Immunological Characterization of a 36kD Polypeptide in Peanuts (*Arachis hypogaea* L.)

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ABSTRACT

Antiserum against a 36 kD polypeptide (a subunit of arachin) that occurs in poor blanching peanuts was prepared. This antiserum was of high titer but was not monospecific for the homologous 36 kD anitgen; it showed cross-reactivity with other peanut proteins. The antiserum was adsorbed by the whole seed protein fraction of a good blanching cultivar to render it monospecific. The adsorbed antiserum could accurately differentiate the protein extracts from good blanching and poor blanching peanuts when tested by the enzyme linked immounosorbent assay (ELISA) and by immunoblotting. Time course studies of protein deposition during seed development revealed that the 36 kD polypeptide was present from the early stages of embryogenesis. In addition, it was present in all parts of the cotyledon and not limited to the surface of seed. Although the function of the 36 kD polypeptide is not known, our results indicate that its presence can be detected uising immunological assays.

Key Words: Peanut, blanchability, ELISA, immunoblotting

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Blanchability in peanut is the measure of the adhesion of the seed coat to the cotyledon or inversely the ease with which the seed coat (skin) separates from the cotyledon. Thus blanchability is one of the important criteria in judging the quality of the peanuts to be used as cocktail peanuts. Mozingo (5) reported that blanchability is a factor related mainly to the genotype, seed size, and maturity stage. Shokraii *et al.* (6) reported the possible involvement of a 36 kD polypeptide in blanchability since its presence appeared to be correlated with poor blanchability in most, if not all, peanut cultivars and genetic lines. This putative marker protein, a subunit of arachin, was purified by preparative sodium dodecylsulfate-polyacrylamide gel electrophoresis (*SDS-PAGE*). An antiserum was raised against the 36 kD polypeptide for possible use in distinguishing the good blanching and the poor blanching peanut samples from each other. Presently the modern techniques of immunological assays are sensitive, simple and would be appropriate for use in the rapid identification of poor blanching peanut samples. The objectives of this study were to investigate (1) the time-course of the synthesis of the 36 kD polypeptide during embryogenesis and its distribution within the seed, (2) the relationship between the amount of this presumptive marker

and blanchability index of different peanut samples and (3) the preparation of a monospecific antiserum for its detection using immunological assays.

Materials and Methods

Plant Materials

Seeds of 22 different peanut cultivars and breeding lines used in this study (Table 1) were from the 1983 crop grown at the Tidewater Research Center and Agriculture Experiment Station in Suffolk, Virginia.

Sample preparation

Protein extraction and test for blanchability were performed as described previously (6). The defatted meal was extracted overnight with 0.5 M NaCl solution containing 6M urea and 5% mercaptoethanal (2-ME) at a weight to volume ratio of 1:10 in the cold (4C).

Table 1. Immunological assessment of blanchability in peanut cultivars and breeding lines based on the correlation between their blanchability index, the presence and absence of the 36 kD polypeptide and the ELISA signal (absorbance at 450 nm).

Cultivar or breeding line	Blanchability (%)[a]	36 kD polypetide[b]	A450
NC 8C	87.6	—	0.14
Florigiant	84.3	—	0.06
VC 1	82.0	trace	0.54
NC-Fla 14	80.5	+	1.21
NC 9	75.8	trace	0.32
Keel 29	74.2	—	0.09
GK 3	74.1	—	0.08
NC 7	72.4	+	1.20
NC 2	72.4	—	0.12
Avoca 11	71.8	—	0.04
VA 81B	71.0	—	0.11
Shulamit	70.5	+	1.25
VA 56R	69.2	+	1.01
VA 72R	69.0	+	1.11
GA 119-20	68.8	+	1.10
VA 61R	67.5	+	1.10
VA Bunch 46-2	62.9	+	1.12
Early Bunch	61.8	+	0.93
NC 5	61.2	+	1.10
NC 17	57.3	+	1.20
NC 4	56.0	+	.89
NC 6	53.8	+	1.20

[a] The mean of 7 different analyses in duplicate using 250 grams each of peanuts white roasted, cooled and blanched for 3 min as described by Wright and Mozingo (1975).

[b] (+) present, (-) absent.

Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoretic analyses were performed according to the procedure of Laemmli (4) using 12% polyacrylamide gel slabs with a slight modification as reported before (6). The samples were prepared by mixing three parts of the protein extract with one part of the 4x concentrated SDS-sample buffer (0.125M Tris-HCi, pH 6.8 containing 10% SDS, 5% 2-ME, 40% glycerol and 0.05% bromophenol blue) and heating the mixture in a boiling water bath for two min before applying to the gel.

Antigen preparation and immunization

Antigen from a good blanching peanut (Florigiant)was prepared by extracting 20 mg of the meal with 1 mL of extraction buffer (0.1 m M Tris-HCl pH 7.5 containing 1.5 M NaCl), and later mixed with 0.32 mL of SDS-sample buffer. A volume of extract so prepared and containing 2 mg protein was used for the first injection and volumes containing 1 mg were used for subsequent injections. To obtain antiserum against the 36 kD polypeptide, it was isolated by preparative SDS-PAGE from the extract of a poor blanching peanut (Early Bunch). The 36 kD band was detected by staining the gel for 15 min, excised from the gel with a razor blade, destained in EtOH, ground into a fine paste in the presence of 0.2% SDS and 1% 2-ME and heated for 2 min in boiling water before use. The immunogens were mixed with an equal volume of Freund's Adjuvant (complete), and a total volume of 1.4 mL mixture was used for subcutaneous injection into New Zealand white rabbits. Incomplete adjuvant was used for subsequent immunizations at 2 weeks intervals, and a total of 5 immunizations were performed in 2-3 different sites each time. Prior to immunization, bleedings were taken to obtain preimmune serum.

Immunological Assays

Enzyme-Linked Immunosorbent Assay (ELISA) was performed as described by Conroy and Esen (3). A total of 100mL of the antigen (extracted with 0.1 M Tris-HCl, pH 7.5/1.5 M NaCl) containing 1 mg protein from different peanut cultivars and breeding lines was applied to the 96 well microtiter plate. Each of the antisera was titered in a checker board scheme where different dilutions of the antiserum (1:100 to 1:6400) reacted with different amounts of the homologous antigen. All assays were performed in duplicate or triplicate, and the reactivity was measured as the absorbance of the peroxidase reaction product at 450 nm in an automated microplate reader (Dynatech MR 600). The antigens were prepared and applied in different solvents for coating; some in its native state (in 0.1 M Tris-Hcl pH 7.5), and some in such denaturants as 8 M urea, 1% SDS (with or without 2-ME) and 5% acetic acid.

Preparation of monospecific antibody

Antibodies with multiple specificities were removed from the antiserum to the 36 kD polypeptide by incubating the antiserum with a nitrocellulose membrance (Millipore Triton-free nitrocellulose) saturated with the cross reacting proteins (10 mg/mL of protein extract) from a good blanching peanut sample. Adsorption was repeated 2-3 times in order to remove all the polyspecific antibodies. The antisera so obtained reacted only with the 36 kD polypeptide and thus was monospecific for it. This method of preparation of monospecific antiserum resulted in the loss of about 30% of the activity of the antiserum. However, this was an insignificant loss in view of the high initial titer of the antiserum.

Western blotting and treatment of immunoblots

The proteins from a good blanching and a poor blanching peanut were fractionated by preparative SDS-PAGE using a 12% acrylamide gels and then were elecroblotted onto a nitrocellulose membrane according to the method of Towbin *et al.* (7). Electroblotting was performed (4C) at 60 volts for 45 min and at 100 volts for 1.5 hr. The blots were rinsed with three washes of PBST (phosphate buffer saline containing 0.05 (v/v)% Tween 20), dried at room temperature and cut into 5 mm strips. The strips were individually reacted with antisera in a reaction trough, and protein A peroxidase conjugate was used as the secondary antibody. The immunoreactive polypeptides were detected by incubating the blot strips in peroxidase substrate (4 chloro 1-naphthol in PBS containing 15% MeOH). In each case a strip was stained in a 1μL/mL dilution of india ink in PBS for the visualization of the total protein pattern.

Tissue-printing experiments

The seeds were dissected in various planes and the cut surface of the sections were blotted passively onto a wet nitrocellulose paper for 1-2 hr as described by Cassab and Varner (2). The blots were then rinsed several times with PBST to wash off loosely bound proteins and residue. Later, these blots were reacted with antisera like the Western blots to detect the distribution of the 36 kD protein in the longitudinal and cross sections of the seeds.

Seed development studies

Developing seeds were harvested from both the good blanching and poor blanching peanuts (Florigiant and Early Bunch) for isolation of the total protein extract. Based on size and stage of maturity the developing seeds were grouped into five different stages of development (immature to fully mature stage) as was reported by Basha *et al.* (1) and the polypeptide profile in the two cultivars was studied using SDS-PAGE and immunoblotting.

Results and Discussion

Electrophoretic studies

Electrophoretic studies using a large number of samples from various cultivars and breeding lines showed that the amount of 36 kD polypeptide vaired among the samples which possessed this polypeptide. The extent of variation of the 36 kD band in the seed of poor blanching cultivars is shown in Fig. 1a (arrow). Although this polypeptide occurred in all poor blanching cultivars there did not seem to be high correlation (r=0.53) between the blanchability index and the quantity of this polypeptide based on more than 22 samples included in the study (Table 1). In other words, the 36 kD polypeptide may not be directly responsible for the tight adherence of the seed coat (skin) to the cotyledon. Prolonging the electrophoresis time gave a better resolution of the

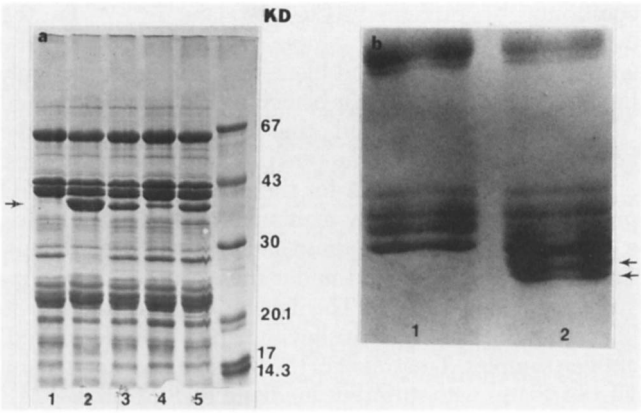

Fig. 1. (a). SDS-PAGE profiles of the proteins of peanut seed meals to show the variation in the quantity of the 36 kD marker protein band. Lane 1, Florigiant, a good blanching peanut in which 36 kD is completely absent; 2, NC-Fla 14, 3; Early Bunch, 4, NC9 5, NC7 and 6, MW marker.

(b). Prolonged SDS-PAGE of the good blanching and poor blanching peanuts samples. Note the 36 kD band is split into two bands indicating that it is actually composed of two separate polypeptides.

polypeptides in the 35-40 kD region and resulted in the splitting of the 36 kD polypeptide into two distinct bands (Fig 1b, lane 2 arrows). Thus it should be determined whether both or only one of these two bands are really involved in blanchability. Studies on the protein extract of the peanut skin (using SDS sample buffer as solvent) showed that the 36 kD protein was also present in the skin of the poor blanching samples, but at much lower concentration (results not shown).

Two different sets of experiments confirmed that the 36 kD polypeptide was a subunit of arachin and not that of conarachin. In one experiment the arachin fraction was precipitated by the addition of $(NH_4)_2 SO_4$ to the whole meal extract to 45% final concentration, then solubilized in sample buffer (in the presence of urea and 2-ME) and subjected to SDS-PAGE. In a second experiment, the arachin band was excised from a native preparative polyacrylamide gel (7%), cut into 5-10 mm rectangles, incubated in the SDS-sample buffer, and then applied directly to the sample well. The results (Fig. 2, lanes 3 and 4) show that the 36 kD polypeptide is a subunit of the arachin. This procedure of using gel peices as sample is preferred because it allows one to determine accurately any polypeptide subunits released upon the dissociation of a native protein after exposure to SDS and a reducing agent such as 2-ME or any other protein denaturant.

Reactivity of anti-36 kD serum with peanut proteins

Anti-36 kD serum showed strong reactivity with the whole protein extract of peanut when measured by ELISA even after the first immunization. Repeated injections of the immunogen resulted in increased antibody titer such that an antiserum dilution of 1/6400 yielded positive reaction. However, the antiserum was not monospecific for the poor blanching peanut samples because there was positive reaction with the good blanching peanut samples equal to about 30% that of the poor blanching ones. The adsorption of the antiserum by the good blanching peanut protein extract rendered the antiserum monospecific for the 36 kD polypeptide. The results (Table 1 and Fig. 3), show the

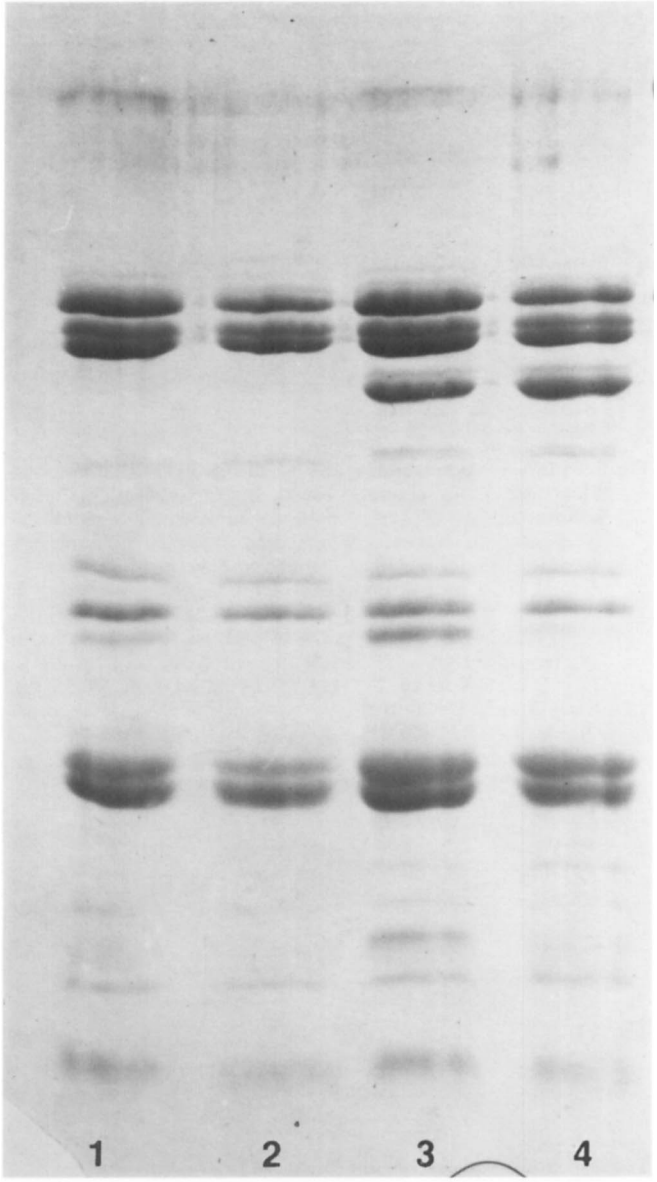

Fig. 2. SDS-PAGE of arachin from a good blanching (Florigiant) and a poor blanching (Early Bunch) samples of peanut in which gel pieces from the presumed arachin bands from a native gel were used as sample protein. Lanes 1 and 2 a good blanching sample; 3 and 4, a poor blanching sample. Lane 1 and 3 were obtained from native gel not exposed to 2-ME, while 2 and 4 are the ones treated with 2-ME during PAGE. The results indicate the marker protein (36 kD) is a subunit of the arachin fraction.

adsorbed antiserum detects specifically the 36 kD protein present in poor blanching samples, indicating the effectiveness of the adsorption in removing non-specific antibodies.

Reactivity of the 36 kD antiserum with the proteins of the good blanching and poor blanching peanuts was also monitored on immunoblots. The results show the reaction of this antibody with numerous polypeptide bands in both samples (Fig. 4a, lanes 3,4). However, when the monospecific antiserum was employed, the 36 kD band was the only one detected by the antiserum (Fig. 4a, lane 6). The presence of a small cross-reacting band in the good blanching sample which persists to react with the monospecific antibody

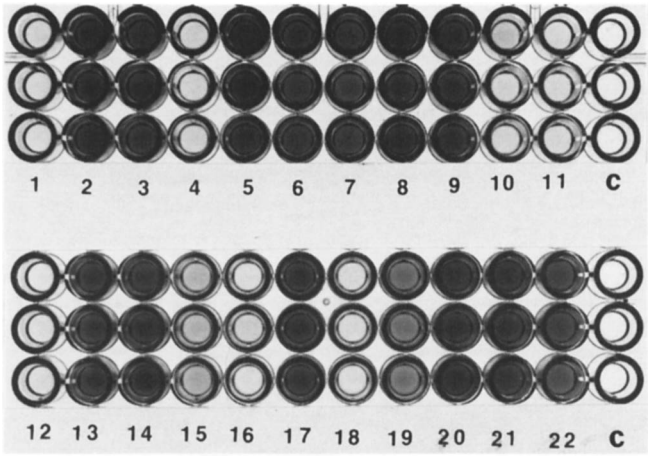

Fig. 3. ELISA reactions showing the use of anti 36 kD serum for the identification of peanut proteins extracted under similar conditions from different cultivars and breeding lines. All samples are in triplicate. No 1 is a good blanching peanut. Nos 2 and 3 are poor blanching peanuts, contain the 36 kD protein. A total of 22 peanut samples in triplicate are presented. 1, Florigiant; 2, VA Bunch 46-2; 3, NC 5; 4, NC 2; 5, NC 6; 6, VA 56 R; 7, NC 4; 8, VA 61R; 9, GA 119-20; 10, NC 8C; 11, GK 3; 12, Avoca 11; 13, VA 72R; 14, NC-Fla 14; 15, NC 9; 16; VA 81B; 17, NC 7; 18, Keel 29; 19, VC 1; 20, Shulamit 6; 21, NC 17; 22, Early Bunch. C=Control

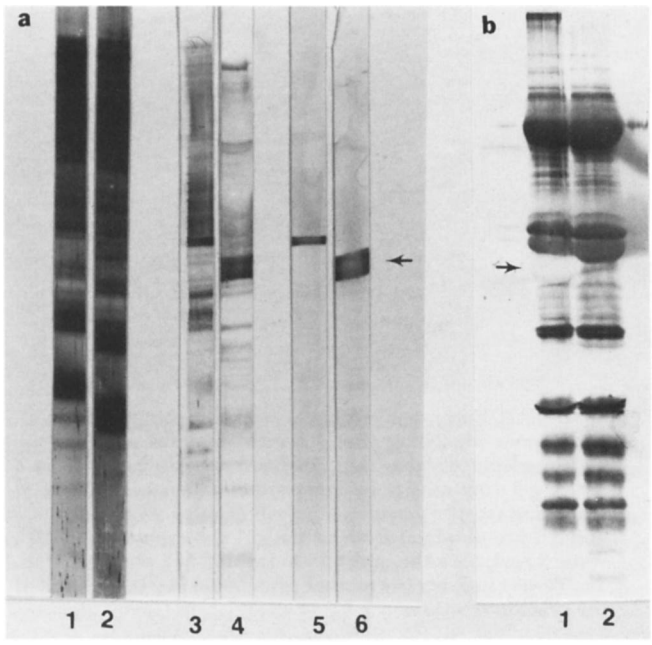

Fig. 4. a) Immunoblots of good blanching and poor blanching samples (Florigiant and Early Bunch), probed using antisera against the 36 kD polypeptide. Lanes 1 & 2, stained with India ink to visualize the general protein file. Lanes 3 & 4, probed with unadsorbed antiserum. Note there is high degree of cross reactivity, but the 36 kD band is still very well recognizable. Lanes 5 & 6 are the blots treated in the presence of the monospecific antibody which only reacts with the 36 kD marker protein. For the presence of the single cross-reacting band (minute in size) in the good blanching sample We offer no explanation.

b) Interaction of antisera raised against the good blanching peanut protein with the poor blanching and good blanching immunoblots. Note the 36kD region in the poor blanching sample (Lane 1 arrow) is not stained and remains blank.

could not be explained (Fig. 4a, lane 5). When the immunoblots of the two samples were reacted with the antiserum against the good blanching peanut extract only the 36 KD band in the poor blanching blot was not stained (Fig. 4b, lane 1 arrow), and otherwise the two blots of the good and poor blanching samples produced similar profiles.

Optimization of ELISA for the detection of the 36 kD protein was carried out by manipulating several variables, e.g., antigen extraction medium and incubation conditions. The ELISA data obtained under these various conditions are presented in Table II. The data indicate that the anti 36 kD serum does not react well with native (non-denatured) antigen samples. Treatment of the bound native antigen (on ELISA Plate) with different media (e.g., 8 M urea, 2-ME and 5% acetic acid) also did not result in an increase of its reactivity with the antiserum. It is conceivable that the native protein did not bind well to the surface of the wells of the microtiter plates since the amount of the antigen bound to the plates is dependent upon the physical state of the antigen and/or the duration, pH, and temperature of the incubation. However, the almost complete absence of any reactivity (ELISA signal) when undenatured samples were used cannot be attributed to lack of any antigen binding to the plate; it should rather be attributed to the unavailability of the epitope for antibody binding under native state. Addition of urea (8M) and acetic acid (5%) to this native antigen prior to its application to the ELISA plates resulted in a rise in the reactivity of the antigen with the antiserum (Table II). Also, the post-coating treatment of the bound antigen on the ELISA plate with various denaturants would affect the extent of the antigen-antibody interaction significantly. For example in the 8M urea coated samples, reactivity enhances 12% after 2 hrs incubation in Tris-HCl while it drops 50% when 2-ME or SDS is added during the incubation period. Changes in the ELISA response of the coated antigen prepared in a 5% Acetic acid were also quite significant (Table II). In this case incubation in 8M urea and Tris-HCl results in a 50 to 60% increase in its reactivity. This increased reactivity is abolished by the addition of 2-ME to the medium. Addition of SDS and 2-ME together to the incubation medium results in an additional 80% decrease in the antigen reactivity, apparently due to the washing off or removal of the plate-bound antigens by SDS.

It was also noted that the dilution of the antibody with a 2 M solution of urea (instead of PBST) caused a 30 to 40% decrease in its reactivity. When the urea concentration was raised to 4, 6, and 8 molar the reactivity of the anitbody was reduced to almost 80% of the control. This observaion indicates the sensitivity of the antibody to denaturation

Table 2. The effect of the composition of the antigen extraction and incubation medium on the ELISA signal (absorbance at 450) from the 36 kD polypeptide (values are the average of 3 replicates).

Post-coating	ELISA signal (A450) at various extraction medium		
incubation medium	Tris-HCl-NaCl	8M. Urea	Acetic Acid (5%)
None	0.02 ± .01	0.75 ± .02	0.50 ± .08
Tris-HCl	0.04 ± .02	0.90 ± .01	0.84 ± .02
8M urea	0.02 ± .03	0.75 ± .01	0.73 ± .02
8M urea + .1% 2-ME	0.01 ± .02	0.49 ± .07	0.50 ± .07
1% SDS + 1% 2-ME	0.01 ± .01	0.51 ± .00	0.13 ± .04
Acetic Acid (5%)	0.01 ± .02	0.72 ± .01	0.54 ± .04

when it is exposed to high urea concentrations.

Several attempts were made to detect the reaction of the antiserum and its antigen using the Ouchterlony double immunodiffusion technique. However, no antigen-antibody precipitine reaction was observed. In view of the strong positive ELISA tests mentioned above it suggests that lack of precipitate formation in this case must be due to the poor diffusion of the 36 kD polypeptide in the agar medium.

Time course studies and Tissue printing (Passive blotting)

The results obtained from the SDS-PAGE analysis of seeds at various stages of development shown if Fig. 5 indicate that the 36 kD band is present from the earliest stages in the poor blanching peanut and is absent in all stages in the good blanching samples. It was hypothesized that excretion of this marker protein from the surface layer cells of the mature poor blanching cotyledon causes the adhesion of the skin to the seed. The results from tissue printing also showed there is a uniform distribution of the 36 kD protein in the peanut cotyledon. Thus it is not limited only to the surface of the seed, may not be directly involved in the adhesion of the skin to the seed and must play, if any, an indirect role in the blanchability.

Summary and Conclusion

Studies were carried out to investigate the utility of immunological assays for use in blanchability assessment and the possible role of the putative 36 kD marker polypeptide on peanut blanchability. The data did show the possibility of the use of immunological assays, but not the direct mode of involvement of this polypeptide in blanchability trait of peanuts. The electrophoretic data revealed that the 36 kD protein actually is not a single polypeptide but made of two distinct subunits (Fig. 1b arrow). Isoelectric focusing (IEF) studies also showed that the 36 kD polypeptide to be composed of a double band (Shokraii et al., 1985). It is possible that only one of these two bands is responsible for blanchability and this explains the lack of high correlation between the quantity of this polypeptide band and the blanchability index. Futute studies must then focus on the complete separation and immunological assay of each individual subunit of the 36 kD polypeptide.

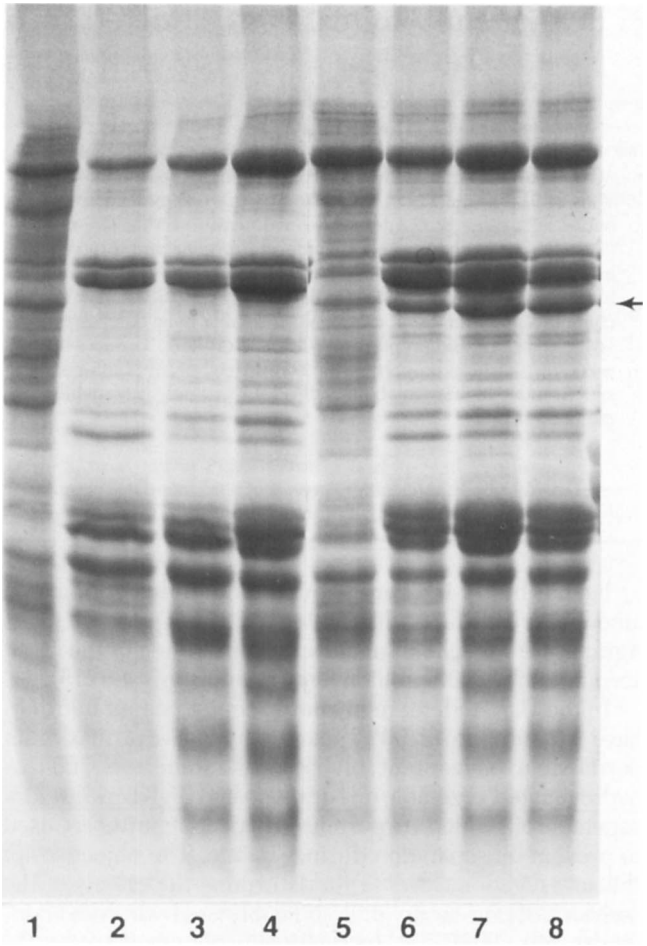

Fig. 5. Time course studies of seed development in peanuts. SDS-PAGE prepared from a good blanching and a poor blanching peanuts (Florigiant and Early Bunch) at different stages of seed development. Lanes 1, 2, 3, and 4 belongs to Florigiant (good blanching sample). Lanes 5, 6, 7, and 8 are from Early Bunch (poor blanching). Note, that the 36 kD band (arrow) is present in the poor blanching sample from the early stage of embryogenesis.

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