Electrophoretic Methods for Detecting Differences in Seed Proteins of Soybean Varieties and Induced Mutants

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With the aim of extending the genetic identification of soybean varieties and mutants, gradient polyacrylamide gel electrophoresis has been employed to detect differences in proteins extracted from seeds of the varieties Lee, Pickett, and Harosoy, and three radiation-induced morphological mutants of Harosoy. Of eight solvents compared, a Tris-glycinate buffer (pH 8.6), with or without 2-mercaptoethanol, extracted the most protein from seed-meals. Sonication extracted more protein into a given solvent than did agitation. Electrophoretic banding patterns of the extracted proteins of a given variety or mutant were qualitatively the same (given bands migrated to the same positions) regardless of solvent or solution method employed, although different relative intensities of bands were observed for the same sample in different solvents. By electrophoresis of the extracted seed proteins on gradient polyacrylamide slab gels, we detected previously unreported differences between Lee and Pickett varieties and discovered that the patterns of two of the mutants were similar to each other but different from that of the other mutant, which was similar to the pattern of the parent Harosoy variety. When the extracts were treated with sodium dodecyl sulfate and 2-mercaptoethanol before electrophoresis on gels containing sodium dodecyl sulfate (1) there were differences in the major proteins or protein subunits of the two mutants that had similar patterns on gradient gels, and (2) the remaining mutant and the three natural varieties shared the same proteins, differing only in the relative amounts present in each.

The increase in genetic knowledge of soybean seed proteins has not kept pace with the chemical and physical knowledge gained in recent years (see, *e.g.*, Catsimpoolas *et al.*, 1971; Eldridge *et al.*, 1970; Koshiyama, 1972; Wolf, 1970, 1972). A rapid method of detecting differences in protein components would be advantageous for the purpose of genetic analysis, which involves mass screening. This report has two purposes: (1) to compare various methods of preparing samples of soybean seed protein for electrophoretic analysis and (2) to describe differences detected among soybean varieties and induced mutants through gradient polyacrylamide gel electrophoresis of their seed proteins. We also report electrophoretic differences on gels containing sodium dodecyl sulfate (SDS) after pretreatment of the extracts with SDS and 2-mercaptoethanol (ME). A previous electrophoretic analysis of soybean proteins showed that 61 soybean varieties could be separated into two major groups on the basis of a difference in only two proteins, component "A" being present in 13 varieties and "B" in 48 (Larsen, 1967).

Three varieties of soybeans, Lee (L), Pickett (P), and Harosoy (H), were chosen for comparison in the present study, because the last (H) was reported to have the "A" component and the first two (L, P) the "B" component (Larsen, 1967). We wished to see whether our methods could distinguish between the Lee and Pickett varieties. Fourth-generation mutants derived from Harosoy seed exposed to 18 krads of γ-rays were chosen on the basis of differences from the parent Harosoy variety in pod color and pubescence characters (mutants H1 and H3) or in time of seed maturation (mutant H2). The aim was to discover whether these visible characters were correlated with differences in seed protein components.

EXPERIMENTAL SECTION

Seeds used in this study were harvested in 1971 at the University of Tennessee–Atomic Energy Commission Agricultural Research Laboratory and were stored in a room with a constant temperature of about 50°F until use. The seeds had an approximately 8% moisture content and contained the following percentage of nitrogen, as judged by Kjeldahl analysis: L, 6.1%; P, 6.5%; H, 5.8%; H1, 6.8%; H2, 6.9%; H3, 6.8%.

Whole seeds of the varieties and mutants were ground for 20 sec in a Mitey-Mill electric food grinder (STURDEE Health Products, Island Park, N. Y.). Each resulting meal was stirred thoroughly, so as to be as homogeneous as possible. Sixteen 0.5-g portions were weighed from each meal sample, and 5 ml of solvent was added to each portion, so that for each kind of meal there were two samples containing each of the eight solvents to be tested (Table I). One of each pair of samples was then agitated for 18 hr at *ca.* 2° in a cold room, and the other was sonicated for three 10-sec bursts with a Sonifier Model 15-75 (Branson Instruments, Inc.). The samples were centrifuged for 1.5 hr at 45,000 rpm in a Beckman Model L2 with a 50Ti rotor. The samples used in this study all come from this single extraction. The sample solution (2–3 ml) was withdrawn from between the lipid layer and the sediment, and 25 μl of 10% NaN_3 was added to each sample to prevent microbial growth. The concentration of protein in the extracts of the seed-meals was determined by the method of Lowry *et al.* (1951), as modified by Elrod (1967). This modification employs a Technicon AutoAnalyzer. In this method the copper tartrate solution was 0.05% (w/v) $CuSO_4 \cdot 5H_2O$ and 0.10% $NaKC_4H_4O_6 \cdot 4H_2O$, the buffer contained 80 g of Na_2CO_3 and 32 g of NaOH in each liter, and the phenol reagent (Precision Laboratories, Cincinnati, Ohio) was diluted with three parts (by volume) of water before use.

Three different solutions were used to prepare the samples for electrophoresis. Solution A was an aqueous solution containing 60% (v/v) glycerine, 0.002% (w/v) Phenol Red, 0.3% (w/v) NaN_3, 0.015 *M* NaH_2PO_4, and 0.015 *M* Na_2HPO_4. The apparent pH was 6.7. Solution B was an 0.08 *M* aqueous sodium phosphate solution adjusted to pH 7.0. It also contained 1.0% (w/v) SDS and 1.0% (v/v) ME. Solution C was an aqueous solution containing 50% glycerine, 0.01% (w/v) Bromophenol Blue, 1.0% (w/v) SDS, and 1.0% (v/v) ME.

The electrophoresis tank (Model 4200) and appropriate

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Table I. Solvents for Dissolution of Proteins

No.	Solvent	pH
1	Distilled water	
2	0.01 *M* ME	6.3
3	0.01 *M* sodium phosphate	7.0
4	0.01 *M* sodium phosphate–0.01 *M* ME	6.7
5	0.01 *M* sodium phosphate–0.155 *M* NaCl	6.7
6	0.01 *M* sodium phosphate–0.155 *M* NaCl–0.01 *M* ME	6.7
7	0.025 *M* Tris–0.19 *M* glycine–0.001 *M* EDTA	8.6
8	0.025 *M* Tris–0.19 *M* glycine–0.001 *M* EDTA–0.01 *M* ME	8.6

accessories were from ORTEC, Inc. (Oak Ridge, Tenn.). For electrophoresis in the absence of SDS a sample of each extract was prepared by mixing 50 μl of the extract with 300 μl of solution A and 500 μl of water. Twenty-five microliters of this prepared sample mixture was applied to a gel that was a 4–17.5% continuous gradient of polyacrylamide (Caton and Goldstein, 1971) with a 1-cm layer of 4% gel above the gradient. This gradient gel, buffered at pH 8.6, contained 0.375 *M* tris(hydroxymethyl)aminomethane (Tris), 0.06 *M* HCl, and 0.001 *M* ethylenediaminetetraacetic acid (EDTA). The electrode buffer for the gradient gels was an aqueous solution (pH 8.3) containing 0.025 *M* Tris, 0.19 *M* glycine, and 0.001 *M* EDTA, to which Bromophenol Blue (0.125 mg/l.) was added. During the electrophoresis of two slab gels the maximum current was limited to 120 mA and the voltage to 175 V by a Hewlett-Packard Model 712C power supply. The time required for the Bromophenol Blue marker to migrate 90% of the gel length (*ca.* 6 cm) was about 2 hr. Electrophoresis of the samples on gradient gels was repeated one to three times.

Samples of the extracted solutions were also incubated with SDS and ME, followed by electrophoresis in much the same manner as described by Weber and Osborn (1969). Here, 25 μl of each soybean extract was mixed with 1 ml of sample preparation solution B and incubated for at least 12 hr in a water bath at 38°. One volume of the incubated sample was mixed with one volume of sample preparation solution C, and a 50-μl portion of this final mixture was used for electrophoresis on gels cast in Pyrex tubes (5 mm i.d., 7 mm o.d.), 9 cm long. Each tube contained 1.2 ml of a gel mixture containing 10% (w/v) acrylamide, 0.1% (v/v) tetramethylethylenediamine, 0.1% (w/v) ammonium persulfate, 0.3% (w/v) methylenebisacrylamide, 1.0% (w/v) SDS, and the buffer concentration indicated below. Approximately 0.2 ml of the same gel mixture was used to cap the sample. Both the gel and electrode buffers were prepared from a stock buffer (pH 7.0) 0.4 *M* in sodium phosphate and 0.01 *M* in EDTA. The electrode buffer was a 1:10 dilution of the stock buffer, made 0.1% (w/v) in SDS. The final concentration of the buffer in the gels was also 0.1 of the stock concentration. All gels were stained with Coomassie Blue (Weber and Osborn, 1969). Electrophoresis of the samples on gels containing SDS was twice repeated, in one case, more than a month apart.

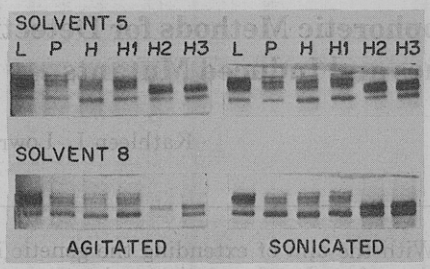

Figure 1. Electrophoretic patterns on gradient gels of soybean extracts in solvents 5 and 8 (Table I). Only the 6–9% portion of the gradient is shown. This portion contained the most intense bands and the only observable differences: L, Lee; P, Pickett; H, Harosoy; H1, H2, H3, mutants derived from Harosoy. The sample migrated from cathode to anode, or from the top to the bottom of the figure.

RESULTS AND DISCUSSION

The concentration of protein in each extract is listed in Table II. Sonication extracted more protein into a given solvent than did agitation, but the electrophoretic banding pattern of a given variety or mutant was qualitatively the same regardless of solution method, *i.e.*, given bands migrated to the same positions (Figures 1 and 2). Thus sonication does not appear to have altered any of the major protein constituents extracted. The banding pattern was also qualitatively the same for a given sample regardless of solvent, although different relative intensities of bands were observed for the same sample in different solvents (Figure 1).

As expected from previous data on oil-free meal (Smith and Circle, 1938; Wolf, 1972), solvents with the highest pH (8.6) extracted the most protein. All extracting solvents used in this investigation had a higher pH than the single solvent (pH 4.8) used by Larsen (1967). With the exception of one water extract (Pickett) and all of the samples extracted by agitation in solvent 5, the concentrations of extracted proteins exceeded the range reported by Larsen, and the absolute amounts extracted were greater in all cases. The solvents with pH 8.6 used in this study (7 and 8) extract a relatively greater amount of the proteins migrating to the region where Larsen observed differences with respect to soybean variety and a relatively lesser amount of the very acidic (fast migrating) and very basic (slow migrating) proteins. Thus, the extraction at a higher pH yields a sample with a simpler electrophoretic pattern and with the added advantage of containing amplified amounts of the proteins that appear to be most significant for detecting varietal and mutant differences.

The electrophoretic pattern of seed proteins of the Lee variety can be distinguished from that of Pickett and Har-

Table II. Lowry Total Protein Analysis of Six Soybean Sample Solutions

	Total protein (mg/ml) present in sample of variety[a]											
	L		P		H		H1		H2		H3	
Solvent	S	A	S	A	S	A	S	A	S	A	S	A
1	32.9	18.4	27.8	10.6	26.1	23.5	20.4	15.9	19.9	14.2	23.0	15.8
2	31.8	19.9	24.8	13.5	29.6	16.1	28.3	15.7	29.9	15.0	32.8	16.6
3	24.5	19.2	20.2	17.9	22.2	13.9	22.4	14.7	19.5	17.0	22.2	17.9
4	21.0	18.0	21.9	15.0	25.3	12.7	22.8	20.3	24.5	20.0	23.4	21.4
5	21.4	7.6	20.1	8.8	19.4	9.0	21.9	9.0	18.0	8.0	21.2	9.8
6	30.5	16.6	27.7	13.5	24.7	13.4	20.7	12.1	22.7	17.3	22.7	19.0
7	41.6	26.0	36.1	27.2	37.9	24.0	36.5	28.4	39.9	26.7	37.8	27.3
8	41.1	24.3	42.5	20.0	37.8	19.5	40.5	22.6	37.9	16.6	39.7	20.2

[a] L, Lee; P, Pickett; H, Harosoy; H1, H2, H3, mutants derived from Harosoy; S, sonicated; A, agitated.

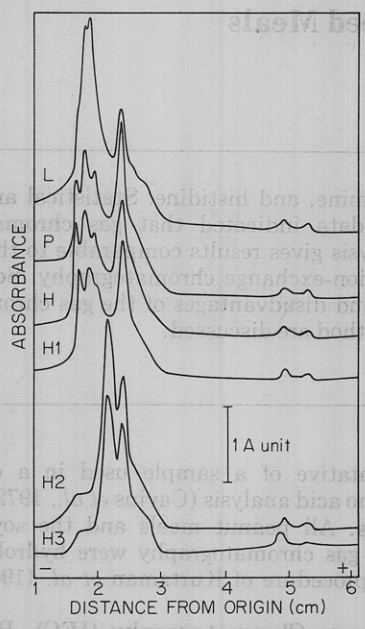

Figure 2. Densitometer tracings of electrophoretic patterns on gradient gels of soybean extracts in solvent 8, sonicated (Table I). Note the similarity of P, H, and H1, and the decided difference of L from those three.

osoy on gradient slab gels (Figures 1 and 2). This finding is of interest, since under different conditions of extraction and electrophoresis Lee and Pickett were reported previously (Larsen, 1967) to belong to a group of varieties with a protein component called B, whereas Harosoy was reported to be in a group of varieties having component A; A and B were considered in that study to be the only components distinguishing the electrophoretic patterns of seed proteins among 61 soybean variations. Mutant H1 has a gradient gel pattern similar to that of the parent Harosoy variety and Pickett. Mutants H2 and H3 have patterns similar to each other but different from the other patterns on the gradient gels. Thus there is no positive correlation between visible characters of the mutants and the electrophoretic properties of their seed proteins on the polyacrylamide gradient slab gels employed in this study.

Results of the electrophoresis on gels containing SDS after treatment of the samples with SDS and ME are shown in Figure 3. Mutants H2 and H3 differ from each other and from all the other samples. From Figure 3 it can be seen that the three original varieties have the same

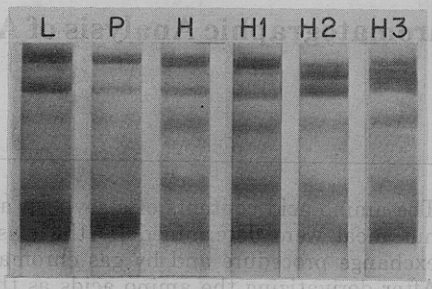

Figure 3. Electrophoretic pattern on gels containing SDS of soybean extracts after pretreatment with SDS and ME. Original extracts were sonicated in solvent 8 (Table I). The sample migrated from anode to cathode, or from the top to the bottom of the figure.

major protein bands, the difference being in the intensities of the bands. Thus, the protein differences between L and P or H shown in Figure 1 apparently arise from different proportions of the same subunits in the slower migrating proteins. Harosoy mutant H1 also has the same subunits as the parent and the Pickett varieties, differing from them only in the amount of each subunit present. Amino acid analysis of acid hydrolysates of the six meals indicated neither significant differences between the varieties or mutants in the types of amino acids present nor any significant differences in the amount of each type.

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