High-Resolution Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of Soybean (*Glycine max* L.) Seed Proteins[1]

S. K. SATHE,[2] G. G. LILLEY,[3] A. C. MASON,[2] and C. M. WEAVER[2]

Cereal Chem. 64(6):380–384

ABSTRACT

Thirty-one soybean (*Glycine max* L.) varieties were electrophoretically evaluated using two high-resolution sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) procedures. Increased tris (tris[hydroxymethyl]aminomethane) concentration in the separating gel (8–25% linear acrylamide gradient) and the running buffer afforded high resolution of soybean-protein subunits comparable to that of the urea SDS-PAGE system. When compared with urea SDS-PAGE, non-urea SDS-PAGE permitted the following: better resolution of polypeptides in the molecular weight range of 29,000 to 32,000; differentiaton of genetically closely matched pairs (Century and Century 84; Wells II and Miami); identification of a unique doublet of polypeptides in the Raiden variety; and in some varieties, identification of a peptide with higher mobility than that of the A_5 subunit of the 11S globulin.

Soybean (*Glycine max* L.) is one of the legumes with high protein content (35–40% protein on a dry weight basis). The major portion of total soy proteins consists of glycinin (11S) and β-conglycinin (7S) proteins (Derbyshire et al 1976). Glycinin has an estimated molecular weight (MW) of about 350,000 and is composed of at least six nonidentical subunits. Each of these subunits contains an acidic polypeptide linked to a basic polypeptide by a single disulfide bond. Molecular weights of the acidic subunits range between 37,000 and 42,000, and those of the basic subunits range between 17,000 and 20,000 (Moreira et al 1979, Nielsen 1985). β-Conglycinin (MW ~180,000) also consists of at least four nonidentical subunits (α′, α, β, and γ) with an estimated MW range from 42,000 to 57,000 (Medeiros 1982, Thanh et al 1975, Thanh and Shibasaki 1977, Davies et al 1985). Both glycinin and β-conglycinin are heterogeneous; this heterogeneity arises because of co- and post-translational modifications (proteolysis and glycosylation) of precursor molecules before packaging of these proteins into protein bodies (Sengupta et al 1981).

Polyacrylamide gel electrophoresis offers sensitivity, ease, economy, high resolution, and the ability to analyze large numbers of protein samples in a short time. The most common high-resolution polyacrylamide gel electrophoresis technique used to study soy proteins (Hu and Esen 1981, Hughes and Murphy 1983) is SDS-PAGE employing a linear gradient of acrylamide concentration according to Laemmli (1970). This method is not useful for the study of polypeptides of small molecular weights (≤10,000), because these small peptides can leach out of the gel during staining and destaining. To overcome this problem, Swank and Munkres (1971) recommend including 8*M* urea in the gels. Inclusion of 6*M* urea in SDS gels improves resolution and elicits changes in relative positions of certain major components of soybean seed proteins (Fontes et al 1984). Although two-dimensional gel electrophoresis (isoelectric focusing in the first dimension followed by SDS-PAGE in the second dimension) permits higher resolution compared to one-dimensional gel electrophoresis (Hu and Esen 1982), it is time-consuming; therefore, large numbers of samples cannot be analyzed in a short time. Recently, Fling and Gregerson (1986) showed that increasing tris (tris[hydroxymethyl]aminomethane) concentration in the separating gel and the running buffer of the Laemmli system affords better resolution and permits visualization of polypeptides with small molecular weights (total range of 1,700–100,000). This improvement of the Laemmli system may permit rapid analysis of large numbers of samples for identification of unique seed varieties as a first step in screening germ plasm collections. The purpose of this study was to explore this possibility.

MATERIALS AND METHODS

Materials

A total of 31 seed varieties was obtained. Twenty varieties from J. R. Wilcox (Dept. of Agronomy, Purdue University, W. Lafayette, IN) included the following: 1) Century; 2) Century 84; 3) Cumberland; 4) Cutler 71; 5) Hack; 6) Hardin; 7) Harper; 8) Hobbit; 9) Lawrence; 10) Miami; 11) Wells II; 12) Oakland; 13) Peller; 14) Pixie; 15) Regal; 16) Ripley; 17) Union; 18) Will; 19) Winchester; and 20) Woodworth (all 1984 crops); the remaining were from R. L. Bernard (USDA, University of Illinois, Dept. of Agronomy, Urbana): 21) Raiden (PI 360.844) from Japan; 22) Banesi; 23) Cloud; 24) Dunfield; 25) Ebony; 26) Kura; 27) Giant Green; 28) Kingwa; 29) Mandarin; 30) Illini; and 31) Jogun (numbers 22, 27, and 28 were 1984 crop; numbers 24, 25, 1985 crop; the rest were from 1983 crop).

Following are the sources of chemicals. Electrophoresis grade acrylamide, bis (*N,N′*-methylenebisacrylamide), TEMED (*N,N,N′,N′*-tetramethylethylenediamine, tris, ammonium persulfate, and glycine were from Bio-Rad Laboratories, Richmond, CA. Urea and SDS were from Fisher Scientific Co., Fairlawn, NJ. Bromophenol blue, Coomassie Brilliant Blue R, glycerol, and β-mercaptoethanol were from Sigma Chemical Co., St. Louis, MO. Hydrochloric acid (HCl) and acetic acid were from Mallinckrodt Inc., Paris, KY. Bulk grade methanol was from Purdue University chemistry stores, W. Lafayette, IN. Molecular weight markers, phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), myoglobin backbone polypeptide (16.95 kDa), α-lactalbumin (14.2 kDa), myoglobin fragment I + II (14.4 kDa), myoglobin fragment I (8.16 kDa), myoglobin fragment II (6.21 kDa), and myoglobin fragment III (2.51 kDa), were from Sigma. Certain purified subunits of the 7S and 11S proteins were provided by N. C. Nielsen, Dept. of Agronomy, Purdue University, W. Lafayette, IN.

Preparation for Extraction

Seeds were ground to a 20-mesh flour in a Wiley-type mill and extracted with the appropriate solvent by one of two methods within 12 hr of grinding.

Extraction Without Urea

Extraction was achieved with 0.05*M* tris-HCl (pH 8.5) containing 0.02*M* β-mercaptoethanol (β-ME) for 30 min (flour-to-solvent ratio of 1:10, w/v) at room temperature (25°C) with

[1]This is Journal paper no. 11167 from the Purdue Agricultural Experiment Station.
[2]Department of Foods and Nutrition, Purdue University, West Lafayette, IN 47907.
[3]CSIRO, Division of Protein Chemistry, 341 Royal Parade, Parkville, Victoria 3052, Australia.

This manuscript was prepared for electronic processing.

vortexing every 10 min. Samples were then centrifuged in an Eppendorf centrifuge for 10 min (25° C, 15,600 × *g*). The supernatants were mixed with an equal volume of SDS-PAGE sample buffer (0.05*M* tris-HCl pH 6.8, 1% SDS, 0.01% bromophenol blue, 30% glycerol, and 2% β-ME) and boiled for 5 min.

Extraction with Urea

On a separate set of samples, extraction was performed according to the method of Fontes et al (1984) using 0.05*M* tris-HCl (pH 8.0), 0.2% SDS, 0.01*M* β-ME, and 5*M* urea as the solvent (flour-to-solvent ratio of 1:10, w/v) for 30 min at room temperature (25° C) with vortexing every 10 min. Samples were then processed as in the non-urea method.

SDS-PAGE Without Urea

Samples extracted with 0.05*M* tris-HCl (pH 8.5), 0.02*M* β-ME were electrophoresed by Fling and Gregerson's method (1986) using 14.5 cm × 16.5 cm × 1.5 mm separating gel and 1.0 cm × 16.5 cm × 1.5 mm stacking gel. The resolving gel (8–25% linear acrylamide gradient) contained 0.75*M* tris-HCl (pH 8.8), 0.1% SDS; the stacking gel was 0.125*M* in tris-HCl (pH 6.8) and 0.1% in SDS. The running buffer was 0.05*M* tris, 0.19*M* glycine (pH 8.5), and 0.1% SDS. The 5*M* urea in the sample buffer recommended by Fling and Gregerson (1986) was eliminated in this study to avoid any possible carbamylation of proteins and the possibility of altered protein mobility due to protein modification. Gels were run at 8 mA/gel while being cooled with running tap water (~15° C) until the dye migrated to the gel edge (16 hr).

SDS-PAGE with Urea

Samples extracted by the method of Fontes et al (1984) were electrophoresed in 10–18% acrylamide linear gradient gels containing 6*M* urea in the separating gel, and 5% acrylamide containing 6*M* urea in the stacking gel according to Fontes et al (1984). Separating gels 11.5 cm × 16.5 cm × 1.5 mm with 4.0 cm × 16.5 cm × 1.5 mm stacking gels were run at 100 V constant voltage for 1 hr followed by 125 V constant voltage until the dye migrated to the edge of the gel (about 4 hr). The gels were cooled with cold running tap water (~15° C).

For both gel systems, a 10 μl equivalent of each original extract was used as the sample load, and electrophoresis was started within 2 hr of extraction. Approximately 10 μg of each purified subunit was electrophoresed.

Gels were stained in 50% methanol containing 10% acetic acid and 0.25% Coomassie Brilliant Blue R for 16 hr. The gels were destained with 50% methanol containing 10% acetic acid for 2 hr followed by 5% methanol containing 7.5% acetic acid.

RESULTS AND DISCUSSION

Electrophoretic patterns of 31 soybean varieties in a 6*M* urea SDS-PAGE system are shown in Figure 1. Molecular weight estimates of lipoxygenase (Lx); β-conglycinin subunits α′, α, and β; and acidic and basic subunits of glycinin were, respectively, 89,130, 79,070, 69,500, 52,480, 34,670, and 19,230 and were in close agreement with earlier reports (Thanh et al 1975, Medeiros 1982, Fontes et al 1984). The A_3 and A_5 subunits (two acidic subunits of glycinin) had estimated MWs of 49,890 and 11,750, respectively. A shift in mobility of the α′ subunit described in Fontes et al (1984) was found in certain varieties (lanes 10, 11, 23, and 28). The MW estimate for the shifted α′ subunit was 80,350. This shift in mobility was reported to be a genetically controlled trend (Fontes et al 1984). The A_5 and A_4 subunits of glycinin were previously reported to be either greatly reduced or absent in the Raiden variety (Staswick and Nielsen 1983, Fontes et al 1984). In this investigation, we found polypeptides in Raiden with mobility identical to these subunits (Fig. 1, lane 21). In addition, based on intensity and band width, we found other varieties that had reduced levels of A_5 subunit (Fig. 1, lanes 1, 12, 14, and 16).

Several new polypeptides were detected in the MW range of 14,400–16,950. These polypeptides were present in certain varieties (Fig. 1, lanes 22, 25, 26, 30, and 31) and were either greatly reduced (Fig. 1, lanes 6, 11, 12, 14, and 17) or barely detectable (Fig. 1, lanes 6 and 21) in others. Polypeptides with small MWs (2,510–6,210) were also detected in this gel system. These were clearly detectable (Fig. 1, lanes 25, 26, 27, and 31), greatly reduced (Fig. 1, lanes 4, 8, 10, and 16), or were almost nondetectable (Fig. 1, lanes 3 and 5). A high-MW component (Fig. 1, arrow) was present in most varieties and was either greatly reduced (Fig. 1, lanes 12, 23, and 28) or nondetectable (Fig. 1, lane 25).

When these same varieties were electrophoresed in SDS-PAGE without urea (Fig. 2), MW estimates for lipoxygenase, α~, α, β, γ, A_3, acidics, basics, and A_5 were, respectively, 93,330, 82,220, 70,630, 48,420, 46,240, 40,740, 33,570, 20,650, and 10,000. The MWs for α′, α, and β are consistent with an earlier report (Derbyshire et al 1976). Molecular weights of A_3 and A_5 subunits are in agreement with those reported by Moreira et al (1979) and Hirano et al (1985), respectively. A shift in mobility of α′ subunit was also observed in this gel system in the same varieties (Fig. 2, lanes 10, 11, 23, and 28). The γ subunit of β-conglycinin, which

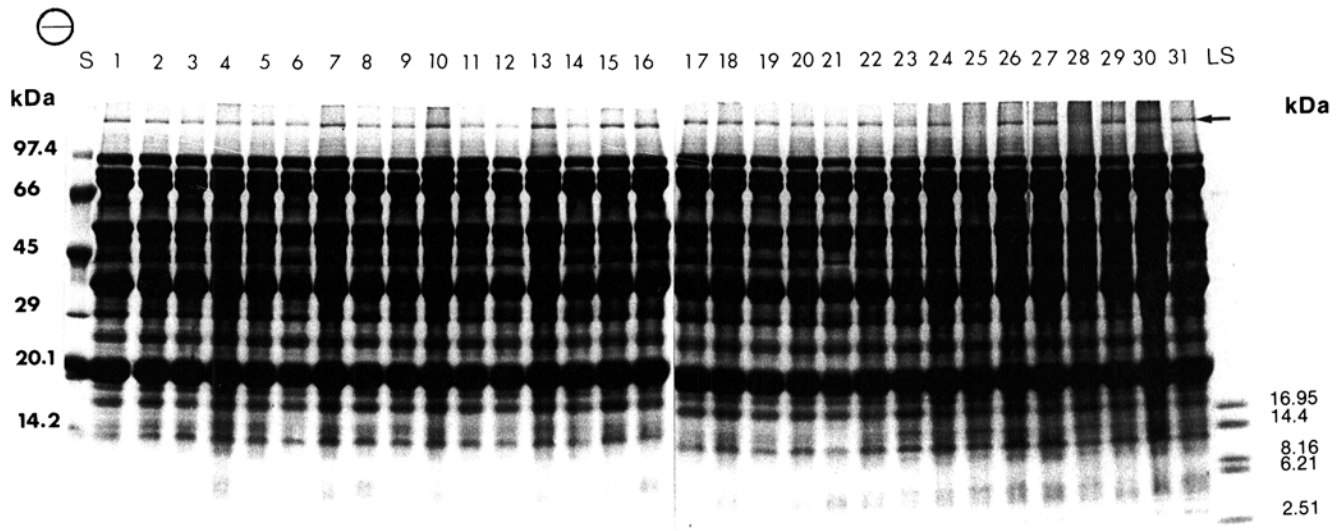

Fig. 1. Electrophoretic profiles of 31 soybean varieties in 6*M* urea sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fontes et al 1984). This figure is a composite of two gels. Numbers on top refer to the variety number identified in materials and methods. S = High-molecular weight (MW) markers, LS = low-MW markers. The MWs of standard proteins are shown in the margins. Direction of migration was from top to bottom. Protein load for each variety was a 10-μl equivalent of the original extract.

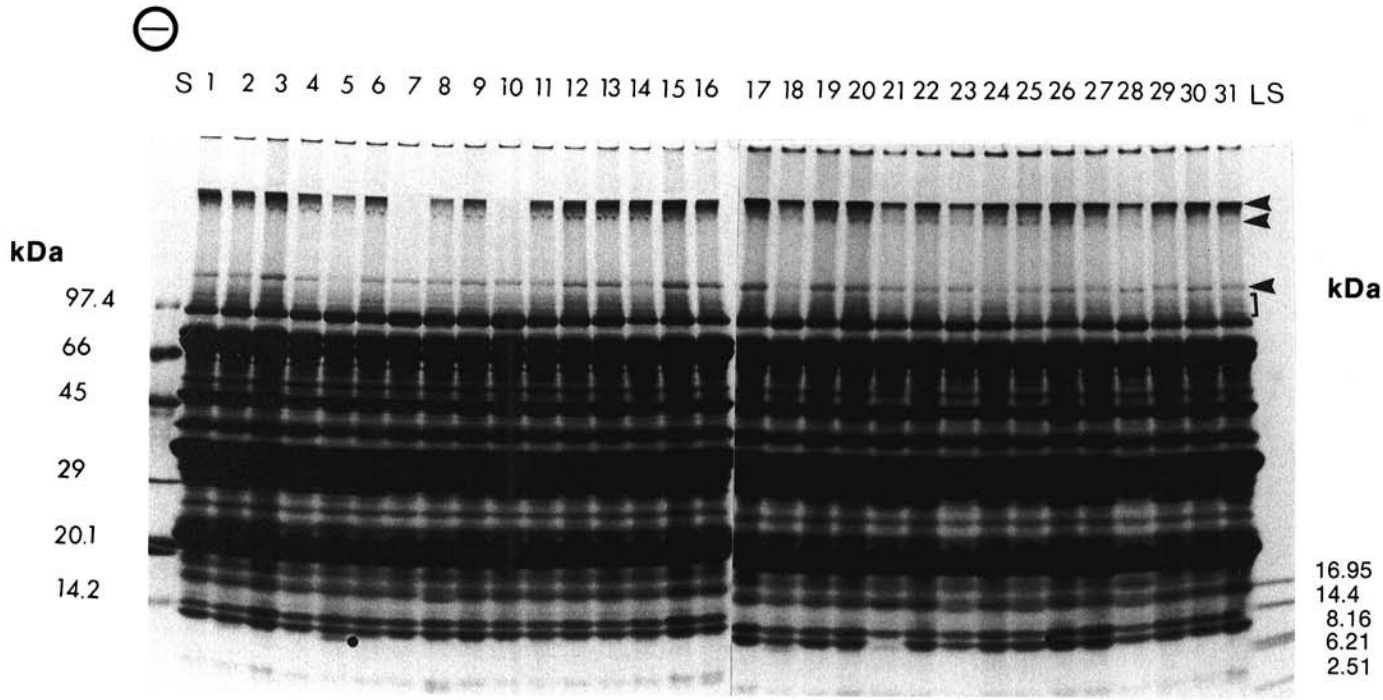

Fig. 2. Electrophoretic profiles of 31 soybean varieties in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fling and Gregerson 1986). Details as in Fig. 1.

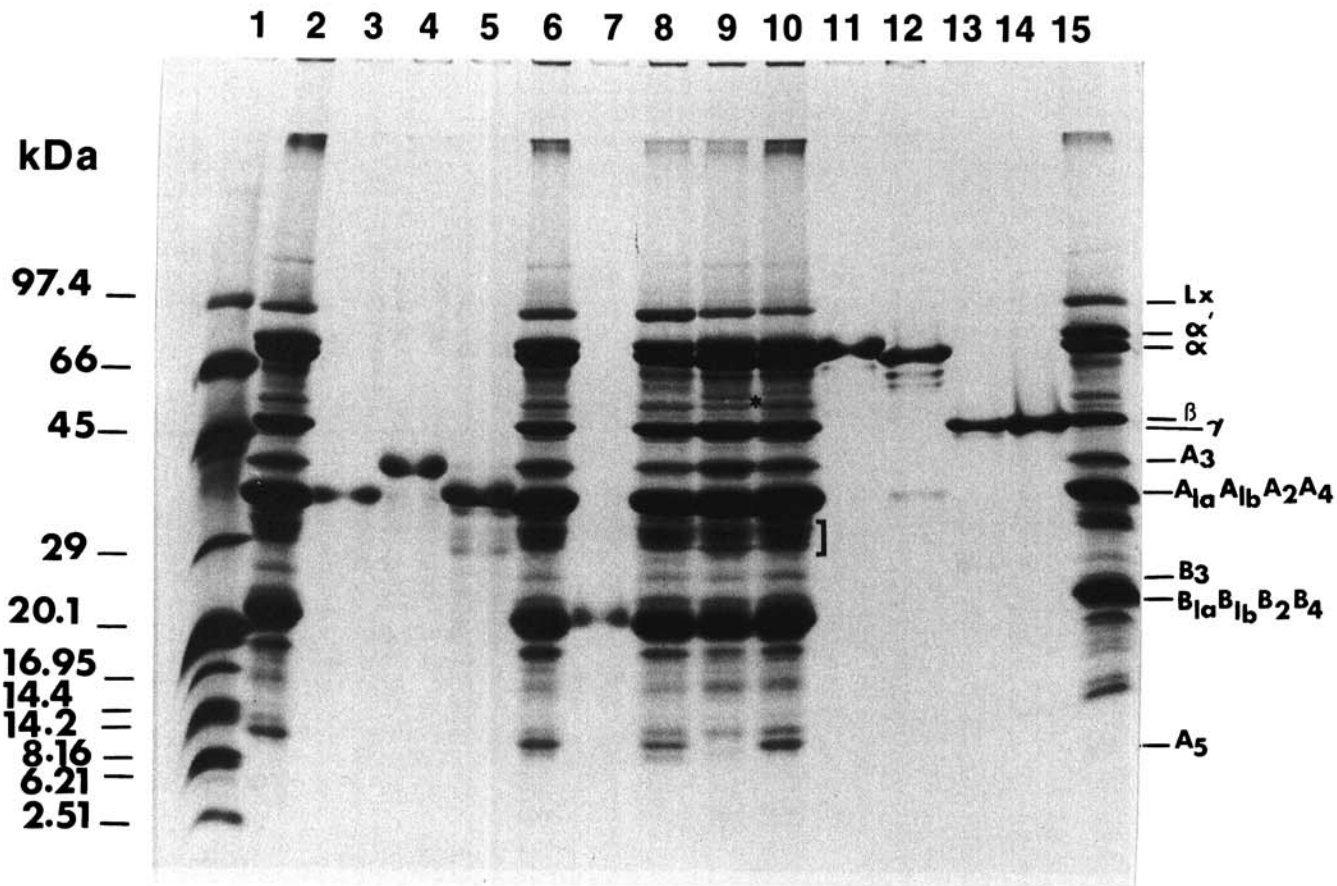

Fig. 3. Electrophoretic profiles of selected soybean varieties and some purified subunits of glycinin and β-conglycinin. Lanes **1,** molecular weight markers; **2,** Century; **3,** A_{1a}; **4,** A_3; **5,** A_4; **6,** Cutler; **7,** B_{1a}; **8,** Hack; **9,** Raiden (PI360.844); **10,** Kura; **11,** α'; **12,** α; **13,** β; **14,** γ, **15,** Kingwa. Approximately 10 μg of each purified subunit was loaded. A 10-μl equivalent of the original extract from each soybean variety was loaded. This was an 8–25% linear acrylamide gradient gel according to Fling and Gregerson's (1986) procedure.

migrates slightly faster than the β subunit, was detected in several varieties and was easily visible in gels when larger sample wells were used for the same-size protein load (Fig. 3). Several polypeptides (MW 29,000–32,000) with higher mobility than the acidic polypeptides of glycinin were resolved in the gels using larger sample wells (indicated by the bracket in Fig. 3). This cluster contained four (Fig. 2, lanes 2–6; Fig. 3, lane 9), three (Fig. 2, lanes 23 and 25), or two (Fig. 2, lane 28) polypeptides. In addition, a polypeptide migrating slightly faster than A_5 subunit was detected in this gel system (indicated with •, Fig. 2, lane 5) in a few varieties (Fig. 2, lanes 2, 5, and 18; Fig. 3, lanes 6 and 8). This SDS-PAGE system revealed an interesting feature unique to the Raiden variety: a doublet just above the β subunit of β-conglycinin was seen (Fig. 2, lane 21; Fig. 3, lane 9, indicated with *). The lower polypeptide was seen in all varieties, but the upper one was present only in Raiden. This feature was not observed in the urea SDS-PAGE system. In the high-MW range (>97,000), a triplet of varying intensity was seen in both the SDS-PAGE and urea SDS-PAGE systems (indicated by a bracket in Fig. 2). In the SDS-PAGE system, three distinct bands were seen in most varieties in various amounts (Fig. 2, arrowheads). The two polypeptides with lowest mobility were not detectable in two varieties (Fig. 2, lanes 7 and 10). The varieties Century and Century 84 (Fig. 2, lanes 1 and 2) are genetically similar as are the varieties Miami and Wells II shown in lanes 10 and 11 of Figure 2 (J. R. Wilcox, *personal communication*). These closely matched pairs can be distinguished as follows: the polypeptide with slightly higher mobility than A_5 subunit is present only in Century 84; comparing Miami and Wells II, the two polypeptides with the lowest mobility are present only in Wells II. These data suggest that because of the high resolution when using SDS-PAGE without urea, single-dimensional gel electrophoresis may be useful in differentiating closely matched varieties.

Swank and Munkres (1971) recommend including urea in SDS-PAGE for two reasons: protein aggregates are dissociated and resolution of polypeptides of MWs from 1,000 to 10,000 is improved due to reduction of effective pore size in acrylamide gels while maintaining the linear relationship between log MW and relative mobility (R_f). Urea, however, is known to be a carbamylating agent of proteins due to cyanate formation (Lundblad and Noyes 1984). Carbamylation of proteins may not be desirable. SDS-PAGE in the absence of urea allows recovery of noncarbamylated proteins from which bound SDS may be removed (Kapp and Vinogradov 1978, Gibson and Gracy 1979, Hanaoka et al 1979). Presence of urea may cause salting out of proteins preventing migration of large proteins into the gel. Also, urea crystallization prevents gels from cooling to ≤4° C. Besides these factors, inclusion of urea at 8 *M* concentration in the SDS gel system (Takagi and Kubo 1979) has the following effects: the maximum amount of SDS bound to proteins is reduced by almost half; consequently, the minimum equilibrium concentration of SDS required to reach saturation is nearly doubled; the apparent α-helical content of a protein is reduced to 50–70% of that in the presence of SDS alone, thus causing conformational change in the protein; and effective size of the SDS-protein complex is smaller than the effective size of the same protein in 8 *M* urea alone but greater than in SDS alone. These effects may cause alteration in protein mobility in urea SDS-PAGE.

In SDS-PAGE, protein separation and consequent mobility are based on two criteria: SDS binding to protein is at saturation, and the intrinsic charge of the protein is negated to give a constant surface charge density. (On a weight per weight basis, the nonspecific affinity of SDS for a protein yields a constant binding ratio of approximately 1.5.) The first criterion is satisfied when SDS is present in sufficient quantity (≥0.1%); however, when the presence of urea doubles the saturation concentration of SDS, the second criterion may not be fulfilled, that is, overcoming the effect of the intrinsic charge of a protein (Takagi and Kubo 1979). This may explain why some polypeptides (e.g., A_4 subunit of glycinin) have reduced mobility in the urea SDS-PAGE system. Increase in size of the SDS protein-complex as well as reduction in gel pore size in the presence of urea may further contribute to retardation in protein mobility. Obviously, subunit A_4 must have certain structural features that respond to the presence of urea in a more extreme fashion than the rest.

TABLE I
Relative Mobilities (R_f) of the Standard Proteins in Urea SDS-PAGE[a] and SDS-PAGE Gels

Protein	Molecular Weight	R_f Urea SDS-PAGE	R_f SDS-PAGE
Phosphorylase b	97,400	0.0179	0.1862
Bovine serum albumin	66,000	0.1850	0.2655
Ovalbumin	45,000	0.3000	0.3310
Carbonic anhydrase	29,000	0.4070	0.4551
Soybean trypsin inhibitor	20,100	0.5132	0.5448
α-Lactalbumin	14,200	0.6195	0.6551
Myoglobin backbone polypeptide	16,950	0.5752	0.5764
Myoglobin fragment I and II	14,400	0.6106	0.6111
Myoglobin fragment I	8,160	0.6770	0.6538
Myoglobin fragment II	6,210	0.6920	0.6655
Myoglobin fragment III	2,510	0.7876	0.7326

[a] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Such an effect was observed in R_f values of all the standard proteins with MW ≥14,000 (Table I). Increase in effective size of SDS-protein complexes levels off in the presence of SDS alone. Such a leveling off does not occur in the presence of urea (Takagi and Kubo 1979). This should result in lower mobility of polypeptides with MW <14,000. Data in Table I show contrary results. Swank and Munkres (1971) suggest that for small MW polypeptides (≤10,000), intrinsic charge and conformation are more important determinants of electrophoretic mobility than size of the SDS-polypeptide complex. Because urea doubles saturation binding of SDS to proteins (Takagi and Kubo 1979), importance of intrinsic charge and conformation may become even greater and may partly explain higher R_f values of small polypeptides in the urea SDS-PAGE compared to the SDS-PAGE system, as observed in this experiment.

CONCLUSION

Data presented in this investigation indicate that the SDS-PAGE system of Laemmli as modified by Fling and Gregerson (1986) afforded good resolution that was comparable to that obtained in the urea SDS-PAGE system but avoided the problems associated with urea. It also afforded better resolution of soy proteins in the MW range of 29,000–32,000 and the ~10,000-dalton region. This system may permit identification of varietal differences using single-dimensional gel electrophoresis and allow screening of large numbers of samples in a short time.

ACKNOWLEDGMENTS

We gratefully acknowledge R. L. Bernard, University of Illinois, and J. R. Wilcox, Purdue University, for seed samples and N. C. Nielsen, Purdue University, for purified subunits of glycinin and β-conglycinin.

LITERATURE CITED

DAVIES, C. S., COATES, J. B., and NIELSEN, N. C. 1985. Inheritance and biochemical analysis of four electrophoretic variants of β-conglycinin from soybean. Theor. Appl. Genet. 71:351.

DERBYSHIRE, E., WRIGHT, D. J., and BOULTER, D. 1976. Legumin and vicilin, storage proteins of legume seeds. Phytochemistry 15:3.

FLING, S. P., and GREGERSON, D. S. 1986. Peptide and protein molecular weight determination by electrophoresis using a high-molarity tris buffer system without urea. Anal. Biochem. 155:83.

FONTES, E. P. B., MOREIRA, M. A., DAVIES, C., and NIELSEN, N. C. 1984. Urea elicited changes in relative electrophoretic mobility of certain glycinin and β-conglycinin subunits. Plant Physiol. 76:840.

GIBSON, D. R., and GRACY, R. W. 1979. Extraction of proteins and peptides from Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gels. Anal. Biochem. 96:352.

HANAOKA, F., SHAW, J. L., and MUELLER, G. C. 1979. Recovery of functional proteins from sodium dodecyl sulfate-polyacrylamide gels.

Anal. Biochem. 99:170.
HIRANO, H., FUKAZAWA, C., and HARADA, K. 1985. The primary structures of the A_4 and A_5 subunits are highly homologous to that of the A_3 subunit in the glycinin seed storage protein of soybean. FEBS Lett. 18:124.
HU, B., and ESEN, A. 1981. Heterogeneity of soybean seed proteins: One dimensional electrophoretic profiles of six different solubility fractions. J. Agric. Food Chem. 29:497.
HU, B., and ESEN, A. 1982. Heterogeneity of soybean proteins: Two dimensional electrophoretic maps of three solubility fractions. J. Agric. Food Chem. 30:21.
HUGHES, S. A., and MURPHY, P. A. 1983. Varietal influence on the quantity of glycinin in soybeans. J. Agric. Food Chem. 31:376.
KAPP, 0. H., and VINOGRADOV, S. N. 1978. Removal of sodium dodecyl sulfate from proteins. Anal. Biochem. 91:230.
LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680.
LUNDBLAD, R. L., and NOYES, C. M. 1984. Pages 134-140 in: Chemical Reagents for Protein Modification. Vol. I. CRC Press Inc.: Boca Raton, FL.
MEDEIROS, J. S. 1982. Characterization of the subunits of β-conglycinin and the application of ELISA to the determination of the contents of β-conglycinin and glycinin in soybean. Ph.D. Thesis. Purdue University, W. Lafayette, IN.
MOREIRA, M. A., HERMONDSON, M. A., LARKINS, B. A., and NIELSEN, N. C. 1979. Partial characterization of the acidic and basic polypeptides of glycinin. J. Biol. Chem. 254:9921.
NIELSEN, N. C. 1985. The structure and complexity of the 11S polypeptides in soybeans. J. Am. Oil Chem. Soc. 62:1680.
SENGUPTA, C., DE LUCA, V., BAILEY, D. S., and VERMA, D. P. S. 1981. Post-translational processing of 7S and 11S components of soybean storage proteins. Plant Mol. Biol. 1:19.
STASWICK, P. E., and NIELSEN, N. C. 1983. Characterization of a soybean cultivar lacking certain glycinin subunits. Arch. Biochem. Biophys. 223:1.
SWANK, R. T., and MUNKRES, K. D. 1971. Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. Anal. Biochem. 39:462.
TAKAGI, T., and KUBO, K. 1979. Sodium dodecyl sulfate-protein polypeptide complexes in 8*M* urea with special reference to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Biochim. Biophys. Acta 578:68.
THANH, V. H., OKUBO, K., and SHIBASAKI, K. 1975. Isolation and characterization of the multiple 7S globulins of soybean proteins. Plant. Physiol. 56:19.
THANH, V. H., and SHIBASAKI, K. 1977. Beta-conglycinin from soybean proteins. Isolation and immunological and physicochemical properties of the monomeric forms. Biochim. Biophys. Acta 490:370.

[Received January 8, 1987. Revision received June 4, 1987. Accepted June 4, 1987.]