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Identification of Cultivar Differences in Seed Polypeptide Composition of Peanuts (*Arachis hypogaea* L.) by Two-Dimensional Polyacrylamide Gel Electrophoresis[1]

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ABSTRACT

Seed polypeptides from several cultivars of peanut (*Arachis hypogaea* L.) have been compared by means of a two-dimensional polyacrylamide gel electrophoresis. Protein was extracted from the defatted peanut meal by homogenizing in 5 millimolar K_2CO_3-9.5 molar urea. After addition of Nonidet P-40 (2%, v/v) and dithiothreitol (0.5%, w/v) the solution was centrifuged at 25,000*g*. This procedure led to solubilization of more than 95% of the total protein. The clear supernatant fraction was then subjected to two-dimensional polyacrylamide gel electrophoresis, employing isoelectric focusing in the first dimension and electrophoresis in presence of sodium dodecyl sulfate in the second. After examining several cultivars, it was possible to construct a composite map to include all of the polypeptide species found among all of the cultivars examined. At least 74 major and between 100 and 125 minor components were detectable by Coomassie blue staining. The majority of these had isoelectric points between pH 4.4 and 8.0, and molecular weights between 16,000 and 75,000. Several different cultivars have been compared using this method and it has been shown that considerable variation exists among the major polypeptides present. The method should prove valuable for analyzing different genotypes and selecting varieties with a particular storage protein make-up, as well as for following compositional changes that occur during seed development and germination.

The peanut (*Arachis hypogaea* L.) is an economically important source of vegetable protein, but is relatively low in its content of methionine, lysine and tryptophan (1, 11). There are a few reports suggesting that certain genotypes exist within the species that contain a better balance of nutritionally important amino acids than found in commercial cultivars (1, 3, 11).

Current methods for screening variants for differences in protein composition are generally tedious and often inaccurate. For example, one-dimensional polyacrylamide gel electrophoresis, although widely employed, resolves only a limited number of polypeptides (4, 8, 10, 11, 19). In the peanut, the proteins are very poorly defined and are contained mainly in two major globulin fractions, arachin and conarachin, which together constitute 97% of the protein of the meal. Each of these globulins consists of several polypeptide species, which are thought to exist together as subunits of large multimeric complexes. In addition, many of the polypeptides appear to have similar molecular weights (2, 20). In this study I have characterized the proteins of peanut meal using a two-dimensional electrophoretic technique which separates the polypeptides according to their isoelectric point in the first dimension and by subunit size in the second. The method has been employed to distinguish different cultivars according to their polypeptide composition. It also allows application of up to 500 μg of protein on a single slab gel, thus providing the potential for purifying significant quantities of individual polypeptide components.

MATERIALS AND METHODS

SEED MATERIAL

Seeds from the following peanut (*A. hypogaea* L.) cultivars were provided by A. J. Norden of the University of Florida from their breeding lines: Florunner, Florigiant, Early Bunch, Jenkins Jumbo Runner, Altika, UF 75102, UF 77318, 439-16-10-3, 439-16-10-3-1, 439-16-10-3-2, and 439-16-10-1-1. After removal of skins and embryonic axes, the cotyledons were ground in a mortar with a pestle. The ground material was then defatted with cold ethyl ether by repeated extractions until the peanut meals were essentially fat-free. The defatted meals were stored at −20 C.

CHEMICALS

SDS (specially pure) was from British Drug House, Poole, UK.; ampholines were purchased from LKB, Uppsala, Sweden; urea was bought from Pierce Chemical Co., Rockford, Ill., Nonidet P-40 was obtained from Particle Data Laboratories, Elmhurst, Ill. Coomassie brilliant blue R-250 and N,N'diallyltartardiamide (DATD) were products of Bio-Rad Laboratories, Richmond, Calif. Protein standards and agarose were from Sigma Chemical Co. Reagents for polyacrylamide gel electrophoresis were products of Eastman Kodak Co.

PROTEIN EXTRACTION

About 200 mg of defatted meal was suspended in 10 ml of 9.5 M urea and 5 mM K_2CO_3 and then homogenized on an ice bath using a Polytron homogenizer. The homogenate was then made to 2% (v/v) with Nonidet P-40 and 0.5% (w/v) with DTT and centrifuged at 25,000*g* for 30 min at 18 C. The clear supernatant fraction was decanted, assayed for protein, and used for further analysis. Alternatively, peanut meals were extracted with 20 mM Tris-HCl (pH 8.2) buffer containing 1 M NaCl as described earlier (2).

ONE-DIMENSIONAL DISC GEL ELECTROPHORESIS

Proteins extracted with 1 M NaCl were diluted and then electrophoresed in 10% (w/v) polyacrylamide tube gels (0.5 × 6 cm) both under nondenaturing (5) and denaturing conditions (16).

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TWO-DIMENSIONAL GEL ELECTROPHORESIS

This procedure was based on that of Horst *et al.* (12) which is a modification of the original method of O'Farrell (18).

Isoelectric Focusing. The IEF[2] gel (0.3 × 11 cm) was prepared by mixing the following solutions: 1.33 ml acrylamide stock solution (31.96% [w/v] acrylamide, 5.64% [w/v] N,N'diallyltartardiamide), 5.5 g urea, 2 ml 10% (v/v) Nonidet P-40, 0.5 ml ampholine mixture (0.25 ml pH 3.5–10 ampholines, 0.18 ml pH 5–7 ampholines, and 0.08 ml pH 9–11 ampholines), 0.30 ml riboflavin TEMED mixture (4 mg riboflavin, 0.8 ml N,N,N',N'-tetramethylethylenediamine in 100 ml water), and 5 μl of ammonium persulfate (0.24 g/ml) in a total volume of 10 ml. About 200 to 400 μg of protein was applied on each gel and focused for 16 to 18 hr toward anode. A blank gel without any sample was also included in each run for subsequent determination of the pH gradient. At the end of run, the gels were removed from the tubes and equilibrated for 10 min with a buffer containing 65 mM Tris-HCl (pH 6.9), 1% (v/v) 2-mercaptoethanol, and 1% (w/v) SDS. After equilibration, the gels were drained and either subjected to electrophoresis in the second dimension directly or stored frozen.

SDS Gel Electrophoresis. The equilibriated IEF gels were electrophoresed in the second dimension in 10% acrylamide slabs (13 × 0.15 × 15 cm) prepared by following the procedure of Laemmli (16). The IEF gel was sealed with a solution of 1% (w/v) agarose, containing 1% SDS, and 1% 2-mercaptoethanol. After electrophoresis, the proteins were fixed in acetic acid-ethanol-water (7:40:53, v/v) and stained with Coomassie blue R-250. The slab gel was calibrated using proteins of known mol wt.

RESULTS

PROTEIN EXTRACTION

Extraction of defatted peanut meal with a buffer containing urea, K_2CO_3, Nonidet P-40, and DTT resulted in almost complete solubilization of the tissue proteins. Thus reextraction of the fibrous residue with hot 1 N NaOH yielded only trace amounts of protein, indicating that the initial extraction had effectively removed over 95% of the original tissue proteins. Unlike the extracts prepared by other methods (2, 6, 17, 22), the proteins solubilized by our method remained in solution even after repeated freezing and thawing thus allowing consistent electrophoretic patterns to be obtained over periods of several weeks. Prolonged storage in urea solution, particularly at room temperature, was avoided however, since this can give rise to multiple spots in the IEF dimension due to carbamylation of the proteins (12, 18).

ONE-DIMENSIONAL DISC GEL ELECTROPHORESIS (1-D PAGE)

To illustrate the value of the two-dimensional electrophoretic technique, peanut proteins were first analyzed by single dimensional procedures. Electrophoresis of peanut proteins from different cultivars under nondenaturing conditions (Fig. 1A) revealed no major differences in composition. Most of the proteins migrated only into the top half of the gel as two major bands. These may correspond with the arachin monomer and dimer, as noted by others (2, 6, 17, 22). However, when these preparations were dissociated using DTT and SDS, and later subjected to electrophoresis under denaturing conditions, the resolution of polypeptides improved considerably. As seen in Figure 1B at least six major and eight minor bands were seen on each gel. Although this method gave better resolution than the nondenaturing gel system, individual polypeptides with mol wt around 40,000, 23,000, and 18,000 (arrowed) were still poorly resolved.

[2] Abbreviations: *g*: gravitational force determined at R_{av}; IEF: isoelectric focusing; 2-D PAGE: two-dimensional polyacrylamide gel electrophoresis.

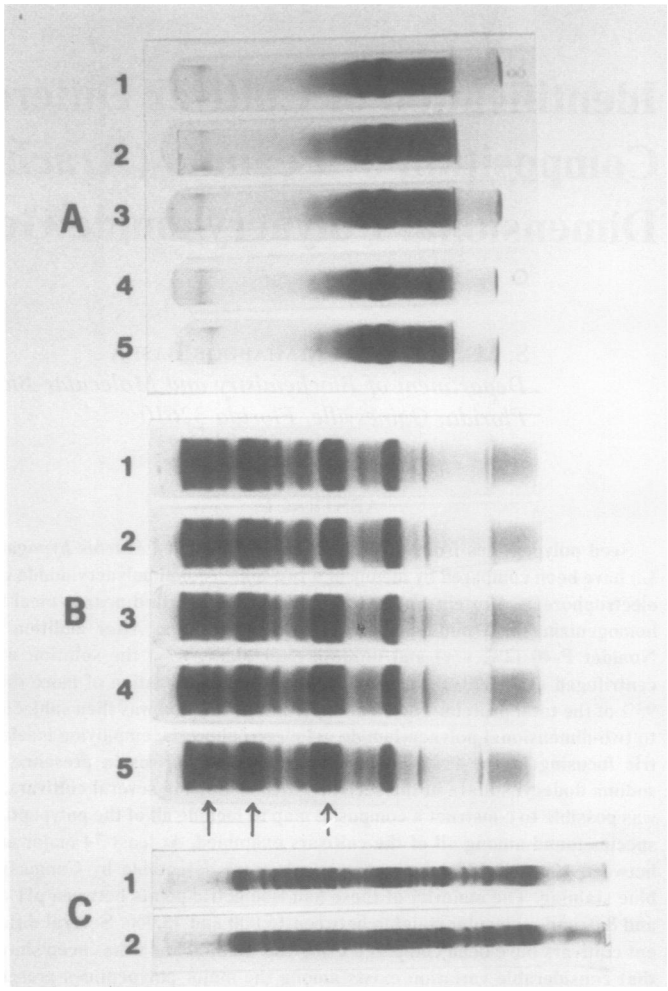

FIG. 1. One-dimensional polyacrylamide gel electrophoresis of seed proteins from different peanut cultivars: A: nondenaturing gels; B: SDS gels; C: IEF. 1: Florigiant; 2: Florunner; 3: 439-16-10-1-1; 4: Jenkins Jumbo; 5: Early Bunch.

TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS (2-D PAGE)

IEF. Isoelectric focusing of peanut proteins gave a dramatic improvement in polypeptide resolution (Fig. 1C), revealing as many as 40 to 50 distinct polypeptide bands. This is to be expected, since IEF is capable of separating proteins which differ only in a single charge. Satisfactory resolution was obtained even when up to 500 μg of protein was applied per 3-mm diameter gel. If higher amounts are to be analyzed, the diameter of the gel can be increased proportionately. IEF patterns of proteins were always reproducible, provided the ampholine ratios and duration of focusing were kept constant.

SDS-PAGE. The resolution of proteins was further improved when the IEF gels were subjected to electrophoresis in the second dimension in presence of SDS. On a single such slab gel at least 150 to 200 polypeptide components were detected by Coomassie blue staining. Moreover, the polypeptides (mol wt 40,000, 23,000, and 18,000) which showed only one to two bands on 1-D SDS gel were resolved into several components by the two-dimensional procedure. For example, the components in the quadrants G, H, N, P, Q, R, S, T, X, and Y (Fig. 2) gave a single broad band on the one-dimensional SDS gel (Fig. 1B) because of their similar mol wt. However, on the two-dimensional slab gels these components were resolved into five to 20 individual spots.

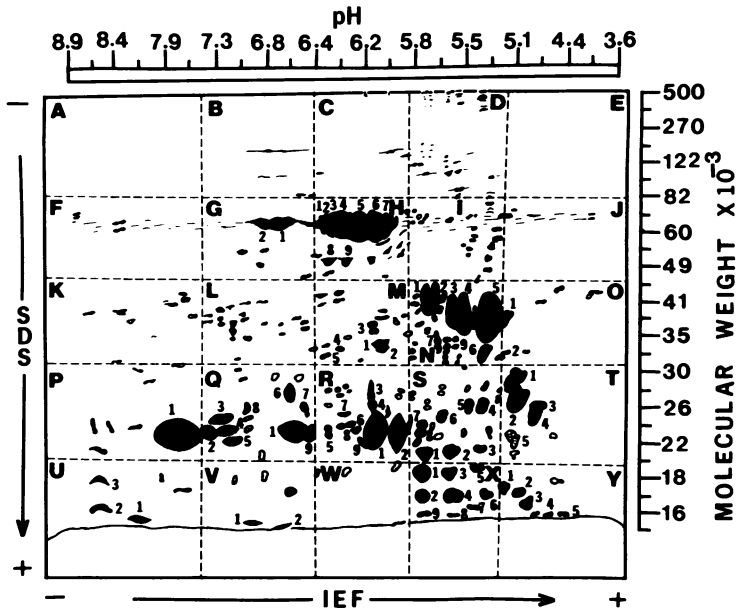

FIG. 2. Composite two-dimensional map showing positions of all polypeptides that have been detected in the 11 different peanut cultivars examined in this study.

COMPOSITE TWO-DIMENSIONAL POLYPEPTIDE MAP

Examination of several peanut cultivars revealed the existence of wide variability in their polypeptide compositions. There was a need to construct a composite polypeptide map to include all of the polypeptides found in all of the cultivars examined, to serve as a basis for the comparison and eventual classification of cultivars. After examining 11 cultivars, a composite polypeptide "map" has been constructed (Fig. 2). These studies have shown that it is possible to distinguish at least 74 major and about 100 to 126 minor polypeptide components as distinguished by isoelectric point (pI) and mol wt. The majority of the proteins focused between pH 4.4 and 8.0 and had mol wt between 16,000 and 75,000. For easy identification, the polypeptide map is divided into alphabetically labeled quadrants. Polypeptide spots within each quadrant are designated by a number. Vertical lines are based on approximate pH values and horizontal lines are used to indicate apparent mol wt. Because the isoelectric points of proteins in urea are not believed to be accurate (12, 18), I have avoided assigning individual polypeptides specific isoelectric points. Instead, I have designated each spot by a specific coordinate based on their position on the composite map. In order to resolve any low mol wt (<16,000) polypeptides that might have run with the dye front on 10% gels I have also employed a second dimension gel containing 15% (w/v) acrylamide. Since this gel system did not reveal any new polypeptides, I now routinely employ the 10% system. Except for the polypeptides in "H" quadrant, all other major components can be resolved into distinct, nonoverlapping spots (Fig. 2). The latter group of polypeptides may represent either a group of distinct polypeptides which have closely similar isoelectric points and mol wt, or, more probably, slight charge variants of a single protein species. On single dimensional SDS gels, these polypeptides gave a single band (Fig. 1B).

TWO-DIMENSIONAL POLYPEPTIDE MAPS OF DIFFERENT PEANUT CULTIVARS

Comparisons of two-dimensional gels prepared from different cultivars have revealed interesting differences in their polypeptide compositions (Fig. 3). For example, cultivar Florigiant did not contain polypeptide spots R1 and R2 while Jenkins Jumbo and Altika accumulated both of these components in relatively similar amounts. Similarly varying amounts of protein were found in R1 and R2 spots among the other cultivars. Other significant differences were noted in spots N1, N2, N3, N4, and N5. Thus Florigiant and UF 75102 did not contain N1, N3, and N5, while Florunner and 439-16-10-3 contained relatively smaller amounts of N5, but lacked N1 and N3. Similarly Altika and Early Bunch contained N2, N3, N4, and N5 but not N1. In contrast Jenkins Jumbo possessed all five components. Figure 4 shows a close up of two regions (mol wt, 40,000; 20,000) on the 2-D map which show major differences among the cultivars in their polypeptide composition. Other differences are also seen among the cultivars in spots R3, S6, T2, T4, and Y2.

DISCUSSION

In this paper I have presented a method which allows the complete extraction of peanut meal proteins without resorting to the use of ionic detergents, and a procedure for the subsequent two-dimensional electrophoretic analysis of these proteins. Previous workers have usually solubilized peanut proteins from full-fat or fat-free meal with either water, buffers, or salt solutions (2, 7, 9, 13, 17, 22). In many cases, it appears that the protein extracts prepared by such procedures were not uniform from experiment to experiment. Thus, considerable amounts of protein precipitate out of solution during storage and freeze-thawing. In part this may be due to the formation of multimeric complexes or disulfide bond interchange (14, 15, 23). Variable amounts of low mol wt components have also been noted (3, 8), possibly the result of proteolysis. In the work presented here, homogenization of the peanut meal in presence of alkaline urea solution, and the subsequent addition of non-ionic detergent and DTT led to almost complete solubilization of all the proteins. All of this material remained in solution during subsequent storage. In part, this was undoubtedly due to the efficient dissociation of protein complexes into subunits. Because solubilization was accomplished without resorting to ionic detergents, the polypeptides could be separated subsequently according to their native charge. IEF was accomplished by a modification of the method of O'Farrell (18), the main difference being the replacement of bisacrylamide cross-linker by N,N'-diallyltartardiamide, which provides a larger pore size in the gel matrix (24).

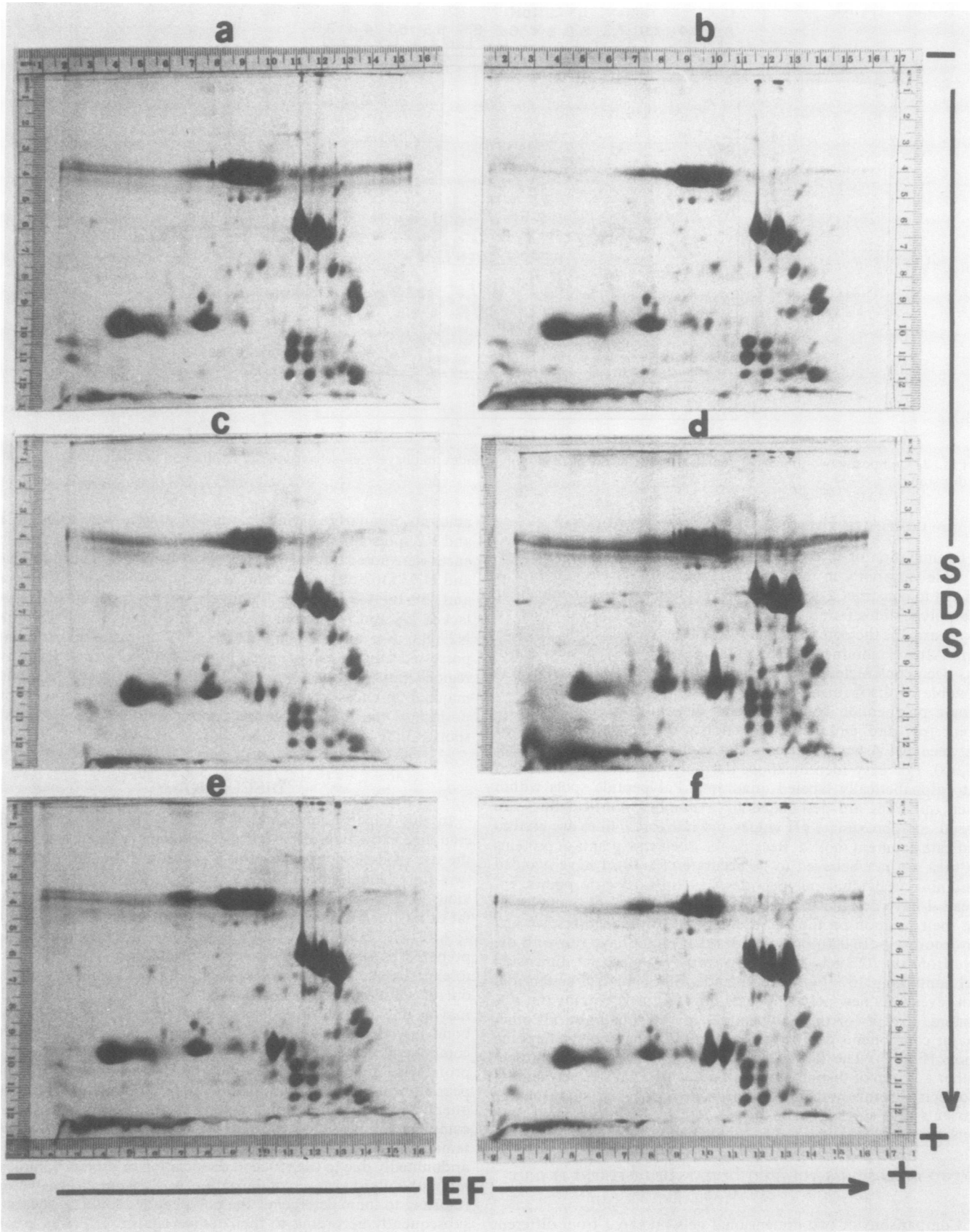

FIG. 3. Two-dimensional gel electrophoresis of the polypeptides in six peanut cultivars: a: Florigiant; b: Florunner; c: 439-16-10-3; d: 439-16-10-1-1; e: Early Bunch; f: Jenkins jumbo runner.

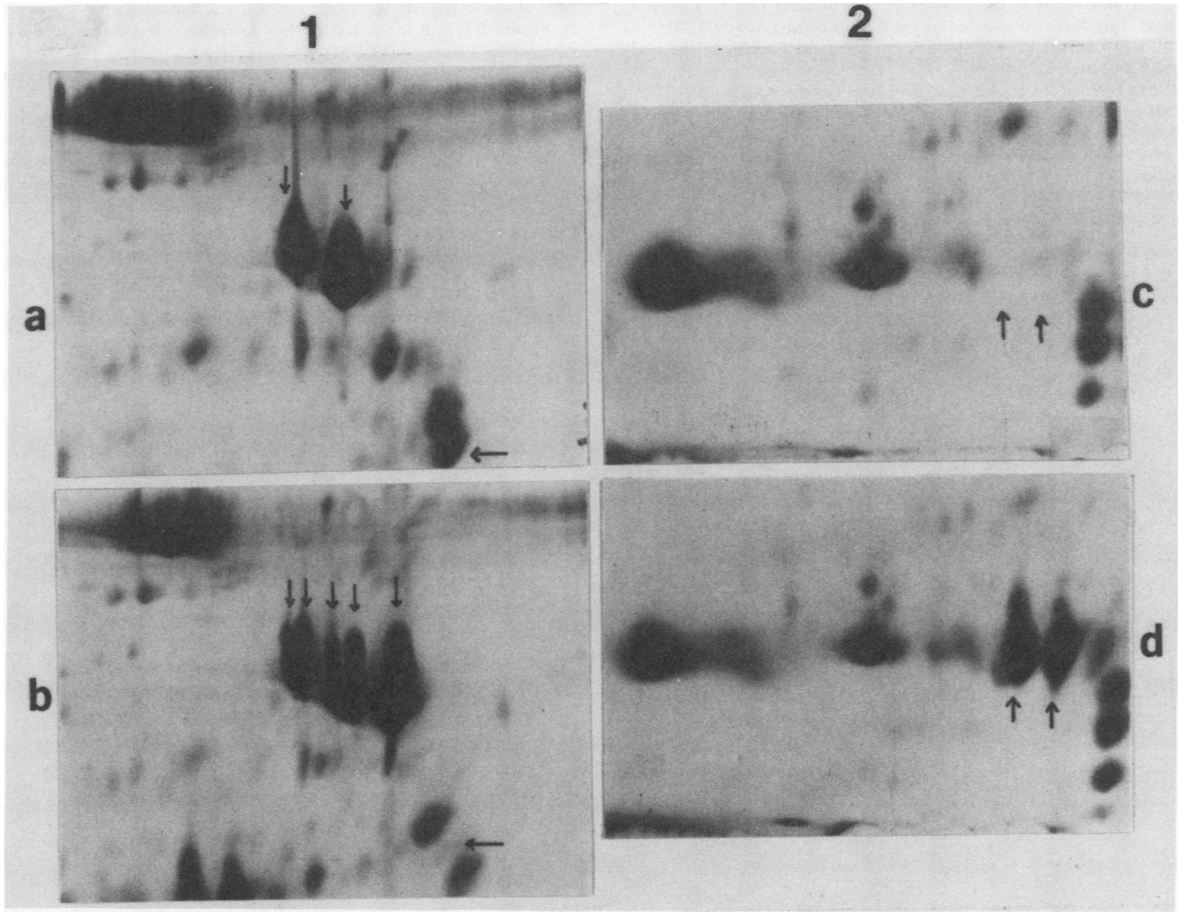

FIG. 4. Two regions of the two-dimensional polyacrylamide gel showing variations in polypeptide composition among peanut cultivars. Regions shown correspond to quadrants 1. 'N' and 'T'; 2. 'R' and 'S'; a: UF 75102; b: Jenkins Jumbo; c: Florigiant; d: Altika. The arrows indicate the polypeptides missing in 'a' and 'c' which are present in 'b' and 'd'.

While one-dimensional SDS electrophoresis revealed about 14 components, the two-dimensional procedure resolved at least 150 to 200 polypeptide spots. Moreover, I was able to analyze up to 500 μg of crude protein on a single analytical slab. Clearly the method has potential for the preparation of homogeneous polypeptide species which can then be analyzed for amino acid composition or used as antigens to prepare monospecific antibodies (21). O'Farrell, using a similar technique in association with autoradiography, was able to identify at least 1100 polypeptides in extracts from *Escherichia coli* (18). The potential exists for separating proteins distinguished by only a single charge difference. It is likely that some rows of proteins noted on the gels represent only minor variations of a single polypeptide type. I do not believe that these horizontal arrays of spots are artifacts resulting from charge modifications during and after extraction, since: (*a*) they were consistent from experiment to experiment; (*b*) the changes were not progressive upon storage of the sample; and (*c*) different cultivars showed distinct and consistent differences in many of these protein spots. Clearly, the two-dimensional technique could provide the plant breeder with an important tool for analyzing different genotypes and for selecting varieties with a particular storage protein make-up. The method should also prove valuable for studying other seed legume proteins and in following compositional changes during seed processing, storage, and germination.

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