characterized by a lack of differentiation of the palisade, hourglass cells, and in the hilum region. Overgrown seeds may be a suitable system to examine whether the presence of extensin is correlated with the differentiation of the testa, and in the control of the seed size.

The technique of tissue printing on nitrocellulose paper is a simple immunolocalization procedure that should be of general use. Tissue printing of developing soybean seeds shows that extensin is primarily localized in the seed coat, hilum, and vascular supply of the seed (Fig. 5 a). These tissue prints differ from those stained with india ink, where the seed coat and cotyledons show a uniform protein stain, and the vascular supply of the seed cannot be distinguished (Fig. 5 b). In cotyledons, india ink stain presumably shows the presence of seed storage proteins, which are very abundant in this tissue (Meinke et al., 1981). Recently, the tissue-printing technique has been used to demonstrate the accumulation of extensin after wounding of carrot root, and the presence of extensin in soybean root nodule cortex (data not shown).

The presence of hydroxyproline in Leguminous seed coats is not unique. Van Etten (1961) reported that hydroxyproline is an abundant amino acid in the seed coat of several plant families. It is not yet known whether this hydroxyproline in other species is in an extensin-type of protein. The fact that hydroxyproline is present in the seed coat of several plant species, and that it is very abundant in this tissue compared with other plant tissues, may indicate that extensin serves a protective and mechanical function in the testa.

As we show here extensin antibody labels specifically both types of sclereids in seed coats. This encourages us to propose that extensin may be a marker for the sclerenchyma tissue of the plant. In general, seed coats contain massive numbers of sclereids, and this may explain the abundance of hydroxyproline in this tissue. On the other hand, immunogold-silver localization of extensin in soybean root nodules showed that sclereid cells are the major cell types that are labeled (data not reported here). The sclerenchyma cells are supposed to enable plant organs to withstand various strains, such as may result from stretching, bending, weight, and pressure, without undue damage to the thin-walled softer cells, such as parenchyma (Esau, 1965). The presence of ex-

tensin in sclereids may be related to their specific function in the plant.

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