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Chickpea Seed Proteins: Isolation and Characterization of 10.3S Protein

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The chickpea proteins were fractionated by gel filtration and DEAE-cellulose chromatography. The major protein fraction, with a sedimentation coefficient value of 10.3S, has been isolated by water dilution of the protein extracted by 10% (w/v) NaCl solution, followed by DEAE-cellulose chromatography. The protein preparation was found to be homogeneous by ultracentrifugation and polyacrylamide gel electrophoresis. The chemical composition, subunits, molecular weights of the protein, and subunit and spectral characteristics were determined. The storage protein role of 10.3S has been confirmed by its isolation in the protein bodies of chickpea.

Chickpea (*Cicer arietinum*), also known as Bengal gram, is a major food legume crop of India. It contains 17–21% protein on dry weight basis (FAO/UN, 1973). The overall composition, nutritional properties (Krishnamurthy, 1975; Jaya, 1978), and a few fractionation studies of the chickpea proteins (Radionova, 1957; Esh and De, 1960; Grigorcha and Klimenko, 1970; Alekseeva and Grigorcha, 1973) have been reported. The major water insoluble globulins of chickpea were reported to be complex with four or six fractions (Leonov, 1968). The trypsin inhibitors constituting less than 1.0% of the total protein have been well characterized (Belew et al., 1975; Belew and Eaker, 1976).

No detailed study of the various protein fractions of chickpea in terms of physicochemical characteristics appears to have been made. While working on the germination modification of storage proteins of legumes (Ganesh Kumar and Venkataraman, 1975, 1978), we isolated one of the major storage proteins of chickpea, employing dilution of the protein extracted with 10% (w/v) NaCl solution. This protein fraction differed from the major chickpea seed globulin, isolated by a different method of Alekseeva and Grigorcha (1973), in its sedimentation behavior.

In this investigation, an attempt has been made to study the fractionation characteristics of the total proteins of chickpea. Further, some of the physicochemical properties of the major storage protein, isolated by water dilution method, are presented.

EXPERIMENTAL SECTION

Materials. Seeds of chickpea (*Cicer arietinum*) were obtained locally. They were decorticated, powdered to 100-mesh size, and defatted with hexane. The defatted flour was used for protein extraction. The chemicals and reagents used in this study were of reagent grade.

Methods. *Extraction of Protein for Solubility Studies.* The defatted powdered flour was extracted with water adjusted to different pH's with either concentrated HCl or 10% NaOH for 1 h at 4 °C with continuous stirring. The slurry was centrifuged at 5000 rpm for 30 min. The N content of the clear supernatant was determined by the micro-Kjeldhal method.

Extraction of Protein for Fractionation Studies. The defatted flour was extracted with 0.01 M borate buffer of pH 7.8 for 2 h at 4 °C. The extract was centrifuged and

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the clear supernatant was dialyzed for 48 h at 4 °C against 0.02 M Tris–glycine buffer of pH 8.3 for polyacrylamide gel (PAG) electrophoresis, against 0.03 M phosphate buffer of pH 7.8 for gel filtration, against the extracting buffer for ion-exchange chromatography, and against 0.02 M Tris–glycine buffer of pH 8.3 containing 1 M NaCl for sedimentation velocity experiments. The concentration of protein in the dialysate was determined by Lowry's method (1951), using bovine serum albumin as the standard.

Preparation of 10.3S Protein. The defatted chickpea flour was extracted with 10% (w/v) NaCl in solute-to-solvent ratio of 1:10 for 2 h on a mechanical shaker. The slurry was centrifuged at 5000 rpm for 30 min. The supernatant was diluted with 10 volumes of distilled water. The resultant precipitate was collected by centrifugation dissolved in 10% (w/v) NaCl solution. Another dilution was performed with 20 volumes of distilled water. The precipitate obtained in this step was further purified by DEAE-cellulose chromatography.

DEAE-Cellulose Chromatography. The DEAE-cellulose column (2 × 20 cm) was equilibrated with 0.01 M borate buffer of pH 7.8 containing 0.25 M NaCl (buffer I). The protein solution (~30 mg/2.0 mL) in buffer I was applied on the column and eluted with the buffer containing 0.25 M NaCl. The fractions collected were discarded. A continuous linear gradient was set up in buffer I from 0.25 to 0.40 M NaCl. The fractions eluting in the range of 0.30 to 0.33 M were collected, dialyzed against distilled water, and lyophilized.

Gel Filtration. Gel filtration was carried out on Sepharose-6B-100 gel in a 2 × 85 cm column. The flow rate was 25–30 mL/h, and 2.5-mL fractions were collected. The fractions were monitored at 280 nm.

DEAE-Cellulose Ion-Exchange Chromatography. DEAE-cellulose was adjusted to the initial pH of the borate buffer (pH 7.8) and packed into a 2 × 13 cm column. One hundred milligrams of proteins was loaded on the column. A linear gradient of 0–0.8 M NaCl concentration was set up using Varigrade apparatus (Virtis Model with nine chambers), and the eluted fractions were monitored at 280 nm. The concentration of NaCl was determined by titration with standard 0.01 N $AgNO_3$, using K_2CrO_4 as indicator (Rieman et al., 1951).

Polyacrylamide Gel (PAG) and $NaDodSO_4$–PAG Electrophoresis. The electrophoretic conditions for PAG and $NaDodSO_4$–PAG were essentially the same as previously reported (Ganesh Kumar and Venkataraman, 1978).

Sedimentation Velocity Experiments. The experiments were perfomed in a Spinco Model E analytical ultracentrifuge equipped with rotor temperature indicator unit and phase plate schlieren optics. A standard 12-mm duraluminium cell centerpiece was used. The experiments were carried out at 28 °C with 1.0% protein solution, using a speed of 59780 rpm. The enlarged tracings of the photographs were used for the calculation of the relative percentage of each fraction. Correction for the radial dilution was not made. The $s_{20,w}$ value of each peak was calculated by the standard procedure (Schachman, 1959).

Proteolytic activity was determined using casein as substrate. The assay mixture included 1 mL of 1% casein (in 25 mM citrate–phosphate, pH 5.7), 0.2 mL of test protein, and 0.8 mL of buffer and was incubated for 2 h at 37 °C. The reaction was arrested by the addition of 1.0 mL of 15% Cl_3CCOOH, and the amino acid content of the supernatant was measured (Yemn and Cocking, 1955).

Amino Acid Analysis. The amino acid analysis was carried out in a Beckman amino acid analyzer. Three milligrams of the protein in 1.0 mL of 6 N HCl acid was hydrolyzed at 110 °C for 24 h. After removal of the excess hydrochloric acid, the residue was dissolved in 2.5 mL of 0.2 N sodium citrate buffer of pH 2.2, and 0.5 mL of the aliquot was used for the analysis. Tryptophan content was estimated by microbiological method using *Lactobacillus arabinosus* (Barton-Wright, 1952).

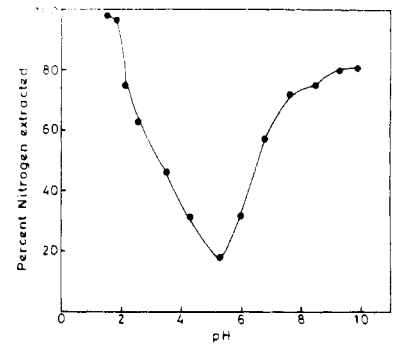

Figure 1. Percent N extractability of chickpea as a function of pH.

Carbohydrate and Phosphorus Estimation. The carbohydrate content of the protein fraction was estimated by the method of Montgomery (1961) and the phosphorus by the method of Taussky and Shorr (1953).

Fluorescence Measurements. A Perkin-Elmer fluorescence spectrophotometer Model 203 was used for fluorescence measurements. Dilute protein solution (0.01%) was used. Excitation spectrum was measured between 250 and 310 nm and the emission spectrum between 300 and 400 nm. All the readings were taken at 25 °C.

Viscosity Measurements. Measurements were made at 30 ± 0.1 °C using an Ostwald viscometer with a flow time of 180 s with distilled water. After temperature equilibration, the flow time was recorded to ±0.1 s with a Rocar stopwatch. From a plot of $(\eta/\eta_0 - 1)/C$ as a function of concentration (C), the intrinsic viscosity (η) was obtained from the intercept on the ordinate where η is the viscosity of the solution, η_0 is the viscosity of the solvent, and C is the concentration of the protein in g/100 mL.

Free and Total -SH Groups. The procedure of Beveridge et al. (1974) was followed for the estimation of -SH and S–S groups of proteins.

Isolation of Protein Bodies. Ten grams of flour was homogenized in 60.0 mL of 80.0% glycerol containing 25 mM citrate–phosphate buffer, pH 5.0. The slurry was centrifuged at room temperature at 2000*g* for 15 min. The supernatant was decanted, layered over 90.0% glycerol, and centrifuged at 35000*g* for 45 min at 15 °C. The sediment was resuspended in 25 mM citrate–phosphate buffer of pH 5.0 containing 2 mM β-mercaptoethanol. The suspension was used as such for detection of the contaminants present in the protein bodies.

RESULTS AND DISCUSSION

Extraction of Proteins. Extraction of chickpea proteins at different pH values is shown in Figure 1. The extractability of N in the pH range 4.0 to 6.0 was very low, whereas increased extraction was observed between pH 6.0 and 9.0. Under the extraction conditions employed (4 °C), denaturation may not be significant.

Fractionation of Chickpea Proteins. *Gel Filtration:* To determine the protein composition of the total extract obtained with 0.01 M borate buffer of pH 7.8, gel filtration on Sepharose-6B gel column was used. The proteins were separated into four fractions with V_e/V_o values of 1.16 (I), 2.11 (II), 2.74 (III), and 3.52 (IV), respectively (Figure 2a).

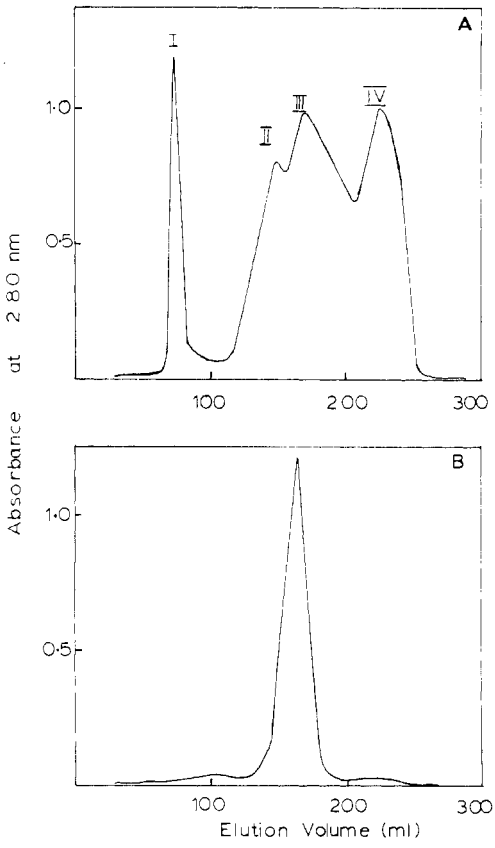

Figure 2. Gel filtration patterns of chickpea proteins: (A) total proteins, (B) 10.3S protein.

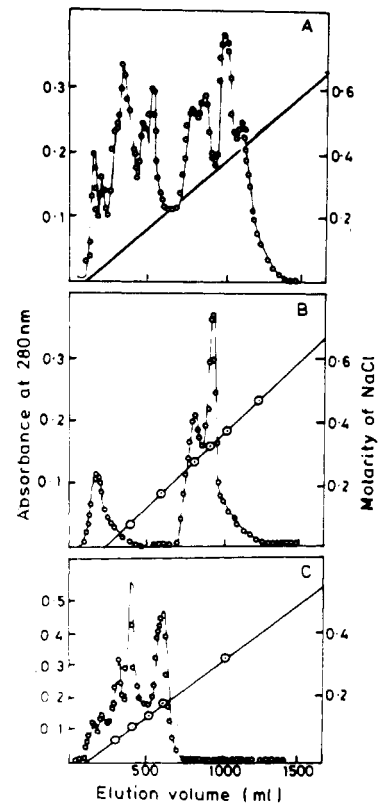

Figure 3. DEAE-cellulose ion-exchange chromatographic patterns of total protein (A), globulins (B), and albumins (C) of chickpea.

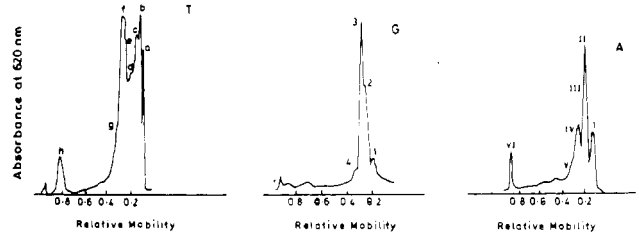

Figure 4. Microdensitometric scanning of PAG electrophoretic patterns of total proteins (T), globulins (G), and albumins (A) of chickpea.

The first fraction was turbid and eluted immediately after the void volume (V_o). Fraction II and III had no nucleic acid contaminants as evidenced by the A_{280}/A_{260} ratio. The aliquots corresponding to the peaks were tested by PAG electrophoresis for purity. None of the fractions were found to be homogeneous, and fractions II and III were cross-contaminated.

DEAE-Cellulose Chromatography. On DEAE-cellulose chromatography, the chickpea proteins were fractionated into at least nine components (0–0.7 M NaCl gradient). The chromatographic pattern (Figure 3A) could be divided into three regions, viz., unadsorbed, weakly bound, and strongly bound. The weakly bound region was comprised of two major fractions with elution constants of 0.09 and 0.18 M NaCl, respectively. There were three prominent protein fractions with elution constants of 0.28, 0.32, and 0.38 M, respectively, in the strongly bound region.

To further characterize the resolved peaks, the total proteins were extracted in 10% (w/v) NaCl solution. The globulins were precipitated by extensive dialysis against distilled water at 4 °C for 48 h. The supernatant contained the water-soluble albumins. The globulins (Figure 3B) on DEAE-cellulose chromatography resolved into three fractions (0–0.6 M NaCl gradient). One was eluted in the unadsorbed region. The other two were eluted at 0.28 and 0.32 M NaCl concentrations, respectively.

On the other hand, the albumins (Figure 3C) on DEAE-cellulose showed the presence of five components (0–0.6 M NaCl gradient). They were eluted at 0.02, 0.03, 0.08, 0.10, and 0.18 M NaCl, respectively. Thus it is possible to identify the weakly bound region of the total protein pattern with albumins and the globulins in the strongly bound region.

PAG Electrophoresis. The total proteins from the seed gave seven bands on PAG electrophoresis with two fast moving and five slow moving components (Figure 4T). The band g was not reproducible. The bands b and f were found to be the major proteins contributing 60% of the proteins (based on microdensitometric scanning).

The globulins were resolved into four bands with R_f values of 0.20, 0.26, 0.30, and 0.36, respectively, and the band with the R_f value of 0.30 was the major globulin (Figure 4G). This could be identified with the f band of the total proteins.

The electrophoretic pattern of the albumins (Figure 4A) showed the presence of six bands. The R_f values of the four prominent bands were 0.11, 0.19, 0.27, and 0.90, respectively. Thus the chickpea proteins are complex, consisting of at least four globulins and six albumin fractions. The smaller number of bands observed in the total proteins may be due to the possible presence of protein fractions with similar electrophoretic mobility.

Sedimentation Velocity Experiments. In the ultracentrifuge the total proteins gave three peaks with $s_{20,w}$ values of 2.2, 6.9, and 10.3S in the approximate proportion of 10:40:50, respectively (Figure 5A). When the globulins were tested in the ultracentrifuge they showed the presence of two components, 6.6S and 10.3S in the approximate proportion of 55:45, respectively (Figure 5B). The albumins showed a major peak with S value of 2.0 (Figure 5C).

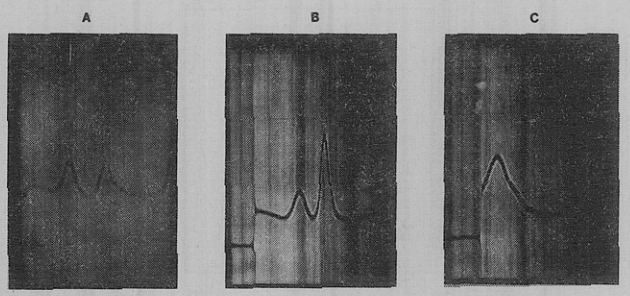

Figure 5. Sedimentation velocity patterns of total proteins (A), globulins (B), and albumins (C) of chickpea.

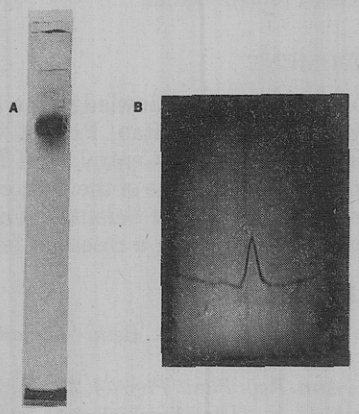

Figure 6. Polyacrylamide gel electrophoretic (A) and sedimentation velocity (B) patterns of chickpea 10.3S protein.

Narayana Rao and Rajagopala Rao (1974) observed variation in the $s_{20,w}$ values of the two globulins of six chickpea varieties studied. The fast sedimenting component has s values ranging from 9.5 to 13.2 and the slow sedimenting fraction from 7.4 to 8.2. It is, therefore, possible that the relatively low s values observed for the globulins fractions in this study may be due to varietal differences.

Homogeneity of the Isolated 10.3S Protein. The 10.3S protein constitutes ~50.0% of the total extractable proteins (Figure 5A). This was isolated by the water-dilution method of the saline extract as described in the Methods section. The preparation, after second dilution, was found to be fairly homogeneous by gel filtration (Figure 2B), PAG electrophoresis (Figure 6A), ion-exchange chromatography (Figure 7B) and about 90% homogeneous by ultracentrifugation (Figure 6B).

Proteolytic Activity. The proteolytic activity of 10.3S protein was tested with casein as substrate. No proteolytic activity was observed.

Chemical Composition of 10.3S Protein. The amino acid composition of 10.3S protein is shown in Table I. It contained large amounts of glutamic and aspartic acids. The content of serine, leucine, and glycine was found to be high compared to other amino acids. Thus, 10.3S protein showed the characteristic amino acid composition of a storage protein (Derbyshire et al., 1976). The carbohydrate content of 10.3S was estimated to be 0.68% as glucose equivalents and it contained very little phosphorus (0.08 mg/100 mg of protein).

Spectral Characteristics. The ultraviolet absorption spectrum of 10.3S was characterized by an absorption maximum of 278 nm with a minimum at 245–250 nm. The ratio of A_{280}/A_{260} was found to be 1.5. This suggested very little contamination with nucleic acids (Warburg and Christian, 1941).

The fluorescence emission spectrum of 10.3S was measured in the range of 300 to 400 nm. The maximum fluorescence intensity was observed at 320 nm. The content of aromatic amino acid of 10.3S protein was found to be phenylalanine-83, tyrosine-37 and tryptophan-12 residues per 276 000 g of protein. The occurrence of the emission maximum at 320 nm suggests the preponderence of tryptophan contribution (Chen et al., 1969).

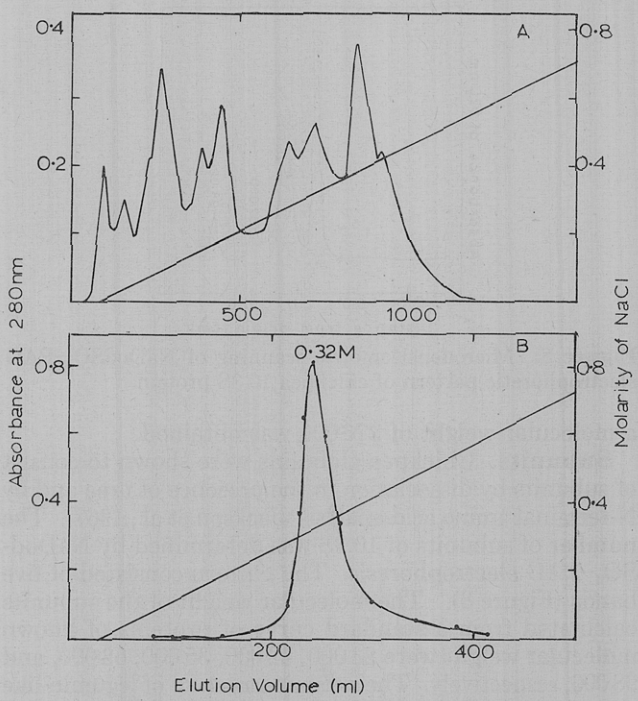

Figure 7. DEAE-cellulose chromatographic patterns of chickpea proteins: (A) total proteins, (B) 10.3S protein.

Table I. Amino Acid Composition of Chickpea 10.3S Protein

amino acid	g/100 g of protein	residue/ 276 000 g of protein[a]
alanine	0.67	113
aspartic acid	8.00	165
arginine	5.33	84
glutamic acid	13.33	249
glycine	3.33	122
histidine	3.33	559
isoleucine	3.67	77
leucine	5.80	122
lysine	4.99	94
phenylalanine	4.99	83
proline	3.99	95
serine	4.40	115
tryptophan[b]	0.90	12
tyrosine	2.43	37
valine	3.47	82
ammonia	3.43	555

[a] To the nearest integer value. [b] Estimated by microbiological method (Barton-Wright, 1952).

Viscosity. The intrinsic viscosity of 10.3S was found to be 0.054 dL/g. Most globular proteins have intrinsic viscosity values ranging from 0.03 to 0.04 dL/g. The observed value therefore was high compared to that of the globular proteins (Tanford, 1961).

Molecular Weight. From the sedimentation coefficient and the intrinsic viscosity values, the molecular weight was calculated using the following equation (Schachman, 1959):

$$MW = \frac{4690(s_{20,w})^{3/2}\eta^{1/2}}{(1-\bar{\nu}\rho)^{3/2}}$$

with the values of $s_{20,w}$ 10; η, 0.054; $\bar{\nu}$, 0.75; and ρ, 1; and

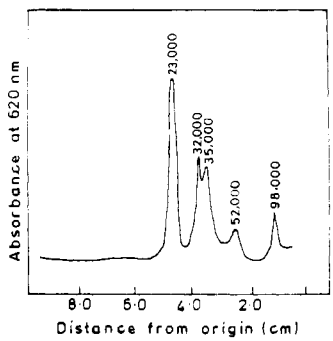

Figure 8. Microdensitometric scanning of NaDodSO$_4$–PAG electrophoretic pattern of chickpea 10.3S protein.

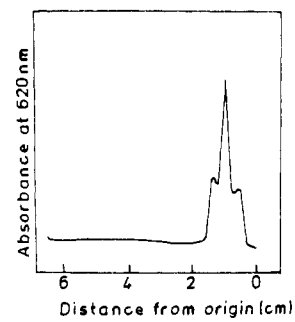

Figure 9. Microdensitometric scanning of PAG electrophoretic pattern of chickpea protein bodies.

a molecular weight of 276 000 was obtained.

Subunits. Chickpea globulins were shown to consist of subunits by dissociation in the presence of urea and by N-terminal amino acid analysis (Jackson et al., 1967). The number of subunits of 10.3S was determined by NaDodSO$_4$–PAG electrophoresis. The pattern consisted of five bands (Figure 8). The molecular weight of the subunits calculated from a standard curve of proteins of known molecular weight were 23 000, 32 000, 35 000, 52 000, and 98 000, respectively. The subunit structure of legumin-like storage globulins, with a characteristic sedimentation coefficient value of 11S from *Glycine max* (Okubo et al., 1969; Catsimpoolas et al., 1971), *Vicia faba* (Wright and Boulter, 1974), and *Vicia sativa* (Vaintraub and Nguyen Thanh Thien, 1968), had been reported. In these globulins, the subunits have molecular weights between 27 000 and 37 000. Since 10.3S protein of chickpea functions as a storage protein (Ganesh Kumar and Venkataraman, 1978), it is more likely that the protein is composed only of the 23 000 and 32 000–35 000 type of subunits in multiple forms, as is the case with other legumin-like reserve proteins (Derbyshire et al., 1976). Further, it is possible that the subunits of molecular weights 52 000 and 98 000 represent artifacts produced by disulfide bond formation between sulfhydryl groups, exposed as a result of disruption of secondary and tertiary structures by the dissociating agent (Tombs and Lowe, 1967).

Free and Total Sulfhydryl Groups. The number of free sulfhydryl groups was 1.3 mol/mol of protein. After reduction with β-mercaptoethanol and dissociation by 8 M urea, the number of free sulfhydryls was 7.4 mol/mol of protein. Thus 10.3S protein had at least three disulfide bonds. The small number of disulfide bonds could partially explain the noncompact structure of 10.3S as evidenced by viscosity data.

Isolated Chickpea Protein Body. It is reported that most of the storage proteins of the seed are located in the subcellular organelles called protein bodies (Ashton, 1976) and the latter was shown to contain various acid hydrolases (Ory and Henningsen, 1969). It was interesting to determine whether the major protein 10.3S is present in chickpea protein bodies or not. For this, the protein bodies were isolated from the defatted meal. The electrophoretic pattern of the chickpea protein bodies (Figure 9) showed one major band (R_f 0.30) and two minor bands (R_f 0.25 and 0.35). By comparison of the R_f values of the globulins (Figure 4G) the major band (R_f 0.30) could be identified with 10.3S protein.

Thus chickpea 10.3S protein is typical of the legume storage proteins. It is oligomeric, nonconjugative, with high molecular weight (~276 000), and composed of at least three subunits. It is not very compact; rather it tends to assume a random coil structure as there are only three S–S linkages in the molecule.

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Estimation of Protein Quality and Quantity in Corn (*Zea mays* L.) by Assaying Protein in Two Solubility Fractions

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The protein from maize seeds can be recovered in two solubility fractions. Fraction 1 includes zeins (low quality proteins), extracted with 60% *tert*-butyl alcohol, and fraction 2, called nonzein (high quality proteins), includes albumins, globulins, glutelins, and others, extracted with a high pH buffer containing an ionic detergent (sodium dodecyl sulfate) and a reducing agent (2-mercaptoethanol). Protein content of the two fractions, determined by a simple Coomassie Blue R-250 dye-binding method, using comparable proteins as standards, gives an estimate of protein quantity of the sample. The zein as percent of total extractable protein or the ratio of zein to nonzein can be used to estimate the protein quality. The method offers promise as a mass-screening tool to identify the maize germ plasm with high protein and/or high quality protein.

For the improvement of protein quality and quantity in major food crops (e.g., cereals and legumes), the plant breeder needs simple, rapid, inexpensive, and accurate screening techniques to identify the germ plasm with high quality and quantity protein. Such techniques are also needed to monitor progress in a breeding program. The discovery of the *opaque-2* maize, which has almost twice the lysine and tryptophan (major limiting essential amino acids in maize) content as compared to normal maize, by Mertz et al. (1964), was the first breakthrough in the search for high quality protein cereals. Subsequently, other maize mutants with high quality protein were found (Misra et al., 1972). Similar mutants were discovered in barley (Munck et al., 1971) and sorghum (Singh and Axtell, 1973). Unfortunately, no such mutant has yet been found in wheat.

Conventional chemical methods for evaluating protein quality (e.g., amino acid analysis) and quantity (e.g., Kjeldahl nitrogen assay) are either time consuming or require sophisticated and expensive instrumentation. Thus, the need for simple and rapid techniques applicable to mass screening has long been recognized. In the case of maize, the high-lysine trait is associated with decreased zein (prolamines) and increased albumin, globulin, glutelin (Misra et al., 1972), and free amino acid levels (Mertz et al., 1974). Consequently, indirect methods based on zein (Paulis et al., 1974; Dalby, 1974; Esen, 1980) and free amino acid determinations (Mertz et al., 1974) have been developed to screen for protein quality. However, no simple and rapid test is available to screen for protein quantity. This report presents data on the application of a protein determination method, recently developed in our laboratory (Esen, 1978), to estimate protein quality and quantity in maize.

MATERIALS AND METHODS

The whole kernel meals used for protein extraction were from the normal (+) and *opaque-2* (o_2) versions of six inbred lines, namely B37, Mo17, Oh43, Mo2OW, W64A, and Mo2RF. The modified *opaque-2* ($modo_2$) version of the inbred Mo2RF was also available and included in this study.

Protein Extraction. Proteins from all of the 13 samples were separated by differential solubility into two fractions zein (fraction 1) and nonzein (fraction 2). A detailed description of zein extraction was described elsewhere (Esen, 1980) and thus only a brief account will be given here.

For zein extraction, 125 mg of meal was weighed and placed in 15 × 100 mm culture tubes (with screw caps) in duplicate. The meal was suspended in 6.25 mL of 60% *tert*-butyl alcohol (C_4H_9OH), the solvent-to-meal ratio (v/w) being 50:1. Tubes, screw caps tightened, were agitated on a rotary shaker at 150 rpm for 30 min, then transferred in a rack to a waterbath and heated at 70 °C for 15 min. Centrifugation was carried out in a table top centrifuge at 1000*g* for 10 min. The supernatants from duplicates of each sample were pooled; this constituted the zein fraction (fraction 1). The pellet was resuspended in 12.5 mL of 0.1 M sodium borate buffer (pH 10) containing 1% (w/v) sodium dodecyl sulfate ($NaDodSO_4$) and 1% (v/v) 2-mercaptoethanol in order to extract the nonzein fraction (fraction 2). In this case, the solvent-to-meal ratio was 100:1, based on the initial weight of the meal, not that of the pellet. The agitation, heating, and centrifugation schedule used for nonzein extraction was the same as that used for zein. The supernatants from duplicates of each sample were pooled; this constituted the nonzein fraction (fraction 2). Both zein and nonzein fractions were stored in 50-mL Erlenmeyer flasks at room temperature prior to analysis of their protein contents. Routinely, analysis was performed either on the day of extraction or the next day.

In a separate experiment, both zein and nonzein extractions were performed twice in order to check the efficiency of single extraction in solubilizing and recovering proteins belonging to each solubility fraction. Following the first zein extraction, the pellet was resuspended in 6.25 mL of 60% C_4H_9OH for a few seconds and immediately centrifuged, and the supernatant was discarded. The

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