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Development of Cystine- and Cysteine-rich Aleurone Grains in Bean Seeds

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ABSTRACT

Histochemical techniques were used to study aleurone grains in developing cotyledons of four cultivars of *Phaseolus vulgaris L.* The ninhydrin-Schiff's reaction and the 2,2'-dihydroxy — 6,6' dinaphthyl-disulfide (DDD) method were used to identify total protein and protein-bound sulfhydryl and disulfide groups respectively. Aleurone grains of the subepidermal and adjacent mesophyll cells showed a more intense staining reaction to DDD 28 days after flower opening than the rest of the cotyledon, and the staining differentiation increased until the seed matured. The greatest concentrations of these deeply staining aleurone grains were in two areas, at the point where the embryo axis attaches to the cotyledons and adjacent to the hilum. A varietal difference was not demonstrated by these techniques.

Additional index words: Phaseolus vulgaris, Histochemical sulfhydryl groups, Protein bodies, Seed proteins.

THE major portion of the protein of mature seeds is made up of reserve materials located in cotyledons or endosperms. Originally considered single entities, storage proteins have now been demonstrated to be mixtures and are subject to the same controls of protein synthesis as are other proteins (8,9). Altschul et al. (1) point out that a wide variety of plant and animal cells accumulate storage proteins in discrete bodies which they suggest "rightfully should be called 'aleurone grains'" when these sub-cellular bodies are located in seeds. Jennings and Morton (5) reported that although amino acid composition of the endosperm proteins of wheat changed considerably during growth and development the composition of aleurone grains changed little. Their data also suggested that there was more than one kind of aleurone grain. Dieckert et al. (3) reported two types of aleurone grains in peanuts. The proteins in both types appeared to be the same when judged by zone electrophoresis and chromatography. Kloz et al. (7), in observations using immunoelectrophoresis, detected new proteins during seed dehydration not seen in earlier stages. They assumed these new proteins to be synthesized from preformed peptide chains. Immunofluorescence techniques were used by Graham and Gunning (4) to study the distribution of two globulins in the cotyledons of *Vicia faba* L. Some aleurone grains in immature cells had neither globulin, a very few had only vicilin but most grains contained both globulins. Immature aleurone grains were found in most stages of seed development.

Opik (10) found that aleurone grains arose from the subdivision of vacuoles in bean cotyledons and found no evidence for an alternate protein-forming system. In the same study it was suggested that cells remain unchanged right up to dehydration as far as changes in the protein synthesizing system of the endoplasmic reticulum and ribosomes were concerned.

The object of the experiments reported here was to determine if proteins rich in cystine and cysteine were differentially distributed in the developing cotyledons of beans.

MATERIALS AND METHODS

Mature and developing seeds of *Phaseolus vulgaris* L. cultivars 'U.I. 111,' 'U.I. 74,' 'N 203,' and 'Common Red Kidney' grown in the greenhouse were used in this study. Flowers were tagged the day they opened and the day of tagging was considered day 1 of the development of the seed. Seeds were harvested after 15, 20, 24, 28, 32, 36, 40, and 44 days. The largest seeds were removed from the pods, and their seed coats removed, and the separated cotyledons were killed and fixed by placing them in a 0.6 M trichloroacetic acid for 24 hours. Fixed material was dehydrated in ethanol, cleared in xylene, and embedded in Tissuemat.[3] The embedded material was cut into sections 10μ thick and mounted on glass slides for staining.

The staining procedures used follow the schedules published by Jensen (6). The ninhydrin-Schiffs' reaction was used as a specific test for total protein and to identify aleurone grains in the bean cotyledons. The deparaffinized sections were placed in a 0.5% solution of ninhydrin in absolute ethanol for 24 hours at 37 C. The sections were hydrated and placed in Schiff's reagent for 20 minutes. Schiff's reagent was prepared from certified basic fuchsin. After rinsing in a 2% sodium metabisulfite solution the sections were dehydrated and cover slips were mounted with a synthetic resin. The specificity of the ninhydrin-Schiff's test was shown by blocking the reaction by deamination of the sections in a mixture of 60 ml of 1% acetic acid and 20 ml of 60% sodium nitrite for 24 hours at room temperature. The second blocking reaction involved acetylation of the sections in 10% acetic anhydride in pyridine for 20 hours at room temperature.

The 2,2'-dihydroxy - 6,6'-dinaphthyldisulfide (DDD) test was used as a specific test for protein-bound cystine and cysteine. The sections were placed in 0.5 M mercaptoacetic acid, adjusted to pH 8.0 with 0.1 *N* sodium hydroxide, for two hours at 50 C. The rinsed sections were then placed in a mixture of 15 ml DDD stock solution and 35 ml of barbital buffer at pH 8.5, made up immediately before using, for one hour at 56 C. The DDD stock solution was prepared by dissolving 100 mg of DDD in 60 ml absolute ethanol. After cooling for 10 minutes the sections were rinsed in water and washed for five minutes each in two changes of 1% acetic acid. The sections were dehydrated in alcohol, washed in absolute ether for five minutes, and returned through the graded alcohol series to water. The sections were placed in a fresh solution of 50 mg diazo blue B (tetrazotized 0-diahistidine) in 50 ml of phosphate buffer at pH 7.4 for 2 minutes at room temperature. The sections were dehydrated and cover slips mounted with a synthetic resin. The DDD and diazo blue B reagents were obtained from Sigma Chemical Co. The specificity of the reaction was tested by blocking the DDD reagent by putting the sections in 0.1 *M* N-ethylmaleimide in phosphate buffer at pH 7.4 for 4 hours at 37 C or in a 0.1 *M* iodoacetate solution adjusted to pH 8.0 with sodium hydroxide at 37 C for 20 hours immediately before DDD treatment. Iodoacetate was obtained from Eastman Organic Chemicals and N-ethylmaleimide from California Corporation for Biochemical Research.

The IKI test was used to stain the starch grains in the mature and developing bean cotyledons.

RESULTS AND DISCUSSION

Fifteen-day cotyledons did not contain visible aleurone grains or starch grains. A large nucleus surround-

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[3] Mention of a trade name does not constitute a guarantee or warranty of the product, and does not imply its approval to the exclusion of other products that may also be suitable.

Table 1. Responses of the granular material in the cotyledonary cells of beans to different staining procedures.

Staining procedure*	Aleurone grains	Starch grains
Ninhydrin-Schiff's	Magenta	Negative
Deamination + ninhydrin-Schiff's	Negative	Negative
Acetylation + ninhydrin-Schiff's	Negative	Negative
DDD	Blue or red	Negative
N-ethylmaleimide + DDD	Negative	Negative
Iodoacetate + DDD	Negative	Negative
IKI	Negative	Positive

* See materials and methods section for details.

ed by strands of cytoplasm enclosing large vacuoles occupied the mesophyll cells at this stage. Cellular organelles were normally obscured in more mature cells by the large number of reserve granules present and by the fact that cells were plasmolized by the killing and fixing treatments. Aleurone grains and starch grains were visible in the mesophyll cells of 20-day cotyledons, but aleurone grains were not visible in the vascular and epidermal cells until later stages of maturity. Subcellular bodies were considered aleurone grains when they were shown to be protein by the ninhydrin-Schiff's test (Table 1). Aleurone grains of 20-day cotyledons were slightly larger (3-6μ) than at later stages of maturity (2-4μ) as also noted by Opik (10). The number of aleurone grains per cell increased regularly at each sampling period from a low of 40 at 20 days to a high of 120 at 44 days. The intensity of staining of aleurone grains with ninhydrin-Schiff's reaction increased during development and reached a maximum at maturity (Fig. 1). Staining was uniform through all areas of the cotyledons indicating a uniform density of protein throughout.

The aleurone grains of bean cotyledons stained blue or red with varying intensity when localized by the DDD reaction. Barrnett and Seligman (2) reported that when diazo blue B was used as the dye with the DDD reaction, a difference in color depended upon whether monocoupling (red color) or dicoupling (blue color) occurs. Widely separated sulfhydryl groups show red or pink coloration, whereas blue coloration indicated a greater concentration of sulfhydryl groups. Aleurone grains stained uniformly red with increasing intensity until 28 days after flowering. Some of the aleurone grains in the subepidermal and adjacent mesophyll cells of 28-day cotyledons stained a deep red with DDD in contrast to the lighter red color of adjacent grains and of grains in the central mesophyll cells. Thirty-two-day cotyledons had blue aleurone grains in the subepidermal and adjacent mesophyll cells. The number of blue-staining aleurone grains increased in these cells until maturity (Fig. 2). These staining behavior responses for protein-bound disulfide and sulfhydryl groups indicated that the primary structure of the proteins being synthesized and stored in the aleurone grains were rich in cystine and cysteine. These aleurone grains were most numerous in the area where the embryo axis attaches to the cotyledons and in the area adjacent to the hilum. Also, the numbers of blue-staining aleurone grains were found to be most numerous in the surface cells with progressively fewer occurring in the interior of the cotyledons and only red-staining ones were found in the center of the cotyledons. Although previous studies have indicated a differentiation of aleurone grains as a function of the time of development, these data describe aleurone grains with specific proteins associated with particular cells. No differences in varieties were observed.

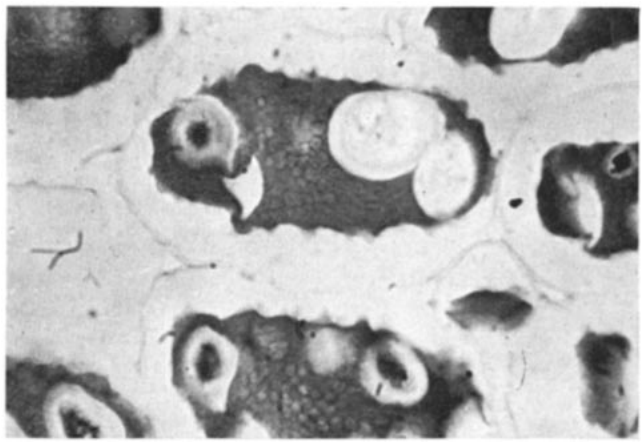

Fig. 1. Cells of a mature bean cotyledon stained with the ninhydrin-Schiff's reaction. The cell contents have shrunk away from the cell wall. The aleurone grains show as small, round to elipsoidal particles surrounding the large unstained starch grains. ca. × 2250.

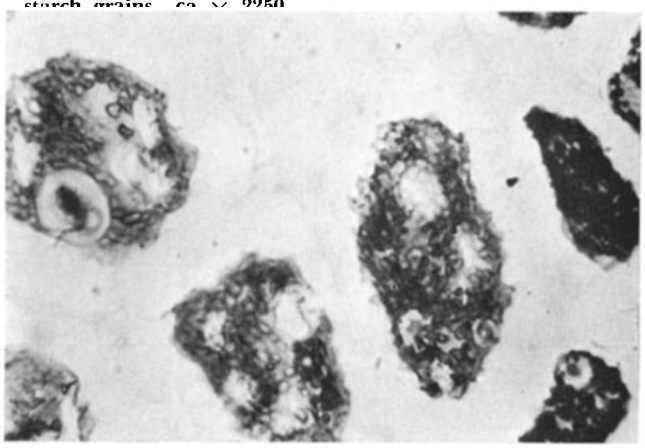

Fig. 2. Cells of a mature bean cotyledon stained with the DDD reaction. Light and dark staining aleurone grains can be seen in each cell. Sub-epidermal cells are the small cells along the right margin. ca × 2250.

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