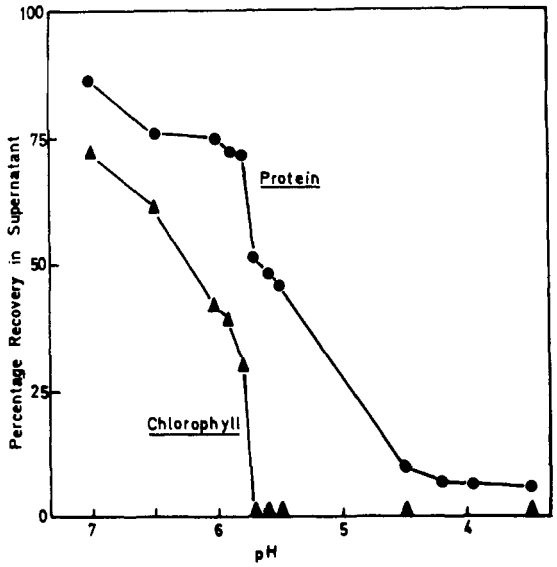

Figure 5. Effect of the acid pH treatment on the recovery of protein and chlorophyll from spinach leaves. Mistol was added at a final concentration of 2 mg/mL. Temperature was 30 °C.

tained (some 30%) with the thermal treatments (Figures 2 and 3).

With leaf juices of some other plants, different alkaline and acid pHs were assayed to obtain chlorophyll-free (≤0.1% with respect to protein) protein preparations by successive treatments at an alkaline pH and an acid pH. Table I shows the results of protein yields, final chlorophyll/protein ratios, and other information such as the pH values selected to optimize the yield of protein. From Table I it seems that the use of successive pH treatments is a good method to obtain a high yield of leaf protein essentially free of chlorophyll. The values of the appropriate pHs depended on the plant, and probably, a study of other factors, e.g., temperature and time, should improve the yields. The addition of Mistol improved by some 10–25% the yield in white protein from juices of some plants. Mistol is not included in Table I for plants in which it did not improve the yield in white protein more than 10%. Together with the high protein yield, the main interest of the method is based on the use of simple and rapid techniques and low centrifugal forces. The white protein of the supernatants may almost quantitatively (around 95%) be recovered by thermal denaturation at 90–100 °C.

Reasons other than the low resuspension of the precipitated thylakoid fragments may also contribute to the high protein yields of Table I. Batley and Bray (1978) found that the treatment of the juices at moderate alkaline pH increases the content of cytoplasmic white protein.

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Studies on Linseed Proteins

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A simple method of dehulling and demucilaging of linseed was developed. Solubility of the proteins of the linseed meal in water, 0.5 and 1.0 M NaCl, and 2% sodium hexametaphosphate solutions was determined in the pH range 2–12. The solubility was minimum around pH 3–6 and maximum around pH 8.0. The solubility minimum shifted to pH 0.5–4.5 in 1 M NaCl and to 0.6–5.3 in 2% sodium hexametaphosphate. The total proteins were characterized by techniques of gel filtration, ion-exchange chromatography, electrophoresis, and ultracentrifugation. The presence of at least three components was observed. The meal extracts showed proteolytic and trypsin inhibitor activities but no hemagglutinating activity. Amylase and amylase inhibitor activities were not detected.

In India, linseed (*Linum usitatissimum*) is one of the five major oilseeds and an important commercial oilseed crop. The deoiled meal obtained after expelling oil contains 25–35% protein. However, due to the presence of several antinutritional factors, viz., mucilage, phytic acid (Peterson, 1958), cyanogenic glycosides (Conn, 1969), antipyridoxine factor (Kratzer, 1947; Klosterman, 1974), allergens (Spies, 1974), and goitrogens (Care, 1954), its use is limited to livestock feed. Earlier work done in this laboratory has given information on these factors and also suggested simple detoxification techniques to render linseed cake fit for poultry feed (Mandokhot and Singh, 1979; Singh, 1979). The current investigation aims at more detailed information, including the nature of changes in

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linseed proteins. In this paper, we report a simple process of demucilaging and the physicochemical characterization of linseed proteins.

MATERIALS AND METHODS

Materials. Linseed, Khategaon variety, was purchased from M/s Flour and Foods, Ltd., Indore, India.

Preparation of Demucilaged, Defatted, and Dehulled Meal. The seeds were cleaned manually to remove the foreign matter and soaked in water (1:3 w/v) for 5 h, with intermittent stirring and an hourly change of water to facilitate the mucilage dispersal and removal. The presoaked seeds were fed into a APV fruit pulper that was fitted with $^1/_{32}$ in. sieve and nylon brushes. Water was flushed through the pulper during pulping of seeds for mucilage removal. After passage of the mass through the pulper 4 times, the seeds were found to be free from mucilage. The pulped seeds of 43–45% moisture content were flaked in a Aktiebolaget Kvarnmaskiner (Type J) flaking machine with a roll gap of 0.4 mm to facilitate the loosening and separation of hulls from cotyledons. The flaked mass was dried at 40 °C for 18 h in a crossflow drier at an air flow rate of 55 m^3/min. The dried flaked seeds were defatted with food-grade hexane by extraction at ambient temperature. After five extractions, the hulls were separated from cotyledons by using a Satake paddy separator. The cotyledons were ground to a coarse mesh, further defatted with hexane at ambient temperature so as to reduce the fat content to less than 1%. The material was powdered by a micropulverizer and passed through a 60-mesh sieve to get defatted, dehulled meal.

Analyses. Crude protein ($N \times 6.25$), ether extract, ash, and crude fiber contents of the seeds and of defatted meal were estimated by AOAC (1980) methods. The mucilage content of the seeds was estimated by the method of Anderson and Jow (1947). The phytic acid content of the meal was estimated by the method of Wheeler and Ferrel (1971) and HCN content by the AOAC (1980) method.

Nitrogen Solubility. Two grams of linseed meal was shaken with 20 mL of solvent [(water, 0.5 M NaCl, 1 M NaCl, or 2% sodium hexametaphosphate (SHMP)] and the pH of the suspension adjusted to the desired pH (in the pH range 0–12) by the addition of 5 N HCl or 5 N NaOH. The suspension was shaken mechanically at room temperature (~28 °C) for 1 h and centrifuged at 6000 rpm for 30 min and the pH of the supernatant noted. Aliquots of 5 mL of supernatant were taken for nitrogen estimation by micro-Kjeldahl method. The solubilized nitrogen was expressed as percentage of the total nitrogen (Srinivas and Narasinga Rao, 1981).

Protein Concentration. This was determined by measuring the absorbance of the solution at 280 nm. $E^{1\%}_{1cm}$ of the protein was established by plotting the absorbance at 280 nm vs. crude protein concentration determined by micro–Kjeldahl method. A factor of 6.25 was used for converting nitrogen content to crude protein. The protein solution was prepared by extracting the meal with 1 M NaCl and extensively dialyzing against the same solution (Srinivas and Narasinga Rao, 1981).

Gel Filtration. Sepharose 6B-100 (Pharmacia Fine Chemicals, Sweden) that had been equilibrated with 1 M NaCl was packed into a column, 1.8 × 100 cm. The proteins, extracted with 1 M NaCl and dialyzed against 1 M NaCl, were loaded on the column and eluted with 1 M NaCl at a flow rate of 21 mL/h. Fractions of 3 mL were collected in an automatic fraction collector, and the absorbance of the fractions was monitored at 280 nm (Fischer, 1969).

Ion-Exchange Chromatography. DEAE-Sephadex A-50–120 (Sigma Chemicals) gel was packed in a column, 2.2 × 20 cm, and equilibrated with 0.01 M phosphate buffer, pH 7.5. The proteins, extracted with 1 M NaCl and dialyzed against phosphate buffer, were loaded on the column, eluted with a linear gradient of 0–0.5 M NaCl, and 4-mL fractions were collected in an automatic fraction collector. The absorbance was monitored at 280 nm. The NaCl concentration was estimated by Mohr's method (Kolthoff and Stenger, 1947).

Polyacrylamide Gel Electrophoresis. Disc gel electrophoresis was performed by the method of Davis (1964) using 7.5% gel. The experiments were done in 0.025 M Tris–glycine buffer, pH 8.3, and the proteins were stained by Amido black 10B. The sample was prepared by extracting linseed meal in 1 M NaCl and dialyzing the protein extract extensively against Tris–glycine buffer. Electrophoresis was also carried out in 0.05 M phosphate buffer, pH 7.6, and on the acidic side in 0.05 M β-alanine–acetate buffer, pH 3.6 (Gordon, 1969).

Ultracentrifugation. The sedimentation velocity experiment was done by using a 1.7% protein concentration in 1 M NaCl at 24 °C at 56 100 rpm in a Spinco Model E analytical ultracentrifuge equipped with the rotor temperature indicator and control system (RTIC) and phase plate schlieren optics. $s_{20,w}$ values were calculated from the photographs by the standard procedure (Schachman, 1959).

Carbohydrate and Phosphorus Content. Total carbohydrate and phosphorus contents of the total proteins were estimated by the methods of Montgomery (1961) and Taussky and Shorr (1953), respectively. Total proteins were extracted from the meal in 1 M NaCl and dialyzed against 1 M NaCl solution.

Proteolytic Activity. Proteolytic activity was determined by using casein hammerstein (BDH Chemicals, England) as the substrate by the method of Chrispeels and Boulter (1975). The extract was prepared by shaking 2 g of meal with 20 mL of 25 mM citrate phosphate buffer, pH 5.7, or with 1 M Nacl. The protein content of the extracts was determined by the method of Lowry et al. (1951).

Amylase and Amylase Inhibitor Activity. The procedure outlined by Granum (1978) was followed to assay amylase and amylase inhibitor activity of linseed meal extracted with 0.02 M phosphate buffer, pH 6.9, containing 0.05 M NaCl. Human saliva was used as the source of α-amylase.

Trypsin Inhibitor Activity. The proteins of linseed meal after extraction with 25 mM citrate phosphate buffer, pH 5.7, or with 0.1 M phosphate buffer, pH 7.6, or with 0.05 M Tris buffer, pH 8.2, were tested for trypsin inhibitor activity by the method of Kakade et al. (1969) using benzoyl-DL-arginine-*p*-nitroanilidine (BAPA) hydrochloride, as the substrate. The protein content of the extracts was determined by the method of Lowry et al. (1951).

Hemagglutinating Activity. Hemagglutinating activity of the proteins was estimated by the method of Paulova et al. (1971) using a 2% suspension of trypsinized rabbit erythrocytes. The proteins were prepared from meal extracted with 0.9% NaCl or with 0.01 M phosphate buffer, pH 7.5, containing 0.9% saline and dialyzed against phosphate buffer saline. Protein content of the extracts was determined by the method of Lowry et al. (1951).

RESULTS AND DISCUSSION

Laboratory trials had shown that when the seeds were soaked in water (1:10 w/v) for 4, 8, 10, and 12 h, progressively more mucilage was removed. After 12 h of

Table I. Effect of Soaking on Demucilaging of Linseed[a]

soaking time, h	residual mucilage on seed, %	nitrogen lost in the soaking medium,[b] %
0	7.45	0
4	3.01	0.7
8	2.34	0.8
10	1.92	0.8
12	1.00	0.9

[a] Seeds were soaked in water and kept on a rotary shaker. Samples were drawn at intervals to estimate the residual mucilage on seed and nitrogen of the soaking medium. All values are averages of triplicates. [b] As percent of the total seed N.

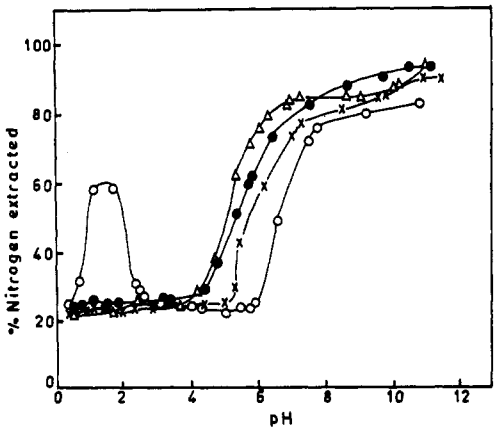

Figure 1. Nitrogen solubility vs. pH profiles: (○) Water; (●) 0.5 M NaCl solution; (▲) 1 M NaCl solution; (×) 2% sodium hexametaphosphate solution.

soaking about 1% mucilage was left on the seeds (Table I). During this period, less than 1% of the original nitrogen of the seed was lost into the soaking medium. In bulk trials, we found that a 5-h soaking of the seeds in water (1:3 w/v) with intermittent stirring and an hourly change of water yeilded a material practically free from mucilage. For flaking the wet seeds, we tried roll gaps from 0.3 to 0.5 mm with an interval of 0.05 mm. We found that a roll gap of 0.4 mm was optimum for the separation of hulls from cotyledons, as judged by visual appearance of the flaked material. A well-flaked material appeared yellowish brown due to the color of the separated cotyledons from the hulls. The dry, flaked material subjected to hexane extraction 5 times was found to loosen the hull from cotyledons and aid further separation. Though there are reports on the preparation of linseed meal, the use of specialized equipment (Smith et al., 1946; Schlamb et al., 1955) or use of acid for demucilaging (Mandokhot and Singh, 1979) may limit their scope. The present method has the advantage of use of commonly available equipment and of avoiding possible acid denaturation of proteins yeilding the meal in native state.

The composition of linseed and meal are given in Table II. Values reported here are in the range of literature values (Peterson, 1958; Mandokhot, 1975). Bolley and McCormack (1952) reported 6.4% phytate content in the meal by multiplying percent organic phosphorus by a factor 4.55 as against the value of 4.2% obtained by us.

Nitrogen solubility profiles of linseed proteins in water, 0.5 M NaCl, 1 M NaCl, and 2% SHMP are shown in Figure 1. Solubility profile of linseed proteins in water exhibits a typical U shape with minimum solubility in the range pH 3–6 and the solubility remained more or less constant after pH 8.0. The solubility curve is similar to that of other oilseeds such as groundnut and soya bean

Table II. Composition of Linseed and Linseed Meal[a]

	linseed, %	linseed meal
(a) crude protein ($N \times 6.25$)	21.9	48.9%
(b) ether extract	42.3	0.25%
(c) crude fiber	4.8	10.85%
(d) ash	2.6	5.5%
(e) mucilage	7.45	
(f) carbohydrate (by difference)	21	34.5%
(g) HCN		0.045%
(h) phytic acid		4.2%
(i) proteolytic activity		
1 M NaCl extract dialyzed against citrate-phosphate buffer		13.8 Kunitz units/mg of protein
citrate-phosphate buffer extract dialyzed against same buffer		14.9 Kunitz units/mg of protein
citrate-phosphate buffer extract, undialyzed		21.7 Kunitz units/mg of protein
(j) amylase and amylase inhibitor activity		ND[b]
(k) trypsin inhibitor activity		
Tris buffer extract		30.3 TIU
phosphate buffer extract		14.7 TIU
citrate-phosphate buffer extract		9.8 TIU
(l) hemagglutinating activity		ND[b]

[a] Items a-h are on a moisture-free basis. [b] ND = not detected.

(Fontaine et al., 1944; Smith and Circle, 1938). However, the curve shows that 22–24% of the total nitrogen is soluble at the point of minimum solubility irrespective of the solvents used. This may be due to either non-protein nitrogen or low molecular weight proteins. The nitrogen dispersion studies of Smith et al. (1946) of linseed meal also show that the pH of minimum solubility of linseed proteins is not sharp. It was found that 1 M NaCl extracted more nitrogen from the meal than 0.5 M NaCl at a given pH probably due to the salting in phenomenon (Kinsella, 1976). About 85% of the total nitrogen was extracted with 1 M NaCl at pH 7.0, and this remained more or less constant at higher pH values. In the presence of NaCl, the pH of minimum solubility shifted to the acidic side, namely, pH 0.5–4.5, probably due to the binding of anions by the proteins (von Hippel and Schleich, 1969). In SHMP, the solubility minimum was in the range pH 0.6–5.3.

The $E^{1\%}_{1cm}$ value at 280 nm for linseed proteins was 10.1 in 1 M NaCl. This was estimated from the plot of absorbance vs. crude protein concentration, which had been determined from micro-Kjedahl nitrogen estimation.

The gel filtration pattern of linseed proteins consisted of three peaks (1, 2, and 3) eluting at 72, 153, and 193 mL (Figure 2). The proportion of these peaks were 3, 67, and 30%, respectively. The first peak, eluting near the void volume (79 mL), had the absorbance maximum toward 260 nm, which is characteristic of nucleic acids and contained 78% phosphorus. This fraction was turbid. This suggests that this fraction contains mostly nucleic acid and some amount of high molecular weight aggregate. The total protein pattern of soyabean on gel filtration reported by Okubo and Shibasaki (1967) showed that the first peak was turbid, containing very little protein. In disc electrophoresis at pH 8.3, the major peak (peak II) showed more than two bands, indicating more than one protein component.

The ion-exchange chromatographic pattern revealed four components and the major peak having a hump suggested that it may be a mixture of two components (Figure 3). Cation exchanger could not be used for fractionation be-

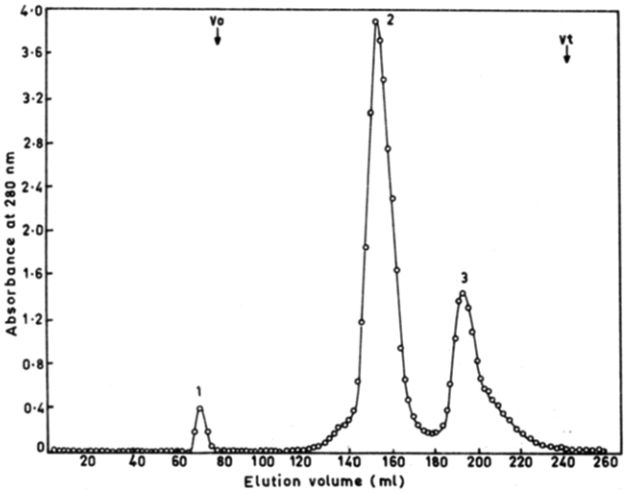

Figure 2. Gel filtration pattern of linseed proteins in 1 M NaCl solution on Sepharose 6B (1.8 × 100 cm).

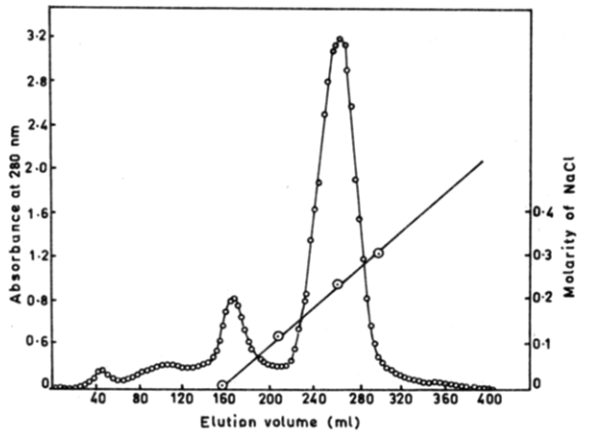

Figure 3. Ion-exchange chromatographic pattern of linseed proteins in 0.01 M phosphate buffer, pH 7.5, on DEAE-Sephadex (2.2 × 20 cm).

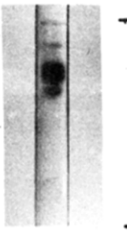

Figure 4. Polyacrylamide gel electrophoresis pattern of linseed proteins (0.025 M Tris–glycine buffer, pH 8.3).

tween pH 4.5 and pH 6.0 due to the precipitation of the proteins in this pH range, during dialysis. The first two peaks appear to be anionic in nature as they elute without salt gradient.

Disc gel electrophoresis at pH 8.3 showed four bands of which two bands were close to each other (Figure 4). Electrophoresis at pH 7.6 showed three bands consisting of one major band and two minor bands. Since there was precipitation of these proteins in the pH range 4.5–6.0, electrophoresis was performed at pH 3.6 in acidic pH in 0.05 M β-alanine–acetate buffer. This on staining with Amido black revealed a number of bands. The major fraction of linseed proteins was found to be oligomeric by sodium dodecyl sulfate gel electrophoresis, and also, the major protein fraction exhibited a number of bands at this pH (Madhusudhan and Singh, 1982). This behavior of total proteins at acid pH may be due to dissociation of the oligomeric major fraction; such an effect has been reported

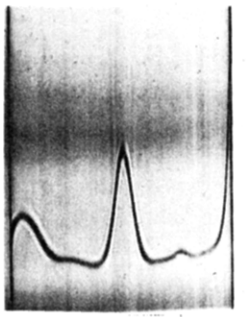

Figure 5. Sedimentation velocity pattern of linseed proteins in 1 M NaCl solution. The photograph was taken after 40 min at 56 100 rpm; bar angle 70°. Sedimentation proceeds from left to right.

in other seed proteins also (Koshiyama, 1972; Prakash and Nandi, 1977; Kishore Kumar Murthy, 1982).

The sedimentation velocity pattern (Figure 5) showed the presence of four components with $s_{20,w}$ values of 1.4, 5.0, 9.0, and 14.0 S and the relative proportion of these peaks were 20, 10, 66, and 4%, respectively. The gel filtration pattern showed the first peak area as 3%, which may coincide with the fast moving component in ultracentrifuge pattern. The proportions of the major peak in gel filtration and sedimentation velocity were almost same. Peak 3 in gel filtration, which may be due to low molecular weight proteins, might have resolved in two peaks in ultracentrifugation. This agrees with our observations in the isolation of the major fraction of linseed proteins (Madhusudhan and Singh, 1982). As is seen from Table II, the highest proteolytic activity was observed in citrate–phosphate buffer extract. This could be due to the fact that citrate–phosphate buffer preferentially extracts low molecular weight fractions. Dialysis of the extract led to a decrease in the proportion of such fractions and a reduction in proteolytic activity. Nath et al. (1978) have reported a similar observation in guar proteins. The presence of proteolytic enzymes in linseed meal has been reported ("Wealth of India, Raw Materials", 1962).

Though diastase enzymes have been reported in linseed meal ("Wealth of India, Raw Materials", 1962), no amylase activity was observed by us in the meal extracts. Tryspin inhibitor activity and hemagglutinating activity are shown in Table II.

The results reported above indicate that the proteins extracted from the linseed meal are free from amylase and amylase inhibitor activity and haemagglutinating activity. However, they are associated with trypsin inhibitor activity. The proteins consist of at least three fractions; the major fraction has a $s_{20,w}$ of 9 S and is possibly high molecular weight protein. The low molecular weight proteins form only a minor proportion (20%).

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Registry No. Trypsin inhibitor, 9035-81-8; proteinase, 9001-92-7; HCN, 74-90-8; phytic acid, 83-86-3.

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Two-Dimensional Electrophoretic Analysis of Soybean Proteins

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Water-extracted proteins from seeds of soybeans (*Glycine max*) were analyzed by two-dimensional gel electrophoresis. Two closely related electrophoretic systems were used: for optimal separation of acidic and neutral proteins, isoelectric focusing was the first dimension; for optimal resolution of basic proteins, nonequilibrium pH gradient electrophoresis was the first dimension. The second dimension was, in each case, polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate. Of approximately 430 spots resolved in the two systems, 35 were identified by comparison with the electrophoretic patterns of purified proteins: glycinin (13 acidic components, 11 basic components), β-conglycinin (6 components), soybean lectin (4 components), and Kunitz trypsin inhibitor. Two-dimensional electrophoresis was used to examine the protein components in a $MgSO_4$–curd and its whey and in the coagulated and noncoagulated fractions produced by boiling a water extract for 30 min. A protein of unknown identity was found to be enriched in both the whey and the non-heat-coagulated fraction. Two acidic subunits of glycinin were also apparently enriched in the same samples. These results illustrate the usefulness of two-dimensional electrophoresis in analyzing complex mixtures of soybean proteins.

Two-dimensional gel electrophoresis in which isoelectric focusing (IEF) in cylindrical gels is followed by polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate (O'Farrell, 1975) is a very powerful technique for analyzing complex mixtures of proteins since it combines two entirely independent electrophoretic approaches that are each individually capable of excellent resolution. As originally formulated, the technique is limited to examining acidic and neutral polypeptides since the pH gradient in polyacrylamide focusing gels does not extend much above pH 7 even though basic Ampholines are used (O'Farrell, 1975). O'Farrell et al. (1977) overcame that limitation, however, through the introduction of nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension.

Soybean seed proteins have been analyzed by various electrophoretic techniques [e.g., Catsimpoolas et al. (1971), Kitamura et al. (1976), Thanh and Shibasaki (1976a), and Mori et al. (1981)] including two-dimensional gel electrophoresis, the first report of which (Hu and Esen, 1982) was published as this paper was in preparation. Our results confirm and extend the conclusion of Hu and Esen that two-dimensional electrophoresis appears to be a promising approach to studying genetic and nutritional aspects of soybean proteins. We have used a variety of electropho-

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