PHYSICOCHEMICAL PROPERTIES OF OILSEED PROTEINS

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ABBREVIATIONS

$(NH_4)_2\ SO_4$	Ammonium sulfate
$CaCl_2$	Calcium chloride
NaOH	Sodium hydroxide
Na_2CO_3	Sodium carbonate
$NaHSO_3$	Sodium sulfite
$Na_2H\ PO_4$	Disodium monohydrogen phosphate
Na_2SO_4	Sodium sulfate
$MgCl_2$	Magnesium chloride
Na_3PO_4	Sodium phosphate
$NaHCO_3$	Sodium bicarbonate
NH_4Cl	Ammonium chloride
KCl	Potassium chloride
NaCl	Sodium chloride
SHMP	Sodium hexameta phosphate
H_2O_2	Hydrogen peroxide
GuHCl	Guanidine hydrochloride
NEM	*N*-ethyl maleimide
D_2O	Deuterated water
SDS	Sodium dodecyl sulfate
ANS	Anilino napthalene sulfonic acid
PCMB	Parachloromercuribenzoate
TNBS	Trinitrobenzene sulfonic acid
N	Nitrogen
CGA	Chlorogenic acid
CA	Caffeic acid
QA	Quinic acid
Arg	Arginine
Ile	Isoleucine
Ala	Alanine
SO_4^{--}	Sulfate ion
Cl^-	Chloride ion
Br^-	Bromide ion
ClO_4^-	Perchlorate ion
SCN^-	Thiocyanate ion

I^-	Iodide ion
CCl_3COO^-	Trichloroacetate ion
Cs^+	Caesium ion
K^+	Potassium ion
Na^+	Sodium ion
Li^+	Lithium ion
Ca^{++}	Calcium ion
Mg^{++}	Magnesium ion
μ	Ionic strength
PAGE	Polyacrylamide gel electrophoresis
UV	Ultraviolet
CD	Circular dichroism
ORD	Optical rotatory dispersion
mol wt	Molecular weight
S_{app}	Apparent sedimentation coefficient
$S_{20,w}$	Sedimentation coefficient corrected for temperature and viscosity and extrapolated to zero protein concentration
$D_{20,w}$	Diffusion coefficient corrected for temperature and viscosity
Å	Angstrom units
nm	Nanometer
g	Gram
m*M*	Millimolar
M	Molar
hr	Hour
NPS	Frequency of nonpolar side chains
P	Ratio of the volume occupied by polar residues to that occupied by nonpolar residues
HQ	Average hydrophobicity
M_n	Number average molecular weight
M_w	Weight average molecular weight

I. INTRODUCTION

Oilseed proteins have in recent years attracted the attention of biochemists, plant physiologists, physicists, and protein chemists because of their unique properties. This has resulted in a sudden surge in the scientific literature. This article makes an attempt to consolidate the known information on the physicochemical properties of oilseed proteins. This attempt, it is hoped, would help in recognizing the generalities in their properties and also in identifying the gaps in our knowledge for further research.

Proteins in plants have many roles to play such as acting as storage (reserve) proteins, source of enzymes needed for many metabolic activities, etc. Plant proteins are generally divided into two groups, namely (1) reserve proteins of the seed and (2) functional proteins of the vegetative part of the plant.[1] The emphasis of this article is on the physicochemical properties of the reserve proteins of seeds, especially oilseeds. The functional properties of the reserve proteins have been reviewed by Kinsella.[2] Wherever the functional properties of the proteins are discussed in this review, it is done so only in relation to their physicochemical properties.

Many oilseeds are known and their list is indeed long. The discussion in this article is

confined to major oilseeds only, namely soybean, groundnut (peanut), sesame seed, sunflower seed, rapeseed, mustard seed, cottonseed, and safflower seed. These oilseeds are commercially important and considerable information is available on their proteins. Poppy seed and linseed, where information is available on their proteins, have also been included.

Storage proteins in oilseeds are known to serve as nitrogen reserves and are utilized during germination of the seed, thus supplying the necessary free amino acids and amide nitrogen to the growing plant during the initial stages of germination.[3] They are described as "definable sets of organ- and tissue-specific gene products sequestered and stored in membrane-bound protein bodies for utilization at a subsequent stage of development".[4] These are a group of proteins which differ in structure and function, amino acid composition, solubility, and other physicochemical properties.[3,4] Ashton,[5] in his exhaustive review on the mobilization of storage proteins, discusses the protein body distribution, structure-composition relationship, their fate during germination, and the various proteolytic enzymes, both exo- and endopeptidases. Of relevance to the present discussion is the location of the storage proteins in distinct organelles which are often called "protein bodies". Several other terms such as "protein vacuole", "protein granule", and "aleurone grain" have also been used.[5-7]

It has been know for many years that the storage organ of seeds contains numerous granules within the cells, and these have been called aleurone grains.[8,9] Since they give the positive stain for proteins, it was suggested that they might be the location of proteins in seeds. This conclusion has been confirmed and is further supported by electron microscopy by various workers. These data support the cytological evidence that subcellular bodies in storage tissue are present in seeds. Strong evidence emerged in the early 1960s that these are indeed protein bodies, and a significant proportion of the proteins of seeds is located in such bodies. Based on these studies, Altschul[10] has drawn some tentative generalizations. According to him, most of the seeds contain what are called protein bodies, and in many seeds the proteins present in these protein bodies represent a majority of the total seed proteins. Thus, the uniqueness of seeds may be that they contain substantial amounts of particle-bound proteins. It is possible that these proteins may possess some unique features which confine them to the protein bodies. Thus, unlike other tissues, seeds are a class of tissues which secrete proteins and the secretions are retained within the cell. Hence, it might be expected that these protein bodies in seeds of the same species or botanical family may have something common in their composition and physicochemical properties. It is possible, however, that there are numerous types of protein bodies in the same species differing in their physicochemical properties.[10]

A considerable amount of work has gone into understanding the protein bodies of soybean and groundnut and their location and function in the seed. In soybean (*Glycine max*), e.g., it has been shown that the protein bodies are filled with large amounts of legumin, some vicillin, and five other components.[11-13] Groundnut (*Arachis hypogea*) protein bodies have been shown to contain mainly arachin, whereas the cytoplasm has been shown to contain conarachin.[14-16] It has also been shown that protein bodies contain, besides storage proteins, other proteins such as enzymes, lectins, etc. in much lesser amounts. The accumulation of storage proteins in protein bodies is akin to starch accumulation in starch granules or oil in spherosomes, all located in reserve tissues. It should also be noted that some proteins like glutelins are accumulated outside the protein body.[17] These days, storage proteins from seeds are treated in the same category as secretory proteins since they undergo post-translational modifications.[17] Another important observation is the heterogeneity of these seed proteins.[18] This may be a result of polymorphism and occurrence of several degrees of oligomerization (resulting in a series of sedimentation coefficients). This may lead to a multiplicity of quaternary structures.[17,19] As Pernollet and Mosse[17] have rightly concluded, these proteins share a common feature with animal storage proteins such as caseins in that they are all trophic secretory proteins able to associate, they exhibit relative multiplicity and polymorphism, and their deposition is close to phosphate reserves in the cell.

Pernollet and Mosse[17] have reviewed in detail the location and structure of storage proteins both in legumes and cereals. According to them, the distribution of the storage proteins falls into two major groups: (1) legumin and vicillin in legumes and (2) prolamine and glutelin in cereals. From the available literature on oilseed proteins, it may be generalized that the storage proteins consist of one group of high molecular weight proteins and the other of low molecular weight proteins. The scope of this article is limited to reviewing the work on the total proteins of various oilseeds, their characterization, fractionation into the high and low molecular weight proteins, description of their physicochemical properties, and their interactions. Similarity in their composition and properties is discussed in detail. An attempt is also made to correlate their structure with their function in the seed.

II. SOYBEAN PROTEINS

Soybean (*Glycine max*) is a legume seed in which the food reserves are stored in the cotyledons. The major portion of the reserve proteins is found in protein bodies which are made up of nearly 90% protein. Oil is located in small spherosomes which are dispersed between the protein bodies.[12] The soy proteins and other constituents present in the seed have been exhaustively reviewed by Wolf.[20,21]

Soybean proteins are soluble in water to the extent of 90 to 95%.[22] The protein isolate is prepared from the defatted flour by alkali peptization and isoelectric precipitation. Isoelectric precipitation results in the precipitation of 92 to 94% of the proteins, and the supernatant whey proteins represent 6 to 8% of total proteins.[23] The globulins, which are precipitated at the isoelectric pH, can be separated in an analytical ultracentrifuge into four components with sedimentation coefficients of 2S, 7S, 11S, and 15S, into four to five components in moving boundary electrophoresis, and seven fractions in gel electrophoresis.[24,26,116] The globulins are classified into four fractions of which the 11S globulin, glycinin, is the most studied. It and the 7S fraction each form about 30% of the total proteins. The 7S fraction is referred to a conglycinin. It is heterogeneous, as it contains at least four different proteins including lipoxygenase and a glycoprotein. The other fractions are the 2S protein(s) and 15S protein. The whey proteins have been shown to contain several electrophoretically distinct components having $S_{20,w}$ values ranging from 1S to 6S.[26-28]

The nomenclature of Naismith[25] has generally been used in identifying the various globulin fractions of soybean proteins, namely 2S, 7S, 11S, and 15S proteins. However, due to variation in the number of components observed in different analytical techniques, Catsimpoolas et al.[29] have proposed the following nomenclature: 2S (α-conglycinin, different from soybean trypsin inhibitor), 7S (β-conglycinin, isolated by the method of Roberts and Briggs),[30] 7S (γ-conglycinin isolated by the method of Koshiyama and Iguchi),[31] and 11S (glycinin, high molecular weight protein component). The 15S protein is possibly a polymer of the 11S protein resulting from intermolecular S–S bonding. Koshiyama[61] did not find any difference in the protein composition of soybean globulin and that of protein bodies.

The reported molecular weight of the 11S protein is 300,000, while that of the 2S protein is ~25,000.[62,64] However, there is one report[63] that four protein fractions with a molecular weight of more than 300,000 were observed in soybean. It is not clear if this is genuinely due to varietal differences of microheterogeneity of the preparation. Utsumi et al.[127] have shown that the 11S protein has microheterogeneity and detected four molecular species with molecular weights ranging from 340,000 to 375,000. According to them, microheterogeneity is an inherent property of the storage proteins of legume seeds. The preparation of an acid-sensitive protein fraction from soybean flour has also been reported.[68]

The properties of soybean proteins are markedly affected by pH, salt concentration, temperature, and storage conditions of the seed and proteins.[56-60,67,118,132,134] The protein isolate stored at −1 to 3°C was insoluble in pH 7.0 buffer, but that stored at −20°C was

80% soluble.[60] However, treatment of the isolate with parachloromecuribenzoate (PCMB) or *N*-ethyl malemide (NEM) increased the solubility. The –SH groups seem to play a role in determining protein solubility. Saio et al.[131] have studied the solubility of proteins from soybean stored as whole, full fat, or defatted meal under controlled conditions of high temperature and moisture. With increased time of storage, the extractabilities of 7S and 11S components decreased, whereas that of 2S component increased. The proteins seem to be more in the native state when the intracellular membranes are intact as judged by their denaturation behavior. Shen[67] determined the solubility of freeze-dried and acid-precipitated soybean proteins as a function of pH and ionic strength. Loss of protein solubility was not related to denaturation of the proteins at pH 12.0. Although pH 12 treatment denatured the proteins, it actually increased protein solubility. Nash and Wolf[118] observed a decrease in the solubility of freeze-dried protein isolate stored in the dark at room temperature for 5 years. The decrease was 6% per year. Storage decreased the solubility of 2S, 7S, and 11S proteins and increased that of the 15S proteins. Disulfide cross-linked polymers were observed. The loss in protein solubility appeared to be linked with polymers formed from 7S and 11S proteins. When the 7S and 11S proteins were stored at 11 or 96% relative humidity at 50°C, polymers were formed.[132] The polymerization of the 7S protein involved hydrogen, hydrophobic, and S–S bond formation, whereas that of the 11S protein was mainly due to S–S bond formation. The solubility of the proteins was also decreased. However, the addition of β-mercaptoethanol restored the solubility, possibly due to the depolymerization of the polymers. Hoshi and Yamauchi[134] also observed the formation of aggregates and decreased solubility of the 11S protein when it was stored at a relative humidity of 96% and 50°C for various intervals of time. Storage at 100°C increased the extent of aggregation. The presence of aggregates was confirmed by electron microscopy. Aggregation was due to intermolecular S–S bond formation.

A. Fractionation of Proteins

Wolf[20] has reviewed in detail the literature on nitrogen extractability of soybean proteins and the methods of their fractionation. The effect of parameters such as pH, temperature, meal-to-solvent ratio, composition of the solvent, etc. on nitrogen extractability has been reviewed. For the fractionation of soybean proteins many techniques such as $(NH_4)_2SO_4$ precipitation,[25] cryoprecipitation,[70] ion-exchange chromatography,[32-37,66] hydroxylapatite chromatography,[38-43] electrophoresis,[44] isoelectric focusing,[49] immunodiffusion and immunoelectrophoresis,[34] affinity chromatography,[51] gel filtration,[39,52-54] and precipitation by metal ions have been used.[55] Since the properties of soybean proteins are markedly affected by pH, salt concentration, and temperature, suitable combinations of them have also been used for the fractionation of soybean proteins.[56-59]

It has been shown that partially purified soybean globulins are soluble even at the isoelectric pH of 4.5 if the salt concentration is 0.25 *M*.[65] Below this critical concentration there was separation into two layers, a protein-poor upper layer and a protein-rich lower layer.

Fractionation of the extract of defatted soybean flour in water or NaCl solution by $(NH_4)_2SO_4$,[25] metal ions,[55] or cryoprecipitation[70] does not lead to homogeneous fractions. Generally a combination of purification procedures has been used to obtain homogeneous protein preparations. To test the homogeneity of the protein, often a single analytical method such as analytical ultracentrifugation, gel electrophoresis, or gel filtration has been used. It is desirable to determine the homogeneity by more than one analytical method and preferably at two or three pH values. The reported differences in the physicochemical characteristics of "pure" soybean protein fractions could be due to the fact that all of them were not homogeneous.

B. Glycinin (11S Globulin)

1. Isolation

Several methods have been described to obtain fairly homogeneous glycinin. Wolf and Briggs[69] obtained nearly 87% pure glycinin by fractional extraction of acid-precipitated soybean proteins in NaCl solutions of different molarity. The cold-insoluble fraction was precipitated between 0.51 and 0.66 $(NH_4)_2SO_4$ saturation and this protein was 92% pure glycinin.[70] Shvarts and Vaintraub[71] have described a method for the isolation of glycinin. The cold-insoluble soybean fraction was separated on a Sephadex® G-100 column by eluting it with solutions of 0.40 to 0.73 $(NH_4)_2SO_4$ saturation. Elridge and Wolf[72] purified glycinin by using pH 4.6 buffers of varying ionic strength at low temperature. A nearly 90% pure glycinin preparation was obtained. Koshiyama[73] extracted the proteins from the meal at 2 to 4°C for 12 hr, dialyzed the extract, and separated it into fractions on Sephadex® G-100 and -200 columns. Since glycinin is precipitated to a greater extent than 7S protein by low concentrations of Ca^{++}, Saio et al.[74] attempted to isolate glycinin by direct extraction of the flour with dilute $CaCl_2$ solution. However, neither the 11S nor the 7S protein fraction was pure. Only enrichment of the fractions occurred. Kitamura et al.[51,75] have purified the 11S globulin of Wolf and Briggs[69] by affinity chromatography on concanavalin A-Sepharose 4B followed by gel filtration on Sepharose 6B. The contaminating 7S protein is bound by concanavalin A. Based on the differential solubility of 11S and 7S proteins in Mg^{++} solutions, Appu Rao and Narasinga Rao[76] have described a method to obtain homogeneous 11S.

2. Amino Acid Composition and Physicochemical Properties

Wolf[20,21] has reviewed the chemical and physicochemical characteristics of the proteins of soybean. The amino acid composition of glycinin is given in Table 1. It is characterized by a high content of aspartic and glutamic acids. Hoshi and Yamauchi[135] have reported that the number of the surface SH, interval SH, and S–S bonds of glycinin are 10.3, 3.6, and 17 mol/mol of protein, respectively. The corresponding values of the lyophilized protein are 5.0, 4.6, and 20.1 mol/mol. The three newly formed S–S bonds could be between the subunits, which results in the formation of aggregates. Draper and Catsimpoolas[108] have reported that the S–S content of glycinin in 8 *M* urea is 20 groups per molecule and that most of them are buried in the interior of the protein molecule in the native state.

The physicochemical parameters of glycinin are listed in Table 2. It has $S_{20,w}$ value of 12.35 at the infinite dilution end. The molecular weight determined by different methods varies between 302,000 and 350,000. The variations may be due to limitations of the methods. The sedimentation equilibrium method (a thermodynamically sound method) gives a value of 317,000, the values from the sedimentation-diffusion method and subunit composition are close to this value.

Diep et al.[128] have reported that the intrinsic viscosity of glycinin has a value of 5.75 mℓ/g, suggesting that it is not globular in shape. The molecular shape has been established to be an oblate ellipsoid with an axial ratio of revolution of 8.11 to 8.38 without any hydration.[103] The lengths of the major and minor axes are 178 to 180 and 22 Å, respectively. The shape factor, β, and the hydrodynamically effective volume are 2.14 and 31.5 to 37.0 $\times$ 10^{-20} mℓ, respectively.

The secondary structure of glycinin has been investigated by many workers.[77,88-91] It is low in α-helix content and has a fair amount of β-structure and aperiodic structure. The reported values are 5 to 8% α-helix, 31 to 35% β-structure, and ~60% aperiodic structure.

The study of the dissociation and denaturation of glycinin has been the subject of many investigations. The effect of acid and alkali,[49,57,77,84,86,105,117,128] urea/GuHCl/detergents,[69,77-81,86,87,88,91,114,120] and organic solvents[83,105] has been studied. At low pH and low ionic strength, the protein dissociates into a slow-sedimenting component.[57,84] Denaturation

Table 1
AMINO ACID COMPOSITION OF 11S (GLYCININ), 7S (β-CONGLYCININ, AND 7S (γ-CONGLYCININ)

	Residues per 100,000 g of protein		
Amino acid	11S[71]	β-Conglycinin[59]	γ-Conglycinin[58]
Aspartic acid	106	110	106
Threonine	44	17	24
Serine	74	47	64
Glutamic acid	169	144	139
Proline	50	42	39
Glycine	64	33	38
Alanine	47	33	42
Valine	43	39	43
Methionine	9	2	2
Isoleucine	45	40	49
Leucine	56	63	78
Tyrosine	24	23	20
Lysine	33	45	48
Arginine	45	53	51
Tryptophan	7	—	2
Phenylalanine	34	41	45
Histidine	17	15	11
Half cystine	7	—	—

Table 2
PHYSICOCHEMICAL PROPERTIES OF 11S GLOBULIN (GLYCININ)[21,140]

Property	Value
Nitrogen content (%)	16.3
Extinction coefficient ($E^{1\%}_{1cm,\ 280\ nm}$)	8.1 ± 0.1
Partial specific volume ($\bar{V}$, mℓ/g)	0.730 ± 0.001
Sedimentation coefficient ($S_{20,w}$)	12.35 ± 0.15
Diffusion coefficient ($D_{20,w}$ cm²/sec)	3.44 ± 0.1 × 10^{-7}
Radius of gyration (R_g)	44 Å
Hydration (g/g)	0.36
Stokes radius	58.5 Å
Number of subunits	12
Acidic	6
Basic	6
N-terminals	Leu 2; Ileu 2; Phe 2; Gly 6
Molecular weight	
Gel electrophoresis	350,000 ± 35,000
Gel filtration	302,000 ± 33,000
Sedimentation equilibrium	317,000 ± 15,000
Sedimentation diffusion	322,000 ± 15,000
From subunit sizes	326,000 ± 35,000
Size	
Electron microscopy	
As observed	100 × 100 × 70 Å
Allowing for hydrophobic region	100 × 110 × 80 Å
X-ray scattering	110 × 110 × 75 Å

of the protein also occurs. It starts at pH 3.5 and reaches a maximum at pH 2.0, completely exposing tyrosine, tryptophan, and phenylalanine residues. At pH 2.6, it dissociates into three fractions of molecular weight 63,000.[86] Hashizume and Watanabe[101] have reported that glycinin treated with HCl (pH 1.5) or NaOH (pH 12.5) for 2 hr at room temperature lost the ability to form a gel. Addition of glucono-lactone restored the ability. Diep and Boulet,[105] however, observed that at pH 2.0 and low ionic strength of 0.01 to 0.02 the protein dissociated into subfractions of 1.0S to 1.1S with molecular weight in the range 15,000 to 20,000; the axial ratio of the molecule increased to between 13 to 17. Increase in ionic strength at constant pH favored reassociation of the subfractions into molecules of higher molecular weight and to a more symmetrical shape. It has also been reported by viscosity measurements that at extreme pH (2.0 or 11.0) and low ionic strength (0.01 to 0.05), the protein dissociates and unfolds; this can be partially reversed by high ionic strength.[128] Alkali treatment of glycinin has been shown to increase its susceptibility to proteolysis. Also, spectrophotometric titration of tyrosine becomes irreversible.[49,77] Obviously the protein is denatured by alkali treatment. Alkali treatment dissociates the protein and the number of fractions entering the electrophoresis gel increases.[117]

Urea and GuHCl have been reported to dissociate and denature glycinin. At pH 7.4, 4 *M* urea dissociates the protein into two subunits of molecular weight 31,000.[86] A stable 4S intermediate also appears to be formed.[87] At low concentrations of urea or GuHCl, tyrosine and tryptophan groups of the protein are exposed. At 6 *M* concentrations, these reagents dissociate the protein into 7S and 3S fractions. Thus there appears to be some incompatibility in the results reported by different workers. Probably the pH and ionic strength used were not the same in all the cases. For example, it has been shown that 5 and 8 *M* urea-denatured glycinin were hydrolyzed more readily in low ionic strength medium than in high ionic strength medium.[114] Obviously, the proteins were less denatured in high ionic strength medium. Renaturation of 8 *M* urea-denatured glycinin has been reported.[104] The renatured protein was indistinguishable from the native protein in gel chromatography, gel electrophoresis, immunological, sedimentation, and circular dichroism (CD) spectral behavior. The subunit composition was also identical. Better renaturation of the protein occurred with 8 *M* urea-denatured glycinin than with 5 *M* urea-denatured protein.[114] This has been attributed to the decreased digestibility of 8 *M* urea-denatured glycinin.

Sureschandra[91] has studied the dissociation/denaturation of glycinin by urea, GuHCl, sodium dodecyl sulfate (SDS) using several physicochemical techniques. The protein has been shown to be dissociated to a 2S component through the intermediate 7S component. The dissociation of the protein by SDS and urea was largely reversible, whereas that by GuHCl is not reversible. It has been concluded that perhaps dissociation and denaturation proceed simultaneously. Difference spectra in the ultraviolet region with characteristic minima at 280, 287, and 292 nm were generated by the addition of the denaturants. Fluorescence quenching and a red shift in the fluorescence maximum were observed. The reduced viscosity of the protein increased with denaturant concentration, and at 6 *M* GuHCl, 8 *M* urea, and 0.5% SDS the value was 27.7, 26.0, and 8.7 mℓ/g, respectively. The optical rotatory dispersion (ORD) and CD results indicated that the tertiary structure of the protein was disrupted with increase in denaturant concentration initially, and ultimately aperiodic structure dominated the CD spectrum. The protein appeared to be stable in the temperature range 15 to 60°C as judged by the far UV-CD and ORD spectrum. In urea and GuHCl solution, the protein appeared to be completely denatured. However, SDS increased the ellipticity values in the far UV-CD, a result similar to that reported by Koshiyama and Fukushima.[88] An analysis of the data obtained by various techniques suggested that denaturation of 11S protein was possibly a two-state process. The free energy change for the reaction had a value of 2 to 3 kcal/mol, indicating that the native structure was only marginally more stable than the denatured state. However, the observation of change in enthalpy of reaction with

change in GuHCl concentration was rather uncommon. The free energy changes and the difference in the number of binding sites for the denaturants were relatively small for glycinin as compared to proteins having high α-helix or β-structure.

The interaction of urea and GuHCl with glycinin was also studied by measuring the apparent partial specific volume of the protein in these solvents under isomolal and isopotential conditions.[137,138] In both urea and GuHCl solutions, the interaction was not preferential with water. The interaction parameter was calculated as 0.10 and 0.11 g of urea and GuHCl, respectively, per gram of protein. A total binding of 545 mol of urea and 525 mol of GuHCl per mole of protein was observed. The changes in volume upon transferring glycinin from dilute salt solution to urea and GuHCl solution have also been calculated.

Kamata et al.[120] have shown that upon removal of 8 *M* urea from the acid-precipitated soybean protein it formed a gel at high protein concentration. The presence of disulfide bridges in the gel structure was also shown and has been attributed to formation during urea denaturation. Glycinin was shown to be a major contributor to the disulfide bridge formation.

Fukushima[83] showed that the denaturing ability of alcohols increased with increase in hydrophobicity of the alcohol. Glycinin denatured with alcohols was more suspectible to proteolysis. Diep and Boulet[105] have reported that butanol at 5% concentration dissociated glycinin into 2S, 6S, and 9S fractions. The role of electrostatic charges in the association-dissociation reaction of the protein is discussed.

The effect of heat on the 11S and 7S proteins of soybean has been studied to determine the thermal behavior and gel-forming ability of these proteins. Catsimpoolas et al.[48] and Wolf and Tamura[82] have studied the effect of heat on the association-dissociation of glycinin. Above 70°C, the protein dissociated into subunits. These dissociated subunits had a tendency to interact and form insoluble aggregates. However, extreme acid or alkaline pH, or high ionic strength decreased the extent of aggregation. Complete cleavage of −SS bonds of glycinin with 2-mercaptoethanol favored the aggregation reaction. The driving force for the formation of insoluble aggregates has been shown to be hydrophobic interactions and stabilization by ionic linkages. Hashizume et al.[100] have shown that glycinin is dissociated by heat to subunits in low ionic strength medium. Yamagishi et al.[119] have examined the heat denaturation of soybean 11S globulin at 70 and 100°C in 0.5 ionic strength buffer. One kind of aggregate contained highly polymerized subunits (nonspecific) and the other monomer of basic subunit, along with seven kinds of oligomers having various proportions of basic to acidic subunits. Iwabuchi and Shibasaki[123] have studied the denaturation of glycinin heated at 100°C for 5 min in solutions of ionic strength 0 to 0.4. Resistance to thermal denaturation increased with increasing concentrations of both KCl and potassium phosphate. Selection of a suitable ionic strength was shown to be critical for the preparation of thermally denatured 11S globulin that had desired amounts of residual structures and was useful for several functional applications.

Yamagishi et al.[121] have used second-derivative spectroscopy to discriminate between buried and exposed tryptophan residues of protein in solvents of different polarity. Half of the tyrosine residues that were buried in native protein were exposed by heating, but tryptophan residues were hardly exposed. This has been interpreted as being due to random cross-linking of SH residues on heating, causing them to retain the hydrophobic region buried in the protein molecule. They have also studied the effect of heat on 11S globulin in the presence of NEM using the technique of differential thermal analysis.[122] The temperature of the endothermic peak of glycinin heated in presence of NEM was slightly higher than that in the absence of NEM. Soluble aggregates were recognized by gel filtration and electrophoresis. With increase in heating time, polymers were formed.

Bikbov et al.[136] have studied the thermal denaturation of glycinin and 7S protein in dilute solution by differential adiabitic scanning calorimetry. The thermograms showed two maxima. The low temperature maximum was consistent with denaturation of 7S component and the high temperature maximum with denaturation of glycinin. The denaturation temperature

changed linearly with salt concentration. The strong influence of NaCl on the temperature of thermal denaturation of the main globulin fraction can be accounted for by a relatively high content of hydrophobic residues in these proteins.

Koshiyama et al.[125] have evaluated the heat denaturation enthalpies of glycinin as 2.0 and 3.2 cal/g at 0.1 and 0.5 ionic strength, respectively. Correlation was not obtained between the heat stability and the content of the ordered secondary structure or a dissociation-association reaction of the protein. However, increase of the hydrophobic region at high ionic strength indicated the possibility of stabilization of the quaternary structure of glycinin by hydrophobic interaction during the process of heat denaturation.

Mori et al.[126] have studied the association-dissociation behavior of glycinin by sucrose density gradient and polyacrylamide gel electrophoresis (PAGE). Soluble aggregates with a molecular weight of 8×10^6 were formed when 0.5 and 5% protein solutions were heated for 1 min at 100°C. At lower protein concentration, subsequent heating caused disappearance of the soluble aggregate, followed by complete dissociation into acidic and basic subunits. At higher concentration, however, subsequent heating caused formation of highly polymerized aggregates and a gel formed after 5 min of heating. The soluble aggregates appear to be transient intermediates in the course of gel formation.

Saio et al.[94,95] have determined the qualitative changes occurring in 7S and 11S proteins of soybean during heat treatment in the range of 70 to 100°C. Heating of protein pastes of 25% protein concentration around 100°C resulted in the formation of an insoluble gel. On heating up to 140°C, the gel gradually became soluble but the gross structure of the subunits remained unchanged. The 11S gel was more soluble than the 7S gel. Above 150°C, the gel became more soluble with a concomitant degradation of the gross structure of subunits (dissociation).

Ishino and Kudo[113] have shown that in order to form aggregates suitable for gelation the proteins must unfold and dissociate into subunits (above pH 11). For the formation of a suitable gel, high ionic strength of the medium, formation of hydrogen, hydrophobic and S–S bonds, and high protein concentration (~8%) are necessary.

Glycinin appears to make much harder tofu gels than 7S protein.[110] These gels are more sensitive to the softening effect of phytic acid. The S–S bonds appear to predominate in glycinin gels. The 11S protein has been shown to precipitate faster and form larger aggregates. It has a higher water-holding capacity than the gels of 7S protein, higher tensile values, higher hardness, and expands more on heating. These results suggest that the functional properties of soybean proteins may be controlled by regulating the binding forces through pH and various additives.

Generally, the gels obtained by 7S or 11S protein or a combination of both are opaque. However, Ishino and Kudo[106] have been able to obtain very firm and translucent gels containing ~2% protein by mixing alkaline solutions of 7S or 11S soybean protein with ethyl alcohol. The 7S protein formed a stronger gel than the 11S protein. The formation of gels has been explained on the basis of dissociation and unfolding of the proteins and the effect of viscosity.

The electrometric and spectrophotometric titration curves of glycinin in native and denatured state have been reported.[85] The irreversibility of the H^+ titration curve of the protein in the pH range 6.5 to 12.0 indicated conformational change of the protein in alkali. However, in urea it was reversible. The number of groups of each class titrated approached the values expected from amino acid analysis data only in the presence of 6 *M* GuHCl and in backward titration in 0.4 *M* KCl. The pK_{int} of carboxyl and ϵ-amino groups were normal in KCl. However, the pK_{int} of imidazole and tyrosyl groups exhibited higher values. The results suggest that some ionizable groups are buried inside the molecule in the native state. The protein has been shown to swell and dissociate into subunits in the acid range.

Okubo et al.[102] have determined the binding of phytic acid to glycinin over a wide pH range. The binding increased as the pH decreased and no binding was detected above pH

6.0. At extreme acid pH, nearly 424 equivalents of phytic acid were bound per mole of glycinin dimer (360,000). This correlated well with the experimentally determined number of basic amino acid residues. It was concluded that the state of quaternary structure was a determinant of the binding. Electrostatic interaction of carboxylate groups with the cationic binding sites hinders phytate binding. Ca^{++} was found to promote dissociation of phytic acid-glycinin complexes at pH 3.0 as determined by dissolution of the insoluble complexes and by gel filtration on Sephadex® G-75. The dissociation has been explained in terms of competition between Ca^{++} and the cationic sites of the protein for the phosphate groups of phytic acid.

Takagi et al.[112] have determined the binding of anilino napthalene sulfonic acid (ANS) to glycinin. The amounts bound by native and heat-denatured glycinin were 15 and 33 mol/mol, respectively. There was an increase in the fluorescence intensity of the heated sample. Possibly, hydrophobic bonding occurs during heat polymerization and gelation of the protein.

Damodaran and Kinsella[124] have studied the binding affinity of 2-nanone to various protein fractions of soybean. The binding by the 7S fraction was very similar to that of the whole soybean protein; glycinin had almost no affinity for 2-nanone. The differences in binding by 7S and 11S has been interpreted as due to structural differences of these two proteins. The binding decreased due to treatment with urea and to succinylation. It has been attributed to structural changes.

Lampart-Szczapa and Jankiewicz[133] have studied the interaction of 11S globulin and wheat proteins; a high molecular weight 11S globulin prolamine complex was observed. The interaction of glycinin and myosin under a variety of solution conditions has been studied.[129,130] The proteins interacted at 85 to 100°C. The acidic subunits of glycinin showed little or no affinity for myosin heavy chain subunits, but the basic subunits interacted. Haga et al.[93] have studied glycinin-myosin B interaction at various temperatures and protein concentrations. At 60°C and low protein concentrations, glycinin had no effect on myosin aggregation; at higher protein concentrations, glycinin promoted myosin B gelation. On the other hand, at 90°C and low protein concentrations, myosin B inhibited aggregation of glycinin; at higher protein concentration, myosin B acted as an aggregation promoter of the protein, indicating the importance of protein concentration and temperature.

Appu Rao and Narasinga Rao studied the binding of Ca^{++}, Mg^{++}, and Zn^{++} by glycinin.[96-99] The binding was negligible at pH 5.5, whereas it was considerable at pH 7.8. Whereas the addition of NaCl or prior EDTA treatment of the protein decreased the affinity of the protein for Ca^{++} and Mg^{++}, it had no effect on Zn^{++} binding. The imidazole groups of histidine residues appear to bind the metal ions. The protein was quantitatively precipitated by 1.0×10^{-2} *M* Ca^{++} or Mg^{++} and 4.0×10^{-3} *M* Zn^{++}. Addition of Ca^{++} did not cause association or dissociation of the protein, but increased its heat coagulation. These results were used to develop a method for the isolation of 2S, 7S, and 11S proteins of soybean.[76]

Catsimpoolas[92] has studied the trypsin and pepsin digestion of glycinin at different pH (8.0 to 11.0), temperature (22 to 47°C), and time (1 to 16 hr) of hydrolysis. The molecular weight of the peptides was determined using Sephadex® microbore columns. The number and weight-average molecular weights were calculated. The molecular weight of the tryptic and peptic peptides was in the range of 8,000 to 10,000. The kinetics of the tryptic proleolysis of glycinin indicate the existence of at least two simultaneous reactions proceeding at different rates.[109] The rate constant suggested that a group of peptide bonds was much more susceptible to proteolytic attack than others. Acidic subunits of glycinin were digested forming 13,500 and 16,000 mol wt fragments, whereas basic subunits were not digested at all.

From carboxyamide methyl 1-glycinin, carboxyamide methyl acidic and basic subunits have been prepared.[107] They were subjected to tryptic hydrolysis and the molecular weight distribution of the fragments was determined.

Measurements with glycinin succinylated to different extents showed that chemical modification dissociated and denatured the protein. Susceptibility to proteolysis increased.[115] Acetylation of glycinin decreased its emulsifying capacity, but increased the emulsion stability.[111]

3. Subunits

Several attempts have been made to dissociate the glycinin molecule in various dissociating and denaturing solvents and to isolate the subunits. The number of subunits, the nature of the subunits, the forces that hold them together, and the primary and secondary structures of these molecules have been determined. Reconstitution of the 11S globulin from these subunits has also been achieved. Hybridization of these subunits with subunits of other oilseed proteins has also been attempted.

Catsimpoolas and Wang[80] have developed a method for isoelectric focusing of the glycinin subunits in sucrose density gradients containing urea and dithiothreitol. They have interpreted the microheterogeneity found in glycinin as due mainly to the differences in the primary structure of the subunits. Catsimpoolas et al.[139] have reported that the major soybean globulin is composed of six different subunits having molecular weights of ~22,300 and 37,200. The "acidic" subunits have a higher content of glutamic acid and proline, whereas the "basic" subunits have higher contents of hydrophobic amino acids, leucine, tyrosine, phenylalanine, valine, and alanine.

Badley et al.[140] have isolated the major storage protein of soybean — glycinin — in a homogeneous form and have characterized it in great detail. The protein had a molecular weight of 320,000 and contained two subunits with different isoelectric points. There were six acidic subunits of ~35,000 mol wt and six basic subunits of ~20,000 mol wt. Detailed analysis of the subunits indicated three different kinds of both acidic and basic subunits. The 12 subunits were shown to be packed in two identical hexagons, placed one above the other, yielding a hollow oblate cylinder of 110 × 110 × 75 Å. The central core hole was blocked, possibly because of the nonspherical shape of the subunits.

Kitamura and Shibasaki[141] have isolated four kinds of acidic subunits and three kinds of basic subunits of 11S globulin. The four acidic subunits were designated A_1, A_2, A_3, and A_4. The molecular weights of A_1, A_2, and A_3, were 37,000; A_4 was 45,000. The N-terminal amino acids were determined as phenylalanine for A_1 and A_2 and as leucine for A_3 and A_4. The amino acid composition of these acidic subunits was roughly similar to each other, but differences were observed in the content of basic acids, namely lysine, histidine and arginine, serine, and proline. Kitamura and Shibasaki[142] have also shown homology between the four acidic subunits of 11S protein. Among the four subunits, the subunit A_4 was quite different from the rest as revealed by immunodiffusion analysis. Kitamura et al.[143] have further shown that the acidic and basic subunits are present in equimolar amounts in the 11S globulin molecule. The four acidic subunits (A_1, A_2, A_3, and A_4) were present in the approximate molar ratio of 1:1:2:2. The four basic subunits were designated B_1, B_2, B_3, and B_4. They were present in the approximate molar ratio of 1:1:2:2. The S–S bonds appear to participate in the binding between the acidic and basic subunits in the molar ratio of 1:1. They proposed a model for the subunit structure of 11S globulin as $(A_1, A_2)(B_3) + (A_3) + (B_1 B_2) + A_4 B_4$. Kitamura and Shibasaki[144] have demonstrated that the acidic subunits of 11S globulin, A_1 and A_2, are distinct polypeptide subunits which have very similar primary structures. Also A_1, A_2, and A_3 subunits are strongly immunologically related, indicating a considerable degree of sequence homology. However, the A_4 subunit was found to be a serologically distinct polypeptide, although some similarities of amino acid sequence were observed.

The acidic and basic subunits of glycinin have been isolated by chromatography on Sephadex® microbore column with a two-step pH change of the elution buffer.[145] Abnormal behavior of the subunits in SDS-PAGE was observed. Yanagi et al.[146] have shown that 11S

glycinin contains acidic and basic subunits and each of the acidic and basic subunits contains three major polypeptides of similar size. The molecular weights of the acidic and basic subunits were shown to be 28,000 and 18,000, respectively. Lynch et al.[147] have shown that the acidic subunits of glycinin are hydrolyzed faster than the basic subunits by pepsin and trypsin. Trypsin hydrolyzed glycinin at ~10% of the rate of hydrolysis of pepsin.

Moreira et al.[148] have purified and characterized the subunits of 11S protein. Six acidic polypeptides and basic polypeptides were isolated. The acidic polypeptides had phenylalanine, leucine, isoleucine, and arginine at the N-termini, whereas the basic polypeptides all had glycine at the N-termini. Considerable homology was observed in the N-terminal sequence indicating that possibly the members of each family arose from a common ancestral gene. Some of the polypeptides were also shown to contain nearly three- to sixfold more methionine than some others. Mori et al.[149] have isolated the acidic and basic subunits of soybean glycinin designated as AS_{1+2}, AS_{2+3}, AS_4, AS_5, and AS_6 and BS, respectively. Reconstitution experiments were carried out and intermediary subunits were investigated. Furthermore, formation of intermediary complexes was observed when native acidic and basic subunits of soybean glycinin and sesame 13S globulin were mixed under reductively denatured condition and subjected to reconstitution procedure. It was concluded that the complexes were probably hybrid intermediary subunits. Utsumi et al.[150] have constituted pseudo and hybrid 11S globulins from native acidic and basic subunits of soybean and broad bean 11S globulin. The subunit structures of these two globulins are known to be similar to each other. Both the pseudo and hybrid 11S globulins were similar in subunit structure to native 11S globulin.

The hybridization of different subunits from different oilseeds has been achieved.[151] The acidic subunit of sesame seed and the basic subunit of soybean have been combined to obtain an intermediary subunit bound by S–S linkages and also a new type of hybrid protein with a molecular weight of 265,000 and a sedimentation constant of 9.6S in 20% glycerol. However, the combination of soybean acidic subunits and sesame basic subunits yielded a new type of protein whose molecular weight was estimated to be 310,000. Staswick et al.[152] have isolated five complexes consisting of one acidic and one basic subunit that were linked, via disulfide bond, from unreduced S-alkylated glycinin. The subunit pairs were $A_{1a}B_2$, $A_{1b}B_b$, A_2B_{1a}, A_3B_4, and $F_2(2)B_3$. They have shown that pairing between subunits is nonrandom, which is consistent with the evidence that glycinin is synthesized as a 60,000 mol wt precursor that undergoes post-translational modification to form the individual linked subunits. Moreira et al.[153] have synthesized five acidic polypeptides of the 11S globulin under the direction of fairly homologous genes. Repeated domains of amino acid sequence within each of the five peptides were observed. Moreira et al.[154] have studied the antigenic properties of purified glycinin and its subunits. Antisera against native glycinin did not react with the isolated subunits, and antibodies prepared against the purified subunits were not active against native glycinin. Their results conclusively show that the acidic and basic polypeptides of glycinin were immunologically unrelated. Hence, immunological tests should successfully differentiate some members of the family of acidic subunits, and other Igs should discriminate between members of the family of basic subunits. Iyengar and Ravenstein[155] have isolated four different acidic subunits of glycinin in presence of urea. Results of peptide mapping, amino acid analyses, and automated sequence analyses of the four acidic subunits show a close similarity in their primary structures. Based on their results, they have concluded that glycinin appears to consist of two identical half-molecules, each consisting of three intermediary subunits and two additional acidic subunits that probably occupy the central hole of each half-molecule of glycinin.

Glycinin is a fairly worked upon molecule and is very well understood at a macroscopic level with reference to its secondary structure, molecular weight, shape, size, etc. Some of its physicochemical properties are summarized in Table 2. Glycinin was first shown to be

composed of 12 subunits of 6 different polypeptide chains, with a molecular weight range of 22,000 to 37,000.[33,80] By a number of techniques, it has been clearly demonstrated that the 12 subunits are packed in 2 identical hexagons placed one above the other.[20,21,80,103,140] In this arrangement, every acidic subunit is shown to be associated with three basic subunits and vice versa. This results in a typical oblate cylinder of 75 and 55 Å radius with a sedimentation coefficient of $S_{20,w}$ = 12.3 and a molecular weight of 300,000. However, some recent work of Kitamura et al.[143] suggests that it possibly consists of four acidic and four basic subunits. Pernollet and Mosse[17] have summarized the legumin structure of soybean, groundnut, pea legumin, and broad bean legumin and have proposed a general model for legumin quaternary structure. The model consists of two hexamers (each made up of three acidic and three basic subunits linked by S–S bonds). It has been shown to have a sedimentation coefficient of 12S. They have also indicated that every acidic subunit is opposite three basic ones and vice versa. Possibly the properties of the 11S protein of soybean could be explained by a modification of the above-proposed model of Pernollet and Mosse.[17]

C. 7S Globulins

1. β-Conglycinin

Even though the general term 7S globulin is used, there appears to be microheterogeneity and various "7S globulins" are known. They have been termed "conglycinins" and designated as α-, β-, and γ-conglycinins. Most of the work has been carried out on β-conglycinin. In order to avoid confusion, the terms α-, β-, and γ-conglycinins are specifically used in the course of the discussion. However, the term 7S globulin or "7S component" or "7S protein" pertains only to β-conglycinin.

a. Isolation

Roberts and Briggs[59] obtained nearly 90% homogeneous 7S by purifying the precipitate obtained at 0.8 to 0.9 $(NH_4)_2SO_4$ saturation by gel filtration on Sephadex® G-100 gel. Koshiyama[52,156] obtained nearly 80% pure 7S fraction by a combination of high ionic strength and low pH for its isolation. His method consists of the following steps: (1) isoelectric precipitation of the total proteins at pH 4.5 with 2 *M* HCl, and (2) dissolving the precipitate in 0.6 *M* NaCl solution and dialysis against 0.4 *M* NaCl at pH 7.6 in phosphate buffer. The protein was nearly 90% pure with 10% contamination of 2S component. Bhosale et al.[157] have reported a simple and quick method for the isolation of 7S and 11S proteins from soybean in highly purified form and in considerable yield. The method essentially utilized the difference in solubility of the two proteins in Mg^{++} solution and $(NH_4)_2SO_4$ fractionation for purification. Orthorhombic crystals of 7S protein were obtained at pH 7.8 and 6°C. Sykes and Gayler[158] have isolated a new protein from the reserve protein of soybean which is particularly deficient in methionine and cystine. The molecular weight of the single polypeptide in SDS gel was 48,000. The amino acid composition, N-terminal leucine, and mobility on gel electrophoresis of this polypeptide were all indistinguishable from the subunit of β-conglycinin. In its undissociated form, the protein behaved as a trimer of 137,000 mol wt. It has an $S_{20,w}$ of 7S, in 0.5 ionic strength, and possesses antigenic determinants in common with β-conglycinin. The results indicate that the protein appears to be a new isomer of β-conglycinin, a homogeneous trimer of β-subunits.

The β-conglycinin has been obtained in a fairly homogeneous form from acid-precipitated soybean globulins by $(NH_4)_2SO_4$ fractionation followed by gel filtration on Sephadex® G-100.

Thanh et al.[159] have suggested, from the results of gel chromatography and disc gel electrophroesis of 7S protein, that multiple components observed in disc gel electrophoresis represent multiple 7S soybean proteins and that they are not artifacts. Lee et al.[164] have separated the 7S globulin at different stages of development of the seed into 7S A and B

Table 3
PHYSICOCHEMICAL PROPERTIES OF 7S GLOBULIN[58,169,174]

Property	Value
Isoelectric point[58]	4.9
Absorption maximum (nm)	278—279
Extinction coefficient ($E^{1\%}_{1cm,280\ nm}$)	5.47
Intrinsic viscosity (mℓ/g)	6.4
Sedimentation coefficient ($S_{20,w}$)	7.9
Partial specific volume ($\bar{V}$, mℓ/g)	0.725
Molecular weight	180,000—210,000
Secondary structure[169] (pH 11.4) (%)	
α-Helix	54
β-Structure	39
Aperiodic	17
Subunits[174]	6

fractions. The two fractions were shown to contain identical subunits. Iibuchi and Imahori[166] have shown that the 7S globulin of soybean seed is a mixture of three kinds of proteins, which were designated A, B, and C and were interconvertible.

b. Physicochemical Properties

The amino acid composition of 7S protein is given in Table 1 and its physicochemical properties in Table 3. The protein dimerizes to a 9S molecule of molecular weight 650,000 if the ionic strength is less than 0.5 *M*.[59] However, dimerization does not occur below isoelectric point of the protein. Diep and Boulet[165] have shown that the 7S protein dissociates at low ionic strength into fractions of molecular weight 100,000 at pH 2.0 and 54,000 at pH 11.0. Increasing the ionic strength from 0.01 to 0.02 at pH 2.0 caused dissociation of the fraction. The effect is attributed to the influence of strong hydrophobic bonds in the 7S globulin and is supported by the fact that *n*-butanol at 5% increased the degree of dissociation of the protein.

Iibuchi and Imahori[166] have shown that the 7S protein purified on concanavalin A-Sepharose 4B existed as monomers at high ionic strength or low pH, whereas it associated to form dimers at low ionic strength and high pH. The sedimentation coefficient of the monomer was 5.6S and that of the dimer was 10S, at a protein concentration of 0.16%. Since the interconversion between monomer and the dimer proceeded fairly rapidly, the ultracentrifugal pattern of the mixture showed a single peak. The apparent sedimentation coefficient was 5.6S or 10S, depending upon the concentration of either the monomer or dimer.

Koshiyama[156] has studied in detail the acid-induced conformational changes of 7S protein using a variety of techniques. The dissociation of the protein into subunits and unfolding of the polypeptide chain were observed. Maximum denaturation occurred at approximately pH 2. Thanh and Shibasaki[168] have reported the association-dissociation of β-conglycinin. The protein had 7S (protomer) conformation at high ionic strength (>0.5) or at acidic pH (pH < 4.8) and 10S (dimer) conformation at low ionic strength (<0.2) in the pH region 4.8 to 11.0. Rapid interconversion between the protomer and dimer was observed in 0.2 to 0.5 ionic strength solutions. At very low ionic strength, the α-subunit dissociated from the protein. The quaternary structures were stable at low ionic strength. Complete reversible dissociation into subunits occurred in 5 *M* urea (μ = 0.01) solution. Reversible dissociation into monomers (3S to 4S) occurred at pH 12.0 (μ = 0.5); also, the dissociation into polypeptides (2S) at pH 2.0 and 12.0 (μ = 0.01) was reversible. However, irreversible dissociation at pH 13.0 was observed and was attributed to alkaline degradation.

Ishino and Kudo[169] have studied by CD and difference spectra the ethyl alcohol concentration-dependent transformation of alkali-denatured soybean 7S protein. Ethyl alcohol increased the ordered structure of the protein. The helical and β-structure content at pH 11.4 and 66% ethyl alcohol were 54 and 39%. The 7S globulin showed a more ordered structure in ethyl alcohol than 11S globulin. Tyrosine molecules are not shown to be incorporated into the newly ordered conformation. The protein is dissociated by acid, urea, detergents, etc., and the changes brought about are reversible.[156]

Iwabuchi and Shibasaki[170] have examined the thermal denaturation of 7S globulin as a function of ionic strength from 0 to 4.0. The nature of thermal denaturation was classified into two main types at an ionic strength of 2.0. At ionic strength near zero, stable dissociation products were observed. At ionic strengths from 0.1 to 2.0, aggregate formation occurred. The stabilizing effect of salt on thermal denaturation appeared at 2.0 to 2.2. In the range of ionic strength 3.5 to 4.0 when 7S protein was heated to 100°C for 5 min, no changes were observed both in antigenic specificity and electrophoresis pattern. Hoshi et al.[171] have shown that modification of soybean 7S globulin with NEM was effective in preventing humidity-induced insolubilization during storage. S–S bonds were involved in the polymerization. A dimer consisting of α- and α'-subunits linked through disulfide bonds was observed in the polymerized 7S globulin fractions, but was not present in the 7S globulin treated with NEM. Although β-subunits had sulfhydryl groups, they did not take part in the formation of dimer.

The effect of heat on the association of myosin and β-conglycinin (7S globulin) has been investigated in the range of 75 to 100°C.[163] The sedimentation coefficient of the β-conglycinin aggregate increased with incubation temperature and its subunit composition became progressively richer in the component of lowest molecular weight. However, at lower temperatures of around 2 and 25°C, complex formation did not occur.

Appu Rao and Narasinga Rao[162] have reported the binding of Ca^{++}, Mg^{++}, and Zn^{++} by the 7S globulin of soybean proteins. The 7S protein binds more of Ca^{++} or Mg^{++} in borate buffer than in Tris®-HCl buffer at the same pH (7.8). The three metal ions appear to bind to the imidazole groups of the histidine residues. Fluorescence spectra, rate of proteolysis, and ORD and CD measurements did not indicate any conformational change in the protein due to metal ion binding; however, it increased the heat coagulation of the protein. The precipitation of the protein followed the order Zn^{++}, Ca^{++}, and Mg^{++} at the same concentration of metal ion.

Yamauchi et al.[160] subjected the 7S soybean protein to pronase digestion and isolated a 3-L-β-aspartimide carbohydrate complex. It contained one asparagine, 2-glocosamine, and 7,8- and 9-mannose residues. Furthermore, 1-L-β-aspartimide-2-acetamide-1,2-dideoxy-β-D-glucose was a product obtained from protein-carbohydrate linkage by partial hydrolysis of Asn carbohydrates. Yamauchi and Yamagishi[167] have determined the carbohydrate sequence of three asparagine-containing carbohydrates of the pronase digest of 7S soybean protein (β-conglycinin).

Lee et al.[161] studied the purified 7S globulin and total proteins from soybean by disc gel electrophoresis. The proteins were dissociated by phenol-acetic acid water containing 5 *M* urea and fractionated. They recognized that 9 of the 32 bands were glycoproteins, 5 of which were subunits of 7S globulin and 1 of agglutinin.

c. Subunits

Similar to the work on the 11S globulin *or* glycinin, there are several reports on the isolation of subunits of β-conglycinin. The major difference between the subunits of 11S globulin and β-conglycinin is the carbohydrate content of β-conglycinin subunits. Reconstitution studies have also been carried out to understand the nature of forces between the subunits that ultimately form the 7S molecule.

Iibuchi and Imahori[166] have shown that the 7S globulin of soybean seed was a mixture of three kinds of proteins that were designated as A, B, and C and were shown to be interconvertible.[166] Differences in sedimentation coefficient, molecular weight, amino acid composition, and carbohydrate content were observed. Each protein consists of three subunits, although proteins B and C consisted of only two kinds of subunits with molecular weights of 68,000 and 52,000. The subunit compositions of A, B, and C were α_3, β_3, and β_2, respectively. The proteins were dissociated into subunits by urea and the process was reversible. Reconstitution studies showed that from fraction A, only A was reconstituted, whereas from B and C, either A, B, or C was reconstituted.

Masaki and Soejima[172] have separated the 7S globulin into five subunits by DEAE-cellulose chromatography in 0.005 *M* Tris® buffer containing 8 *M* urea. The subunits showed differences in their molecular weight, amino acid composition, and N-terminal amino acid residues. Thanh and Shibasaki[173] have separated the subunits of β-conglycinin into six components designated B_1- to B_6-conglycinins. They were found to be isomers containing varying proportions of three kinds of subunits (α, α', and β). The subunit structures of these isomers are $\alpha'\beta(B_1)$, $\alpha\beta(B_2)$, $\alpha\alpha'\beta(B_3)$, $\alpha\beta(B_4)$, $\alpha\alpha'(B_5)$, and $\alpha(B_6$-conglycinin). β-Subunit is a major constituent of B_1- and B_2-conglycinins, whereas B_3- to B_6-conglycinins are composed predominantly of α-subunits. The six β-conglycinins were all glycoproteins containing mannose and glucosamine and differed in the N-terminal amino acid composition. The isolated B_1- to B_4-conglycinins were immunologically identical with one another and with the total β-conglycinin. B_5- and B_6-conglycinins that do not contain β-subunits were partially identical with the total protein. It is presumed that some antigenic determinants lacking in the B_5- and B_6-conglycinins are expected to be located on the β-subunit. Thanh and Shibasaki[174] have examined the subunit structure of β-conglycinin. The $S_{20,w}$ values of the protein were 7.2 and 10.7 at 0.5 and 0.1 ionic strength, respectively. The estimated molecular weight of the monomer was 150,000 to 175,000; that of the dimer was 370,000. The protein consisted of six isomers (B_1- to B_6-conglycinins). The subunit structures of the six conglycinins proposed are B_1, $\alpha'B_2$, B_2; αB_2, B_4, $\alpha_2 B$, B_5, $\alpha_2\alpha'$, and B_6, α_3. The model could explain similarities and differences in the properties of the conglycinins and is consistent with their sugar, N-terminal, and amino acid compositions. The proposed model of the 7S protein has a cyclic structure and the 9S form consists of two identical cyclic structures facing one another. The unfolding of α,α'- and β-subunits of β-conglycinin in phosphate buffer containing 6 *M* urea is reported.[175] Upon removal of urea, self-association of the subunits occurred. The α-subunit reassociated to form a 7S protein identical to B_6-conglycinin. Most of the subunit molecules combined to give a 7S aggregate that had no ability to dimerize at 0.1 ionic strength. The β-subunit associated to form a 16S aggregate at 0.05 ionic strength and pH 8.4. The six molecular species of β-conglycinin (B_1 to B_6) could be reconstituted by mixing the three subunits under proper conditions. The results are discussed with regard to ten possible molecular species ($\alpha_x\alpha'_yB_2$), i.e., their permutation and combinations (e.g., x = 1, 2 . . . ; y = 1, 2 . . .).

Udaka and Nagumo[176] have separated the soybean proteins by gel filtration on Bio Gel® A, and the subunits were separated by gel electrophoresis in alkaline urea or AcOH urea gel. The γ-conglycinin, β-amylase, and β-conglycinin were obtained as enriched fractions. Beachy et al.[177] have isolated mRNAs from immature soybean seeds with sedimentation values of 21S and 25S, which coded for the in vitro translation of polypeptides with electrophoretic mobilities similar to those of the α- and α'-subunits from the 7S protein. Column fingerprinting confirmed that the polypeptides produced in vitro were closely related to authentic α- and α'-subunits.

Pernollet and Mosse[17] have critically reviewed the work on the subunits of 7S globulins and they concluded that the protein generally appears to be present in three forms, namely α-, β-, and γ-conglycinins. Among them, the structure of β-conglycinin has been elucidated

by a number of workers.[159,160,167,174,175] It has been shown to be a glycoprotein and made up of isotrimers (α, α', and β) of molecular weight in the range of 14,000 to 175,000. The subunits α and α' are very similar in amino acid composition, have the same molecular weight of 54,000, and do not contain cysteine. On the other hand, the β-subunit does not contain methionine. All three subunits are glycoproteins containing nearly 4.5% carbohydrates. These contain six forms of three trimers from B_1 to B_6. The combination of α, α', and β determines the quaternary structure of these heterotrimers. They have been shown to reversibly dimerize at low ionic strength or varying pH. They form a superdimer or so-called hexamer of two trimers facing one another. The subunits B_1 to B_6 can be reconstituted to form the native molecule under proper conditions. Two more minor subunits have been isolated and are related to B_1 and B_4. These have been excellently reviewed by Pernollet and Mosse.[17]

2. γ-Conglycinin

a. Isolation and Properties

The γ-conglycinin has been obtained from the total protein by precipitating the 11S and 15S proteins by $CaCl_2$ and subsequently removing the 2S component by gel filtration on Sephadex® G-100.[31] Catsimpoolas and Ekenstam[34] have used hydroxylapatite chromatography to obtain a fairly homogeneous form of γ-conglycinin. This has also been shown to be a glycoprotein containing 12 glucosamine and 39 mannose residues per molecule of protein. It has a nitrogen content of 15.9% and is free from phosphorus. Its amino acid composition is given in Table 1. Koshiyama[178] has reported nine subunits for the protein and this confirms earlier results.[48,84] They are probably not linked through disulfide bonds and are stabilized by hydrophobic and hydrogen bonds. The protein has very little α-helix structure and consists mostly of β and aperiodic structure.

The γ-conglycinin has been shown to undergo a dimerization reaction at low ionic strength and neutral and acidic pH.[179,182] High ionic strength prevents dissociation. In acidic pH and at low ionic strength, the protein unfolds due to the loss of tertiary structure as has been evidenced by UV difference spectra and ORD studies. In the presence of 8 *M* urea or 4 *M* GuHCl, the protein completely dissociates into subunits with $S_{20,w}$ value of 1.1 to 1.4S and a molecular weight of 22,000 to 24,000.[178] However, high ionic strength of buffer decreased the dissociation of the protein. Kim and Lee[181] have isolated γ-conglycinin from the 7S globulin in a homogeneous form and studied the various physicochemical properties. It had an isoelectric pH of 5.4 and contained 16.1% N, 4.2% mannose, and 1.21% glucosamine. The amino acid composition indicated higher contents of lysine, dicarboxylic acids, and ammonia and lower contents of S-containing amino acids and tryptophan. The subunits of γ-conglycinin isolated in the pH regions 4.6 to 5.9 and 5.0 to 5.5 were glycopeptides of 38,000 mol wt and simple peptides of 32,000 mol wt, respectively.

D. Other Protein Fractions

Apart from the main globulins, 7S and 11S proteins, soybean proteins also contain other protein fractions such as 2S fraction (or α-conglycinin), hemagglutinin, trypsin inhibitors, β-amylase, crude cytochrome C, acid phosphatase, lipoxygenase, and lipid-interacting proteins.

1. 2S Fraction

The 2S fraction constitutes one of the major fractions of the soybeans proteins. Several procedures are available for the isolation of the 2S fraction.[31,34,40,52,76] It is called α-conglycinin. Vaintraub and Shutov[41] have reported the isolation of two components from the 2S fraction with the $S_{20,w}$ values of 2.3S and 2.8S. They differ in overall charge, amino acid composition, and N-terminal amino acids. The molecular weight of 2.8S fraction was reported as 36,000 and the isoelectric point at pH 4.4. Aspartic acid has been shown as the N-terminal amino acid.

Table 4
AMINO ACID COMPOSITION OF SOYBEAN AGGLUTININ, TRYPSIN INHIBITOR B-1C, TI[1]; TRYPSIN INHIBITOR KUNITZ, TI[2]; CYTOCHROME C (CYT C); 2.8S GLOBULIN; AND β-AMYLASE[a]

	No. of residues per mole					
Amino Acid	(TI[1])[27]	(TI[2])[28]	(Cyt C)[189]	Agglutinin[185]	2.8S Globulin[20,41]	β-Amylase[42]
Lysine	5	12	12	13	14	32
Histidine	1	2	2	6	2	12
Arginine	2	10	2	7	13	20
Aspartic acid	12	29	10	38	38	83
Threonine	2	8	7	17	11	23
Serine	9	12	7	24	15	33
Glutamic acid	7	19	10	17	24	70
Proline	6	11	7	19	15	40
Glycine	0	17	10	15	23	53
Alanine	4	9	8	23	12	42
Half cystine	14	4	1	0	6	5
Valine	1	15	3	20	22	38
Methionine	1	3	2	1	2	9
Isoleucine	2	15	3	16	24	31
Leucine	2	15	7	26	18	62
Tyrosine	2	4	7	5	4	30
Phenylalanine	2	10	4	15	15	22
Tryptophan	0	2	1	6	4	12
Half cysteine	0	0	1	2	—	9

[a] The number of amino acids are calculated for molecular weight of TI[1], 7,975; TI[2], 21,500; cytochrome C, 12,000; agglutinin per subunit, 2.8S globulin, 32,600; and β-amylase, 61,900.

Koshiyama et al.[182] have isolated the 2S fraction by isoelectric precipitation of the proteins at pH 4.5 and their separation on Sephadex® G-100. All the chromatographic peaks had some inhibitory activities against trypsin and/or α-chymotrypsin. Two of the three major fractions (designated as α_3- and α_4-protein) were immunologically identical to each other, but entirely different from the remaining one (designated as α_2-protein). The α_3-protein was shown to be identical to the Kunitz trypsin inhibitor. The protein, α_4, was observed to be the same-sized isomer of α_3-protein. The amino acid composition of the 2S proteins is given in Table 4.

2. Trypsin Inhibitors

Wolf[183] has reviewed the literature on the physical and chemical properties of soybean trypsin inhibitors. The Bowman-Birk soybean trypsin inhibitor has been shown to be a single polypeptide chain consisting of 71 amino acid residues with a molecular weight of 7861 and has been completely sequenced. The molecule has seven disulfide bonds and is remarkably stable to heat, acid, and proteolytic digestion. It associates to dimers and trimers at protein concentrations greater than 0.1%. This protein has the unique ability to inhibit both trypsin and chymotrypsin simultaneously. The other trypsin inhibitor, Kunitz trypsin inhibitor, has also been sequenced.[184] It consists of 181 amino acid residues. The molecule has only two disulfide bonds. The active site has been shown to be ^{63}Arg-^{64}Ile. The molecule has been shown by X-ray crystallography to have a diameter of 35 Å and to consist of two spherical structures forming a strong complex. The inhibitor has an α-helical structure and is rich in β-pleated sheet structure. The amino acid composition of the trypsin inhibitors is given in Table 4.

Table 5
PHYSICOCHEMICAL PROPERTIES OF SOYBEAN AGGLUTININ[185]

Property	Value	
	Agglutinin	Subunit
Extinction coefficient ($E^{1\%}_{1\text{ cm, }280\text{ nm}}$)	12.8	—
Isoelectric point (PI)	5.81	—
Molecular weight		
Gel filtration	120,000 ± 10,000	30,000 ± 1,500 (0.1% SDS)
Sedimentation diffusion	122,300	30,000 ± 1,500 (0.1% SDS)
Sedimentation equilibrium	122,000 ± 1300	30,300 ± 400 (6 *M* GuHCl)
Frictional ratio	1.3	—
NH_2-terminal residue	4 Alanines	1 Alanine
Diffusion coefficient ($D_{20,w}$ cm²/sec)	5.0×10^{-7}	—
Sedimentation coefficient ($S_{20,w}$)	6.0S	2.3S (6 *M* GuHCl)
Partial specific volume ($\bar{V}$, mℓ/g)	0.745	—

3. Soybean Agglutinins

Wolf[183] and Lis and Sharon[187] reviewed the literature on soybean agglutinins. The protein has been purified to a high degree of homogeneity by affinity chromatography. Lotan et al.[185] have reported its isolation and characterization. The amino acid composition and the physicochemical properties are given in Tables 4 and 5, respectively. Four alanine residues for a molecular weight of 120,000 were found by amino terminal analysis. Therefore, it was concluded that the agglutinin is a tetramer composed of identical subunits. Two binding sites for *N*-acetyl-D-galactosamine were found per 120,000 daltons by equilibrium dialysis and gel filtration, with an association constant $K = 3.0 \times 10^4 \ell$/mol. Lotan et al.[186] have shown that soybean agglutinin is made up of two types of subunits and both of them contained carbohydrates in the ratio 1:1. It is stable over the pH range of 2.2 to 10.8. It is made up of four identical subunits, each of 30,000 mol wt, and is easily dissociated by 0.1% SDS or 6 *M* GuHCl. Each subunit appears to contain a carbohydrate side chain of 2000 mol wt. An important observation has been made by Turner and Liener[188] that the soybean agglutinin contributes very little to the deleterious effects on unheated soybean products. Since, in the earlier days, this was believed to be the cause of poor nutritive properties of raw soybeans, this observation is important. The function of agglutinin is still not very clear, but it has been shown to combine specifically with a soybean-nodulating bacterium, which may involve a polysaccharide-agglutinin interaction.

4. Whey Proteins

Attempts have been made to isolate several other proteins such as β-amylase, crude cytochrome C, crude hemagglutinin, acid phosphatase, and lipoxygenase from the soybean whey.[43,49,142,189-194] The amino acid composition of crude cytochrome C is given in Table 4 for comparison with other whey proteins.

a. β-Amylase

Aibara et al.[195] have determined the structure of soybean β-amylase in trigonal crystals at 4.5-Å resolution by X-ray crystallography. The shape of the enzyme molecule and the locations of mercury-binding sites are presented. The molecule appears to be made up of two domains; the larger domain contains one mercury-binding site on its surface and the smaller domain has another mercury-binding site. The small domain site has been called an essential "sulfhydryl group". They have proposed that a distinct cleft formed between the domains near the latter sulfhydryl groups may be a substrate-binding region. The amino acid composition of β-amylase is given in Table 4.

5. Lipid-Protein Interactions

Mangold[196] has made an exhaustive review of lipoproteins and lipid-protein interactions in oilseeds. It is pointed out that the study of lipoproteins and lipid-protein interactions in seeds and other plant tissues requires model building and use of rather sophisticated physical techniques. The proteins of soybean have been utilized as models for studying protein-lipid interactions. Kamat et al.[197] have shown that soybean protein interacted with phospholipids and neutral lipids under cavitational force fields for assembly into lipoproteins. Native soybean globulins do not form lipoproteins, whereas dissociated and denatured proteins do. This may be due to the greater proportion of hydrophobic residues necessary to activate the globulin to form lipoproteins. Yamashita et al.[198] have studied the interaction of lecithin with 7S globulin of soybean and its conformational response to lecithin binding. The results indicate that lecithin interacts with globulin hydrophobic regions rather than with specific amino acid residues, with a concomitant decrease in β-structure of globulin.

From the foregoing review, it may be seen that soybean proteins have been studied extensively. Glycinin has typical physicochemical properties, but is unique in its structure and composition of subunits. Even though the number of subunits ranges from 6 to 12 (including identical as well as nonidentical), it is now fairly well established that the molecular organization is more complex. Six nonidentical acidic polypeptides and four basic nonidentical polypeptides have been detected. The N-terminal sequences and the amino acid composition have also been determined. The N-terminal sequences show considerable homology between the various subunits indicating the origin of these subunits from a common ancestor. Also, the fact that the N-terminal sequences of basic subunits act as signal peptides is significant. This aspect of homology of the N-terminal sequences of the subunits of *Vicia faba, Glycine max,* and *Cucurbita* species has been reviewed by Mosse and Pernollet.[19] They suggested that the primary structure of the C-terminal moiety of these subunits must be quite different, as has been observed with wheat gliadins.[19] These results are confirmed by physical studies on the quaternary structure of glycinin by several workers. The structure involves an oblate cylinder of radius 55 Å and thickness 75 Å. It is made up of two identical hexagons placed one above the other. They are arranged in a unique way where every acidic subunit is associated with three basic subunits and vice versa. Such a molecule is supposed to have $S_{20,w} = 12.3$ and a molecular weight of 320,000.[17] The role of disulfide links in these molecules has been the subject of many investigations.

Among the conglycinins, the β-conglycinin (the major of the three known conglycinins) has been studied in great detail.[159,166,168,173–175] It has been shown to be a glycoprotein.[160,167] It consists of isotrimers of molecular weights ranging from 140,000 to 175,000, and is made up of three kinds of subunits called α, α′, and β′. Several multiple forms of the subunits have been shown to exist.[174] Furthermore, they have been shown to reversibly dimerize (six trimers) at low ionic strength and at alkaline pH.[168] At extreme pH, the trimers also dissociate into their respective individual subunits α, α′, and β; they can be reconstituted to trimers by mixing them in urea solution.

The other conglycinins, α- and γ-conglycinins, are low molecular weight components. There is no direct evidence that α-conglycinin components have quaternary structures.[34,48] γ-Conglycinin is a 7S component and has been shown to be a glycoprotein with nearly 5 to 6% carbohydrate[66,103] with a sedimentation coefficient of 6.6S and a molecular weight of 104,000. However, similar to α-conglycinin, there is no proof yet that the protein has any subunits, although one can envisage a subunit assembly on the basis of its high molecular weight and in analogy with other seed proteins of the same sedimentation coefficient. The other components such as trypsin inhibitors, β-amylase, and agglutinin have been adequately summarized in the previous pages and critically reviewed.[183,187]

III. GROUNDNUT (PEANUT) PROTEINS

Groundnut or peanut (*Arachis hypogea*) is one of the major oilseeds of the world and is generally grown in tropical and subtropical countries. Several species of groundnuts are available. However, most of the work on the proteins of groundnut has been done on the *Arachis hypogea* species. Deshelled groundnut contains 50 to 60% oil and nearly 30% protein. The major proteins of peanuts are the storage globulins, arachin and conarachin, and they make up nearly 75% of the total proteins. The oil-free meal contains nearly 55 to 60% protein of good nutritional quality. These are easily extractable, and probably groundnut proteins are the only ones which do not contain significant amounts of any intrinsic anti-nutritional factors. The classification of groundnut proteins, their extraction in various solvents, isolation and characterization of arachin, conarachin I, and conarachin II, and their physicochemical properties are discussed below.

A. Nitrogen Solubility and Total Proteins

Irving et al.[199,200] investigated the total extractable groundnut proteins and characterized the various fractions by electrophoresis. They observed two main components (A and B), constituting 87% of the proteins, and two minor components. Naismith and McDavid[201] obtained three protein fractions with sedimentation coefficients of 2S, 8S and 13S. They were designated conarachin I, conarachin II, and arachin, respectively. Dechary et al.[202] obtained four fractions in DEAE-cellulose chromatography. The unadsorbed fraction was designated albumin, fractions II and III as conarachins, and fraction IV as arachin. Tombs[203] separated the total groundnut proteins in PAGE and designated the bands as α-, β-, γ- and δ-regions, depending upon their mobility. The α-region, which constituted nearly 3% of total proteins, contained low molecular weight components. The β-region, which constituted nearly 16%, contained a group of quite distinct proteins and was characterized by a yellow color. The γ-region consisted of two bands that were labeled arachin "monomer" and "dimer", along with a minor component termed α-conarachin. This constituted nearly 71% of the total proteins. The δ-region, comprised mostly of very high molecular weight proteins, was designated as high polymers of arachin. Cherry et al.[204] have separated the groundnut total proteins into eight major fractions using DEAE-cellulose chromatography followed by PAGE. They located the trypsin inhibitor in the albumins and low molecular weight globulin fraction by immunoelectrophoretic method. High concentrations of thiol reagents such as dithiothreitol and β-mercaptoethanol had a profound effect on the electrophoretic mobilities of groundnut proteins and on the activities of some of the enzymes.[205] Freezing also had a similar effect. These results indicate that thiol-reducing compounds and/or frozen storage promote many in vitro changes such as change in molecular weight, conformation, and structural and functional properties.

The effects of SDS and heat on the physiochemical properties of groundnut total proteins have been studied in detail.[206,207] It is observed that arachin dissociated to molecules approximately one sixth of its molecular weight, and the dissociative effect of SDS was greater on the arachin "monomer" than on the "dimer". Conarachins did not appear to dissociate. The protein is denatured in SDS solutions as shown by increase in specific viscosity. Difference spectral results indicate perturbation of chromophores in SDS solution. The Scatchard plot of SDS binding was anomalous; possibly binding occurred in a cooperative way.[206] Thomas and Neucere[207] have studied the effect of wet or dry heat on the total proteins and several enzymes. With increasing temperature, no new bands were observed in starch gel electrophoresis and no difference in electrophoretic mobility of arachin was observed. It is interesting to note that most enzymes exhibited the same activity when groundnuts were dry heated up to 130°C. Cherry et al.[208] have studied the effect of moist heat from 50 to 120°C at time intervals of 15 to 210 min on the proteins of groundnut and have specifically

evaluated the changes in solubility and dissociation. Heating at 50 or 75°C did not cause any significant changes in the properties of groundnut protein. However, heating at 100°C for 15 to 75 min partly dissociated arachin; in the gel electrophoretic pattern, new fast-moving bands were observed. Arachin was completely dissociated when the heating was continued up to 210 min at 100°C. At 120°C, similar changes were observed within 15 to 90 min. However, groundnuts heated at 120°C for 105 to 210 min gave a broad diffuse band of intermediate migration. Although moist heat at high temperatures denatured the proteins, many of the new structural components remained soluble even when the groundnuts were heated at 120°C for 210 min.

Kaneko and Ishii[209] have separated the groundnut proteins by $(NH_4)_2SO_4$, $CaCl_2$, and acid precipitation. The digestibility studies with pronase have shown that conarachin, $CaCl_2$-soluble protein, and pH 3.9-precipitated fractions were more susceptible to the enzyme than arachin, $CaCl_2$-precipitated, pH 5.0-precipitated, or isoelectric-precipitated fractions. Neucere and Conkerton[210] have separated peanut proteins into five fractions using ion-exchange chromatography. These were further characterized by immunochemical and electrophoretic methods. The bulk of the protein was concentrated in two fractions, representing arachin and conarachins. One of the five protein fractions contained relatively high concentrations of methionine, lysine, and cystine. Hydrogen peroxide treatment to destroy aflatoxin in groundnut protein has been studied.[211] The treatment reduced nitrogen solubility at pH 2 to 6, increased apparent viscosity at neutral pH, and markedly reduced the methionine, cystine, and lysine content. Arachin isolated from the total proteins after the H_2O_2 treatment did not show any difference in its properties. Satyanarayana et al.[212] have described a procedure of extraction of groundnut proteins using ion-exchange resins. Varying experimental conditions were determined to optimize the extraction of proteins. Nearly 92, 96, and 66% extraction was obtained using Amberlite® IRC-50, Zeokant-225, and Zeocant-225 resins, respectively.

Work on the nitrogen solubility and classification of total groundnut proteins indicate that four kinds of proteins exist in the seed. They are α-, β-, γ-, and δ-regions, as indicated by Tomb on 2S, 8S, 13S, and 16S proteins. The three fractions of interest are arachin (14S), conarachin II (8S), and conarachin I (2S) and they constitute the bulk of the proteins. The isolation of the various fractions, namely arachin, conarachin I, and conarachin II, are reviewed describing a variety of procedures available in the literature.

B. Arachin

1. Isolation

Several methods are available for the isolation of arachin. The claims of purity of arachin obtained by some of the earlier methods are not borne out by modern methods for determining the homogeneity of proteins. However, no single method of isolation gives a pure fraction since the composition of the various protein fractions in groundnut is highly variety dependent.

Johns and Jones[213] made the first attempt at isolation of arachin by precipitating 10% NaCl extract of total proteins at 0.2 $(NH_4)_2SO_4$ saturation; the precipitate was termed arachin. Later, Jones and Horn[214] showed that arachin was not completely precipitated until the concentration of $(NH_4)_2SO_4$ reached 0.4 saturation. Johnson and Naismith[215] obtained another preparation of arachin by precipitating the total proteins (10% NaCl extract) at 0.32 $(NH_4)_2SO_4$ saturation. This was found to be ultracentrifugally homogeneous. Naismith and McDavid[201] reported a method for isolating homogeneous arachin without the use of $(NH_4)_2SO_4$. Arachin was precipitated from the total proteins by the addition of flavine hemisulfate solution (0.2 g/100 mℓ). The precipitate was washed with alcohol to remove the amines. In the analytical ultracentrifuge, it showed a major peak of $S_{20,w}$ of 13.3, along with a minor peak in phosphate buffer of pH 8 containing 0.5 *M* NaCl.

Arachin prepared by the above methods has not been found to be homogeneous by some of the modern techniques.[216,217] Hence, either modification of the above procedures or newer methods of isolation are necessary.

Table 6
AMINO ACID COMPOSITION OF ARACHIN A AND B[203]

Amino acid	Residues per 100,000 g of protein	
	Arachin A	Arachin B
Aspartic acid	111	111
Threonine	19	28
Serine	57	59
Glutamic acid	171	164
Proline	40	44
Glycine	59	62
Alanine	40	55
Valine	34	41
Methionine	1	2
Half cystine	8	3
Isoleucine	25	32
Leucine	60	64
Tyrosine	28	31
Phenylalanine	31	44
Lysine	26	21
Histidine	17	17
Ammonia	—	—
Arginine	92	92
Tryptophan	11	11

Tombs[203] separated arachin A and B by fractional precipitation. Water dialysis of arachin preferentially precipitated arachin B, while A remained in solution. The amino acid composition of A and B is given in Table 6. Arachin A was dissociated and subjected to electrophoresis; it gave four bands (chains) called α, β, γ, and δ. Arachin B gave only β-, γ-, and δ-bands. It was concluded from the electrophoretic results that the structure of arachin A was $\alpha_2\beta_2\gamma\delta$; that of arachin B was $\beta_4\gamma\delta$; and that of arachin A_1 was $\alpha_4\gamma\delta$. Also, the α- and β-chains were found to have glycine as N-terminal amino acid. Neucere[218] has reported a method for isolation of arachin by cryoprecipitation. The total proteins extracted in phosphate buffer of pH 7.9, and ionic strength of 0.2, are dialyzed for 24 hr and cooled at 2°C for 17 hr when a precipitate is obtained. The precipitate is dissolved in the same buffer and reprecipitated. The final precipitate is dissolved in the same buffer and precipitated at 0.4 $(NH_4)_2SO_4$ saturation. The precipitated arachin was found to be homogeneous by various physicochemical techniques. Dawson[216] prepared arachin by precipitating it between 0.1 and 0.2 $(NH_4)_2SO_4$ saturation from 10% NaCl extract of groundnut flour. Its amino acid composition was very similar to that of arachin obtained by Tombs.[203]

Shetty and Narasinga Rao[217] have shown that arachin is precipitated quantitatively by 23% $(NH_4)_2SO_4$ from 1-*M* NaBr extract of groundnut flour. The protein was found to be more homogeneous compared to the arachin prepared by the method of Tombs[203] and Dawson,[216] as judged by analytical ultracentrifugation, DEAE-cellulose chromatography, and PAGE. Basha and Cherry[219] have homogenized peanut meals with various extraction media to determine optimum conditions for qualitative and quantitative recovery of proteins. Maximum recovery of both arachin and nonarachin proteins was accomplished with 29 m*M* phosphate buffer of pH 7.0 containing 1 *M* NaCl. A method has been reported to obtain pure isolates of arachin from the above extract using a series of simple steps involving differential solubility, cryoprecipitation, and dialysis. Both the arachin and nonarachin components were found to be glycoproteins containing both neutral and amino sugars and differed significantly in their amino acid composition.

2. Physicochemical Properties

Jones and Horn[214] showed that arachin in 10% NaCl did not coagulate even at 100°C and was resistant to heat. However, no information is available on the conformation of arachin in these solutions. Dean[220] has shown that the UV absorption specturm of arachin changes in the pH range 7.3 to 11.0. These changes are reversible and are due to the ionization of the tyrosine groups. Johnson and Shooter[221] studied the association-dissociation of arachin. Arachin undergoes reversible dissociation as follows.

$$A_n \rightleftharpoons nA$$

where A_n is the 14S protein and A is the 9S protein. The equilibrium position depended on pH, salt concentration, and the nature of salt. It was shown that low pH and high salt concentration favored association of arachin, whereas low salt concentration favored dissociation. They also determined some of the hydrodynamic properties of the parent as well as dissociated molecules. From the results, they showed that the molecular weight of 9S component was one half of the parent molecule. Thus, the general equation for arachin can be written as

$$A_2 \underset{\text{low salt}}{\overset{\text{low pH, high salt}}{\rightleftharpoons}} 2A$$

They also concluded that the dissociated molecules were of greater asymmetry than the parent molecule. Johnson and Joubert[222] studied the effect of SDS on the dissociation equilibrium of arachin. SDS increased association up to a concentration of 0.02 *M* (0.05%), and above this concentration caused dissociation to a 3.5S component whose proportion increased with increase in SDS concentration.

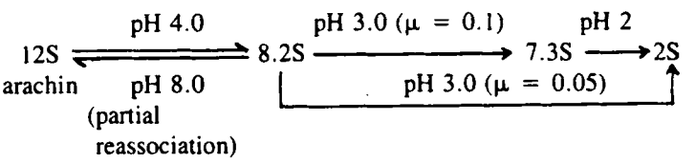
The effect of urea, GuHCl, and amines on arachin has been studied mainly by employing the technique of viscosity.[223] It is observed that in all cases viscosity increased with increase in the concentration of the denaturant, reached a plateau region, and then decreased. The increase in viscosity has been attributed to increase in asymmetry of the arachin molecule, and the final decrease in viscosity is possibly due to dissociation of the protein. Also observed was a relationship between viscosity increment and the ability of arachin to be spun into fibers.[223] Naismith and Williams[224] studied the effect of alkaline pH on the dissociation of arachin. The following scheme was proposed:

$$\underset{\text{arachin}}{14S} \rightarrow 9S \rightarrow 2S$$

They isolated the 9S component by differential pH dialysis. Johnson and Naismith[225] studied the effect of GuHCl on the dissociation of arachin. The following scheme was proposed by them for the dissociation reaction:

$$\underset{\text{arachin}}{14S} \rightarrow \underset{\text{swelling}}{12S} \rightarrow 7.6S \rightarrow \underset{\text{subunits}}{2S}$$

The dissociation was shown to be reversible. Unlike alkali or GuHCl, urea directly dissociates arachin to a 2S species. Naismith and Kelley[226] studied the effect of low pH on the dissociation and denaturation of arachin using the technique of viscosity, sedimentation velocity, and optical rotation. As the pH was decreased from 8.0 to 4.0, the protein dissociated to 8.2S component. With further decrease in pH, it dissociated to 2S component. When the pH was increased from 4.0 to 8.0 by slow dialysis, the 8.2S component associated to the parent molecule only partially. Schematically, the dissociation reaction can be represented as



It was concluded that dissocation and denaturation of arachin were facilitated by low pH and low ionic strength, and arachin underwent drastic structural changes upon decreasing the pH from 8.0 to 2.0. Kinner and Naismith[227] investigated the effect of formaldehyde on arachin in buffer and in the presence of urea and GuHCl. Formaldehyde prevented dissociation of arachin mediated by low ionic strength and high pH. The rate of dissociation of arachin by urea and GuHCl was retarded by formaldehyde. From the above results, they concluded that the halfmers of arachin were cross-linked by formaldehyde. Tombs[203] monitored the spectral properties of arachin in 8 *M* urea at various pH values. The spectrum was characterized by two peaks, one at 245 nm and the other at 296 nm. All 24 phenolic groups were ionized without any involvement of thiol groups. Arachin was precipitated by the addition of 1 *M* $CaCl_2$ to 10% NaCl extract of groundnut flour.[203] This was found to be homogeneous by ultracentrifugation and gel electrophoresis. However, Shetty and Narasinga Rao[217] showed the presence of conarachins in Tomb's preparation of arachin by DEAE-cellulose chromatography. Tombs[203] elucidated the complete structure of arachin after depolymerizing the parent molecule in 10 *M* formamide and in the presence of β-mercaptoethanol. Among the four chains (α, β, γ, and δ), α- and β-chains make up the major part of the arachin molecule. The α- and β-chains have molecular weight of about 35,000 and the γ- and δ-chains of about 10,000 each. The N-terminal amino acid of α- and β-chains has been shown to be glycine, whereas that of γ- and δ-chains as leucine and glutamic acid, respectively.

Arachin has been investigated in great detail and has been shown to be a simple, unconjugated protein. The nitrogen values of arachin prepared by different methods varies between 14.3 to 14.9%.[203,216,218] The amino acid composition of arachin prepared by different methods differs and is reported by Dawson[216] and Neucere.[218] They are given in Table 7. Some of the physical and chemical data on arachin are shown in Table 8.

Tombs[203,228] and Tombs and Lowe[229] showed that arachin exhibited polymorphism. Arachin showed two bands in electrophoresis which have been termed "monomer" and "dimer" bands. The "monomer" with higher mobility was termed arachin A and the dimer with lower mobility was termed arachin B. They reinvestigated the N-terminal amino acids of arachin B by dissociating it into 12 subunits in presence of 8 *M* urea. The ratios of the N-terminal residues showed that the parent molecule contained eight chains with N-terminal glycine, two with valine, and two with isoleucine. They have reported a total of 11 disulfide bonds per 330,000 g protein. Neucere and Ory[230] have reported the effect of organic solvent on arachin. When groundnut protein is treated with organic solvents, the isoelectric point changed to a pH nearer neutrality and there was a distinct separation of arachin into two closely related components on ion-exchange chromatography. Neucere et al.,[231] in studying the effect of dry roasting on the proteins of groundnuts, observed that upon roasting the electrophoretic mobility of arachin increased and it retained its antigenic property. Daussant et al.[232,233] have shown by immunoelectrophoresis that arachin is microheterogeneous and contains four fractions, the major one being α-arachin. Neucere[234] has studied the effect of wet (40% moisture) and dry (<5% moisture) heat on groundnut proteins. The major protein, α-arachin, and another protein remained antigenic in both wet- and dry-heated seed within the temperature range of 110 to 155°C. Disc gel electrophoresis showed greater differences in protein migration for the dry- than for the wet-heated seed, and protein solubility was inversely proportional to dry heat, whereas a sigmoidal curve was obtained with wet heat.

Table 7
AMINO ACID COMPOSITION OF ARACHIN ISOLATED BY VARIOUS WORKERS

	Residues per 100,000 g of protein			
Amino acid	Tombs[203]	Dawson[216]	Neucere[218]	Shetty and Narasinga Rao[217]
Aspartic acid	84	95	91	75
Threonine	21	19	21	14
Serine	44	45	39	35
Glutamic acid	117	133	133	124
Proline	40	44	21	24
Glycine	51	57	47	48
Alanine	45	47	43	35
Valine	37	42	37	61
Methionine	4	4	1	—
Half cystine	6	5	0	—
Isoleucine	26	29	25	14
Leucine	46	53	48	39
Tyrosine	23	25	18	17
Phenylalanine	35	36	32	25
Lysine	17	17	15	12
Histidine	16	15	13	12
Ammonia	127	135	80	42
Arginine	66	71	54	99
Tryptophan	3	3	—	4

Table 8
PHYSICOCHEMICAL PROPERTIES OF ARACHIN[203,215-217,221,261-263]

Property	Values
Sedimentation coefficient ($S_{20,w}$)	14.6
Molecular weight	330,000
Diffusion coefficient ($D_{20,w}$ cm²/sec)	$3.86 \pm 0.1 \times 10^{-7}$
Partial specific volume (mℓ/g)	0.720 ± 0.005
Frictional ratio (f/f_0)	1.216
Extinction coefficient ($E^{1\%}_{1\,cm,\ 280\ nm}$)	7.98
Isoelectric point	5.1 ± 0.1
Carbohydrate (%)	0.3
Nitrogen content (%)	(14.3—14)
Intrinsic viscosity (mℓ/g)	
1 *M* NaBr	4.7
1 *M* NaCl	6.2
b_0 (degree)	100
Subunits	6
Secondary structure (%)	
α-Helix	5—10
β-Pleated	25—30
Aperiodic	65—70

The maximum solubility of α-arachin occurred after wet heat at 120°C. Neucere and St. Angelo[235] have shown that α-arachin has the same association-dissociation properties in sucrose as in low ionic strength phosphate buffer. It is more soluble in 1 *M* sucrose than in low ionic strength buffer. Sucrose has no effect on the immunological behavior, electro-

phoretic properties, and conformational state of α-arachin. It appears to stabilize the conformational status of α-arachin even in the presence of heat.

Yotsuhashi and Shibasaki[236] have studied the effect of denaturants like urea and GuHCl on the dissocation of arachin into subunits. They conclude that the dimer (14S) dissociates to 9S in low ionic strength buffers. Arachin dissociates into a 1.5S molecule in the presence of either urea or GuHCl. However, the dimer form appears to be more resistant to dissociation than the monomer form. Six major bands and other minor bands were detected in starch gel electrophoresis under the denaturing conditions in 8 *M* urea. It was concluded that the subunits of arachin are not held together by disulfide bonds.

Jacks et al.[237] have studied the effect of heat on the antigenicity and conformational stability of arachin. The reactivity of arachin with antiarachin serum decreased after arachin was heated. The infrared amide IV and VI bands were altered in relative intensities proportional to temperature of treatment. Also, with increase in temperature of heating of arachin, more of the unordered structure was observed with a concomitant decrease in α-helical and pleated sheet structures. These results indicate that the loss of antigenicity is due to a major conformational change in arachin. Van Huystee[238] has determined the effectiveness of various proteolytic enzymes on the degradation of arachin using the technique of PAGE. Papain was the most efficient proteolytic agent. Shetty and Narasinga Rao[206] observed that SDS caused dissociation of arachin. However, no associative effect of SDS was observed even at low concentration as was observed by Johnson and Joubert.[222] Schematically, they represented the reaction as

$$\underset{\text{arachin}}{11S} \xrightarrow{< 5 \times 10^{-3}\ M\ \text{SDS}} 7.4S \xrightarrow{>5 \times 10^{-3}\ M\ \text{SDS}} 3.1S$$

The molecular dimension and subunit structure of arachin have been determined.[239] The purified arachin has been characterized in both its monomeric (9S) form and dimer (14S) form. The monomer had sedimentation coefficient of 9.2S, molecular weight of 180,000, frictional ratio of 1.29, and intrinsic viscosity of 0.06 dℓ/g. An axial ratio of 3.5 and 4.6 was calculated assuming a prolate and oblate ellipsoid (30% hydration) of revolution, respectively. The parameters for the dimer were 14.7S, 350,000 mol wt, 1.25, axial ratios of 3.0 and 3.2, and 0.044 dℓ/g for the viscosity, respectively. They have also shown that arachin consisted of six kinds of principal subunits having an average molecular weight of 29,000 of which three had glycine, two had isoleucine, and one had valine as the N-terminal amino acid.[239] Tombs[240] has used arachin as a model protein in studying the mechanism of gel formation. According to him, globular proteins like arachin form gels as a result of aggregation to form strands followed by interaction of the strands to form the gel mesh. However, a limited random aggregation (other than ordered strands) may also lead to gelation, but the major mechanism is the above-proposed model of strand formation, which is dependent on protein concentration. Tombs et al.[241] have shown that solutions of arachin showed liquid-liquid phase separations under certain conditions of pH and ionic strength. Phase boundaries indicated closed boundaries corresponding to salting in and salting out of globulins. Arachin, under these conditions, underwent self-association as revealed by concentration dependence of the sedimentation coefficient and gel chromatography. However, in D_2O the association was more extensive. Phase separation has been shown to be due to oligomer formation leading to thermodynamic instability of the solutions. At high protein concentration, deformed, helical, crystalline spherulites were produced. The diameter of oligomer was found to be 400 Å by electron microscopy. Shetty and Narasinga Rao[217] have compared arachin obtained by different methods of isolation for their physicochemical properties. Arachin prepared by the methods of Tombs,[203] Dawson,[216] and Shetty and Narasinga Rao[217] differed in their rate of hydrolysis by α-chymotrypsin, heat coagulation, and dissociation into sub-

units. The phosphorous and carbohydrate content of the three preparations did not differ significantly. However, SDS and GuHCl denatured the protein and perturbed the chromophores to the same extent as judged by difference spectroscopy.

Neucere and Cherry[242] have reported the effect of heat on the antigenic and electrophoretic changes of α-arachin in vitro. After dry heating for 1 hr, α-arachin showed indistinct electrophoretic and immunologic properties above 145°C. It had no antigenic property after heating above 175°C. The decreased solubility of α-arachin followed an exponential curve between 110 and 195°C. They have concluded that the above results suggested modifications in the steric arrangements of determinant groups in α-arachin. Srikanta and Narasinga Rao[243] have shown that wet heating of groundnut proteins dissociates (degrades) arachin into lower molecular weight proteins as judged by various physicochemical techniques. Neucere[244] has studied the effect of freezing in the presence of β-mercaptoethanol on the immunological properties of arachin and conarachin. Both arachin and conarachin were completely inactivated after freezing under reduced conditions. The conarachin proteins were more sensitive to reduction than arachin. Slight changes in electrophoretic mobility were observed in both proteins after treatment with β-mercaptoethanol. This treatment could maintain some intra- and/or interchain disulfide bonds. Arachin has been shown to degrade upon storage.[245] Similar changes are brought about by the action of α-chymotrypsin on arachin. The "monomer" appears to be degraded more easily than the "dimer". Also described is a method of preparation of dimer of arachin by utilizing its property of resistance to hydrolysis.

This raises the question as to whether there is an equilibrium between monomer and dimer of arachin. Since the so-called "dimer" has been isolated in a pure form, possibly there is no equilibrium between the two, and the formation of the dimer from the monomer is irreversible under these experimental conditions.

Puri and Bala[246-248] have determined the electrometric titration and flocculation with electrolytes and binding of metal ions of groundnut and other proteins. The heat of ionization in different regions was determined by performing titration at two different temperatures. The titration curves enabled estimation of acid- and base-binding capacities as well as a number of various acid groups. The results agreed with the analytical data for various groups.[246] The flocculation of a few vegetable protein solutions stabilized at pH 11 and 7.5 by electrolytes has been worked out.[247] Higher flocculation values were obtained for the cations Ca^{2+}, Sr^{2+}, and Ba^{2+}, whereas lower values were obtained for Zn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, and Ag^{+}. Protein fractions solubilized in NaCl yielded more stable solutions than whole proteins extracted from the same source with NaOH. Flocculation values of various electrolytes for the solutions stabilized at pH 2.5 showed that for anions with a given valency those with a larger size were more effective flocculents.[247] The binding of Zn^{2+}, Co^{2+}, and Ni^{2+} by equilibrium dialysis technique with the glycinin (soybean), arachin, conarachin, and gliadin (wheat) was also studied by equilibrium dialysis.[248] The number of moles of the metal ions bound per 10^5 g of protein increases with increase in metal ion concentration in the solution and attains a maximum value that is very close to the number of imidazole groups in the proteins. The intrinsic binding constants (R) as well as the standard free energy (ΔF°) and entropy (ΔS°) changes of the process were calculated.[248]

Binding of aflatoxin B_1 by arachin and conarachin II has been investigated.[249] The results show that in the case of arachin more aflatoxin is bound to the protein in low compared to high ionic strength buffer. On the other hand, with conarachin II, the binding of aflatoxin is less in low than in high ionic strength buffer. These results are interpreted in terms of association-dissociation equilibrium of arachin and conarachin in low and high ionic strength buffers.[249] Shetty and Narasinga Rao[250] have reported the effect of succinylation on the oligomeric structure of arachin. Succinylation up to 60% has been shown to lead to dissociation of the parent 14S molecule to 9S and 4S fractions. Above 60% succinylation, only 4S fraction occurred predominantly. All the measured properties — reduced viscosity,

fluorescence intensity at 320 nm, absorbance change at 280 nm, specific rotation at 232 nm, and specific ellipticity at 210 nm — changed up to 20% succinylation; then the values remained constant up to 55% succinylation, and then changed again. The observed changes have been interpreted as due to the formation of a new organized structure before the 4S fraction is denatured. Neucere et al.[251] have described the effects of phenols on arachin digestibility, solubility, and conformation. Arachin was exposed to concentrations of catechol and pyrogallol in the range of 0.05 to 0.3 *M*. The reagents were removed by dialysis. Conformational studies indicated that there was increase in α-helical content of arachin. This modified arachin showed changes in antigenic reaction as compared to native arachin. In vitro pepsin hydrolysis showed that arachin exposed to 0.1 *M* catechol or pyrogallol was more susceptible to hydrolysis than the native arachin.

Yamada et al.[252,253] have been able to separate arachin into two components and have termed them as A_I and A_{II}. The two components have molecular weights of 170,000 to 180,000 and 340,000 to 350,000, respectively. The ratio of these two components has been shown to depend upon the variety. A_I undergoes a reversible interconversion between 9S and 14S, depending upon either ionic strength or pH. On the other hand, A_{II} undergoes only a partially reversible interconversion betwen 9S and 14S. However, the amino acid compositions of A_I and A_{II} are identical. They exhibit the same subunit pattern with six main bands. The six subunits of arachin are labeled S_1, S_2, S_3, S_4, S_5, and S_6. They were isolated by isoelectric focusing in sucrose density gradient. All six subunits were characterized for their isoelectric point, molecular weight, molar ratios, N-terminal amino acids, and amino acid composition. S_1 to S_3 were characterized as acidic subunits having molecular weight ranging from 35,500 to 40,500, and the N-terminal amino acids were valine and isoleucine. S_4 to S_6 were characterized as basic subunits and had the same N-terminal amino acid, namely glycine, and the same molecular weight. The amino acid composition of S_1 to S_3 was significantly different. They had a higher content of glutamic acid and glutamine, but a lower content of alanine, valine, and leucine. The amino acid composition of S_4 to S_6 was rather similar. S_5 and S_6 did not contain cysteine and methionine. It was concluded that arachin (9S) consisted of six different subunits in equimolar concentration and that these were bound noncovalently. These subunits are isolated from urea solution.[254] The subunits were reconstituted by removing urea by dialysis without agitation at 25°C against 20 m*M* Na phosphate buffer of pH 7.9 containing 30% sucrose, 0.1 *M* NaCl, and 7 m*M* β-mercaptoethanol. The reconstitution yield was 90%. The reconstituted molecule was indistinguishable from native arachin in its disc electrophoretic mobility, subunit composition, sedimentation behavior, conformational status, UV absorption spectrum, fluorescence emission spectrum, stability against heating, effect of protease, and GuHCl, suggesting that it was similar to the native arachin. However, attempts to obtain native arachin with only four or five subunits were not successful, indicating the thermodynamic stability of arachin as a hexamer only.

Navin Kumar et al.[255] have reported that arachin forms a gel at pH 3.9, and the gel is thermolabile. Factors such as protein concentration, heating temperature, heating time, cooling temperature, pH, ionic strength, and the dielectric constant of the medium have a profound effect on the gelation phenomenon. Urea has been shown to decrease the gel strength. It was concluded that the cross-links involved in gelation of arachin may be predominantly noncovalent in nature. Shyamasunder and Rajagopal Rao[256,257] have reported the properties of acylated arachin. Acylation affected the solubility at different pH values. The degree of acylation and the nature of the acylating group also influenced the solubility characteristics. The emulsifying capacity of arachin increased after acylation.

The conformational changes of arachin induced by both hydrogen peroxide (H_2O_2) and rancid oil were investigated.[258] Neither rancid oil nor 3% H_2O_2 changed the properties of arachin, but 30% H_2O_2 increased electrophoretic mobility, abolished antigenicity, and re-

duced the ordered structural modes of the protein. The effect of 30% H_2O_2 on the proteins from peanut, pumpkin, and soybean, namely arachin, cucurbitin, and glycinin, respectively, was reported.[259] The changes in solubility of arachin and cucurbitin were 8.5- and 40-fold in aqueous media, respectively; in glycinin the change was 200-fold in acidic media. The effects of various organic solvents on the conformational stability of arachin have been investigated by Jacks et al.[260] Arachins exposed to hexane, acetone, and hexane-Me_2CO-H_2O were virtually identical to native arachin in conformational properties, indicating that these solvents had no effect. However, exposure to acidic hexane resulted in loss of reactivity to antiarachin and increased electrophoretic mobility in nondenaturing gels. These changes have been interpreted as due to irreversible dissociation of arachin by acidic hexane into subunits, each of which maintained the native secondary structure of the multimeric form. The antibody reactivity demanded the quaternary structure of the protein, possibly with the antigenic determinants embracing the separated subunits.

Pernollet and Mosse,[17] based on the subunit composition data available on arachin, have concluded that arachin is a heterohexamer which is itself a "supermonomer" partially dimerzied, forming a heterodecamer (a "superdimer" of 12 subunits in the 14S component).

C. Conarchin II (α-Conarachin)

1. Isolation

Conarachin II (or α-conarachin, as it is called) sediments as 8S component in the total proteins of groundnuts. It forms about 15 to 25% of the total proteins, depending upon the variety. Several methods are available for the isolation of conarachin II.

Johnson and Naismith[215] showed by ultracentrifugation that conarachin could be further divided into two fractions, one of sedimentation constant 2S and the other of 8.4S, based on their $(NH_4)_2SO_4$ precipitation behavior. These were later designated as conarachin I and conarachin II, respectively, by Johnson and Naismith.[225] These workers obtained a preparation of conarachin II which was fairly homogeneous, but it contained conarachin I as a contaminant.[215] Their method involved the following steps: (1) extracting of the groundnut flour in 0.4 saturation of $(NH_4)_2SO_4$; (2) increasing the $(NH_4)_2SO_4$ concentration of extract to 0.65 saturation; (3) separating the precipitated protein; (4) increasing the $(NH_4)_2SO_4$ concentration of the supernatant to 0.85 saturation; and (5) separating the precipitate. It contained mostly conarachin II with a considerable proportion of conarachin I as judged by analytical ultracentrifugation.[215]

Naismith and McDavid[201] extracted the proteins in 10% NaCl and precipitated at 0.85 $(NH_4)_2SO_4$ saturation. The precipitate was dissolved in pH 7 phosphate buffer and the pH lowered to 4.7 in the same buffer ($\mu = 0.025$). The supernatant was cryoprecipitated at 4°C. The protein was not found to be homogeneous in analytical ultracentrifuge in phosphate buffer of pH 8 containing 0.5 *M* NaCl. They purified the precipitate obtained between 0.65 to 0.85 $(NH_4)_2SO_4$ saturation of Johnson and Naismith[215] by reprecipitation with alum. The alum-precipitated protein still contained conarachin I.

Dechary et al.[202] have shown that conarachin II is microheterogeneous and consists of two fractions called α_1- and α_2-conarachins. They isolated α-conarachin by DEAE-cellulose chromatography. The protein precipitated between 0.4 to 0.85 $(NH_4)_2SO_4$ saturation of 10% NaCl extract was dissolved in 0.02 *M* phosphate buffer of pH 7.9 and dialyzed against the same. The protein was chromatographed on a DEAE-cellulose column and the protein eluting between 0.1 and 0.2 *M* NaCl was collected, concentrated, and rechromatographed. A single peak was obtained suggesting that the protein was homogeneous. However, the homogeneity of the preparation was not checked by various methods. Shetty and Narasinga Rao[263,264] reported a method for the isolation of conarachin II. The method involved collecting the precipitate between 32 to 42% $(NH_4)_2SO_4$ saturation and removing the contaminants by gel chromatography on a Sephadex® G-100 column. The protein was found to be homogeneous

Table 9
PHYSICOCHEMICAL PROPERTIES OF CONARACHIN II[90,202,215,261,262,264,270,271]

Property	Value
Sedimentation coefficient ($S_{20,w}$)	8
Nitrogen content (%)	14.0—15.3
Molecular weight	295,000
Isoelectric point (pH)	3.9 ± 0.1
Secondary structure (%)	
α-Helix	12
β-Structure	28
Aperiodic	60
Fluorescence emission maximum (nm)	345
Intrinsic viscosity (mℓ/g)	4.6
Extinction coefficient ($E^{1\%}_{1\,cm,\ 280\ nm}$)	6.25
Subunits	6—8

by various physicochemical techniques. They have also reported the electrometric titration curve of the protein and observed that the side chain carboxyl, imidazole, and ε-amino groups titrated with normal pK_{int} values and their number agreed with the analytical values obtained from amino acid composition. Abnormal pK_{int} values of 10.5 were observed for the tyrosyl phenolic groups.

2. Physicochemical Properties

Conarachin II isolated by the method of Dechary et al.[202] contains nearly 15.3% nitrogen and 1% mannose, even though a value of 14% nitrogen is reported.[261] The various physicochemical properties of conarachin II have been determined and are tabulated in Table 9. The amino acid composition of the protein is given in Table 10. Conarachin II also exhibits association-dissociation equilibrium dependent upon pH and ionic strength of the buffer. Low ionic strength favors association of conarachin II to a 13S component.[202] Jones and Horn[214] showed that conarachin in 10% NaCl solution coagulated upon heating at 80°C. Naismith and Kelley[226] investigated the dissociation and denaturation behavior of conarachin II as a function of pH and ionic strength. With decrease in pH, the protein dissociated and denatured, and at pH 2.0, a 1.8S component was observed in the analytical ultracentrifuge. The process was irreversible since the protein completely precipitated upon dialyzing back from pH 3 to 8.0. Schematically, the dissociation can be represented as

$$8S \xrightarrow[\mu = 0.1]{\text{pH } 4.0} \text{"8S"} \xrightarrow[\mu = 0.05]{\text{pH } 3.0} 2S \xrightarrow[\mu = 0.05]{\text{pH } 2.0} 1.8S$$

The cross-linking action of formaldehyde on conarachin II in the pH range 6 to 11 has been studied using analytical ultracentrifugation.[227] Formaldehyde at pH 8.0 and 0.3 g/g of protein induced aggregation of conarachin II, resulting in a higher sedimenting species with sedimentation constants of 12S, 16S, and 20S. At higher ionic strength, the extent of aggregation decreased since conarachin II dissociates under these conditions. At lower pH values (6.5), even higher aggregates with S values of 30 and 50 were observed. Naismith[265] observed that addition of metal ions such as Al^{+++}, Fe^{+++}, Cr^{+++}, Ca^{++}, and Zn^{++} caused aggregation of conarachin II. Possibly salt linkages between the protein and the metal ion were responsible for aggregation since the complexes were readily broken down by phosphate ions. The extent of cross-linking increased with increase in metal to protein ratio, but decreased with increasing pH. McDavid and Naismith[266] observed that addition of the

Table 10
AMINO ACID COMPOSITION OF CONARACHIN II[263]

Amino acid	Number of residues per 100,000 g of protein
Aspartic acid	94
Threonine	13
Serine	48
Glutamic acid	163
Proline	33
Glycine	44
Alanine	33
Valine	27
Methionine	2
Half cystine	—
Isoleucine	25
Leucine	36
Phenylalanine	24
Lysine	39
Tyrosine	4
Histidine	15
Ammonia	77 ± 5
Arginine	126
Tryptophan	7

extract of the testa of the groundnut to groundnut proteins induced an association reaction resulting in a sedimentation constant of 32S.

It was concluded that the 32S component was a complex between conarachin II and the tannin present in the groundnut testa. Conarachin fractions extracted from heat-treated groundnut flour showed marked differences in amino acid composition.[267] Dawson[268] observed that the electrophoretic pattern of conarachin fractions extracted from heated groundnut flour was changed drastically compared to the unheated sample. The effect of organic solvents on conarachin showed that the solubility was altered and the isoelectric point shifted towards neutral pH upon treating conarachin with organic solvents.

Yotsuhashi and Shibashi[269] have shown that conarachin contained conarachin I and conarachin II in equimolar concentrations and that these have different physicochemical properties. Conarachin I (2S) showed no association-dissociation reaction upon change in ionic strength at pH 8.6 and had no subunit structure. However, it was made up of a group of proteins as shown by gel electrophoresis. Conarachin II exhibited association-dissociation phenomenon as a function of ionic strength. It associated to an 18S component at low ionic strength. However, in denaturing solutions, conarachin II dissociated into subunits of sedimentation coefficient of 2S. The subunit structure of the 8S form was more stable and resistant to dissociation than that of the 18S form of conarachin II. Shetty and Narasinga Rao[263,264] have reported the physicochemical properties of conarachin II. The protein has absorption maximum at 278 to 279 nm, $A^{1\%}_{1\,cm}$ = 6.25; fluorescence emission maximum at 345 nm; and intrinsic viscosity of 0.046 dℓ/g. ORD and CD measurements suggest that the protein has mainly β-conformation and aperiodic structure. It consists of seven subunits with molecular weights ranging from 18,000 to 62,000. The protein exhibits association-dissociation equilibrium, which is influenced by pH and ionic strength.

D. Conarachin I

Conarachin I constitutes nearly 30% of the total groundnut proteins and sediments as a

2S component in the analytical ultracentrifuge. Johnson and Naismith[215] prepared conarachin I by extracting the groundnut flour with 0.4 saturation of $(NH_4)_2SO_4$. The protein consisted of one major peak of 2S and a minor peak of 13S in the analytical ultracentrifuge. Reprecipitation did not improve the homogeneity of conarachin I. Naismith and McDavid[201] modified the method of Johnson and Naismith[215] for isolation of conarachin I. Conarachin I obtained by the method of Johnson and Naismith[215] was dissolved in 10% NaCl solution and the solution was saturated to 0.2 $(NH_4)_2SO_4$. After discarding the resultant precipitate, the supernatant was brought to 0.65 saturation. The precipitated protein was redissolved and reprecipitated. It gave a conarachin I preparation of better homogeneity. Not much information is available on the physicochemical properties of conarachin I.

E. Enzyme and Other Minor Proteins in Groundnut

Irving and Fontaine[272] observed proteolytic activity in groundnut meal. The enzyme was named as arachidin. It is a thermolabile enzyme (T_i <40°C) and has optimum activity in the pH range of 6.5 to 7.5. Mosely and Ory[273] purified the enzyme by $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography. Other enzymatic activities such as esterase, catalase, peroxidase, acid phosphatase, alcohol dehydrogenase, ATPase, INT-oxidase activity, and trypsin inhibitor could be detected.[274-277]

Lotan et al.[278] have isolated and characterized a lectin from *Arachis hypogea*. Fish et al.[279] have reported some of the macromolecular properties of groundnut agglutinin. At pH 8, the protein exists as a compactly folded tetramer of molecular weight 98,000. Between pH 4.75 and 3.0, the molecule reversibly dissociates to a dimer. In the presence of denaturants such as SDS and GuHCl, the molecule dissociates to its four equal-sized constituent polypeptide chains. Binding of lactose to the lectin brings about changes in the near UV-CD bands, whereas the secondary structure of the lectin appears to be unaltered. Dissociation of the lectin to a dimer produces subtle changes in both the near and far UV-CD spectrum. Shyamasundar et al.[280] have identified at least six ninhydrin-positive major acidic peptides in groundnut. They have been identified as γ-glutamyl-L-phenylalanine, γ-glutamyl-L-tyrosine, and γ-glutamyl glycine. The amino peptidase activity in the peanut cotyledons has been shown to be associated with conarachin II fraction.[281] Basha and Pancholy[282] have isolated basic proteins from the total proteins of groundnuts. They were found to be rich in lysine (8.5%), glycine (27.9%), and methionine (1%), but low in aspartic acid (5.3%) and glutamic acid (5.6%). They were glycoproteins as evidenced by the presence of neutral (3.5% glucose and mannose) and amino (0.2% glucosamine) sugars. SDS-gel electrophoresis indicated six major and seven minor polypeptide chains. The apparent molecular weights of the 6 major polypeptides were in the range of 20,000 to 55,000.

Even though work on groundnut proteins started as early as 1880,[287] it was only in the 1930s that fractionation into arachins and conarachins was achieved.[213,219] Later it was shown that these two categories of proteins could be fractionated by varying salt concentration for extraction, by varying $(NH_4)_2SO_4$ concentrations, or by ion-exchange chromatography.

Arachin, which constitutes nearly 70% of the extractable protein, has been shown to be located in the protein bodies.[7,14,15,17,19,283] The existence of polymorphism and quaternary structure and also the association-dissociation phenomenon have been well documented for arachin.[202,221,277,284] The protein has been shown to be sensitive to changes in ionic strength and pH in terms of association and dissociation. Yamada et al.[252,253] have clearly shown that arachin is made up of six subunits constituting both acidic and basic polypeptide chains. All studies have shown that arachin is possibly made up of a ringlike structure that has been confirmed by electron microscopy of the heterohexamer.[17,285]

The other proteins, conarachin I and II, have been isolated by various methods to a fair degree of purity. An important observation by Neucere and Ory[286] has shown that part of conarachin is cytoplasmic and accumulates outside protein bodies. This is very unique among

oilseed proteins. More detailed study of the 7S component and its specific location in the cell in other oilseeds and legumes is indicated. Mosse and Pernollet[19] conclude that arachin and conarachin are legumin- and vicillinlike proteins, a model proposed by Derbyshire et al.[283]

IV. SESAME SEED PROTEINS

Sesame seed (*Sesamum indicum* L.) contains nearly 25% protein, and the defatted meal contains about 50% protein. Except for the presence of oxalates in the hull, there are no known toxic, antinutritional, or coloring principles in sesame. The proteins have long been used as a rich source of methionine. They have a tendency to coagulate upon heating and are also insoluble in water, unless high salt concentration is maintained.

The work on sesame seed total proteins has mostly centered around nitrogen solubility, extractability of the protein as a function of various extractability parameters, the fractionation of proteins, and isolation and characterization of the major protein component (α-globulin). A few of the physicochemical properties of the total proteins have also been reported.

A. Nitrogen Solubility

The earliest work was by Ritthausen,[287] who obtained several preparations of proteins from sesame seed by extracting the protein from oil-free cake under variable conditions of alkali, NaCl concentration, and temperature. The different preparations were analyzed for elementary composition, e.g., carbon, nitrogen, etc. Adolph and Lin[288] determined the solubility of sesame seed proteins from the fat-free meal in NaCl, NaOH, and Na_2CO_3 solution. They showed that prior treatment of the meal with methanol *or* a temperature of 110°C decreased the solubility considerably. Later, Basu and Sen Gupta[289] carried out similar solubility studies with defatted sesame seed in water at various pH values and in the presence of NaCl and $NaHSO_3$. Their studies showed that 6% NaCl at pH 9.0 extracted nearly 87% of the proteins from the meal.

Nath and Giri[290] carried out peptization studies of sesame seed protein with 10% NaCl solution. The effect of particle size of the meal, ratio of sample weight to solvent volume, and other extractability parameters were studied. Sesame proteins were shown to be soluble only below pH 3.0 and above pH 7.0.[291] They also reported an isoelectric point of pH 4.5 from precipitation studies. Guerra and Park[292] carried out similar extractability studies of sesame proteins in various salt solutions such as $CaCl_2$, Na_2SO_3, and Na_2HPO_4, and observed that low salt concentrations increased the solubility and high salt concentrations decreased the solubility of the proteins. Prakash[293] studied the effect of various extractability parameters for maximum extraction of proteins from sesame seed flour and observed that a 60-mesh flour (with less than 0.8% fat) in phosphate buffer of 0.01 *M* with pH 7.5 containing 1 *M* NaCl and 1 hr shaking can yield nearly 85% protein in one extraction and up to 95% protein upon repeated extractions. Johnson et al.[294] have reviewed the composition, fractionation, toxic constituents, and production of sesame protein from sesame seeds. Some of the extractability parameters such as time of shaking, solute-to-solvent ratio, and pH for maximum extractability of proteins from sesame seed have been studied.[295] They observed that a 1:40 flour-to-solvent ratio and an extracton time of 15 min at pH 11.0 extracted more than 90% protein. The alkali-extracted protein isolate was more soluble in water and the salt-extracted protein isolate was less soluble. Nearly 85% of the protein can be extracted in a single extraction at neutral pH containing 1 *M* NaCl.

B. α-Globulin

1. Isolation

The available information in the literature indicates the presence of four different fractions

in the total proteins of sesame seed. There has been some ambiguity in nomenclature of the different fractions.[296-298] For the present discussion, we will adopt the nomenclature of Nath and Giri.[297] The major protein has been designated α-globulin.[296] The other three fractions, constituting about 30% of the total, have been named β-, γ-, and δ-globulins.

Jones and Gersdorff[296] obtained different fractions of sesame proteins by $(NH_4)_2SO_4$ fractionation and heat coagulation. They obtained α-globulin both in crystalline and amorphous form. From a study of the elementary composition and amino acid analysis of crystalline and amorphous α-globulins, they concluded that it was a homogeneous protein. Nath et al.,[299] in their studies on the physicochemical properties of sesame seed proteins, investigated the properties of amorphous α-globulin and showed it to be electrophoretically homogeneous in the pH range 3 to 12 and identical to the α-globulin of Jones and Gersdorff.[296] Electrophoretic, ultracentrifugal, and solubility studies on crystalline α-globulin prepared from sesame seeds of white, brown, and black varieties have been made,[300] prepared by the method of Jones and Gersdorff.[296] Moving boundary electrophoresis in the pH range 2.45 to 4.1 in buffers of high ionic strength gave a single peak and two partially resolved peaks at pH 2.45. Sedimentation velocity experiments with crystalline and amorphous α-globulin in the pH range 4.5 to 9.0 gave two sedimenting species with sedimentation coefficients of 13S and 19S in all varieties. It was also observed that, below pH 4.5, α-globulin underwent denaturation as indicated by solubility and sedimentation behavior. Salem and Beckheit[298] evaluated different procedures for the fractionation of protein components of sesame seed and reported the electrophoretic behavior of the fractions. Guerra and Park[292] extracted the total proteins from sesame seed and separated them into 7 fractions in SDS-PAGE and estimated the molecular weights to be 51,000, 31,000, 28,500, 25,000, 21,800, 20,500, and 17,000, indicating for the first time the oligomeric nature, possibly of the major fraction, namely α-globulin. Prakash and Nandi[301] reported isolation of α-globulin by a modification of the procedure of Nath and Giri.[297] The extracted protein in 1 *M* NaCl was diluted 1:10 with water when a precipitate was obtained. This was repeated twice and the final precipitate that was dissolved in water was dialyzed against 1 *M* NaCl solution. This procedure gave high yields of α-globulin, with a homogeneity better than 95% as judged by gel filtration, gel electrophoresis, ion-exchange chromatography, analytical ultracentrifugation, and isoelectric focusing techniques. The amino acid composition of α-globulin prepared by the above method is given in Table 11.

The supernatant from α-globulin precipitate was saturated with 40% $(NH_4)_2SO_4$ and the precipitate obtained was termed β-globulin. This fraction was found to be contaminated with α-globulin as judged by electrophoretic measurements.[297,302] The supernatant obtained from the precipitation of β-globulin was made 60% with respect to $(NH_4)_2SO_4$ when a precipitate was obtained. This was designated as γ- and δ-globulins. Okubo et al.[303] also isolated α-globulin by dialysis of the 10% NaCl extract against water and found the protein to be homogeneous by various physicochemical techniques.

The major protein fraction of α-globulin isolated by dilution was termed "amorphous" α-globulin by Jones and Gersdorff.[296] The precipitate obtained by adding 20 to 30% $(NH_4)_2SO_4$ when dissolved in 2% NaCl solution at 60°C and cooled to room temperature yielded crystalline α-globulin.[297] Most of the detailed studies have been on the major fraction, α-globulin, and very little work has been reported on the other minor fractions. The physicochemical properties of α-globulin will be discussed in detail. Some of the properties of the total proteins are given in Table 12.

2. Physicochemical Properties

Sinha and Sen[300] reported a value of 13S for the sedimentation coefficient of α-globulin in 10% NaCl solution. At pH below 4.5 and above pH 9.0, the protein dissociated. This effect was independent of the variety of the sesame seeds from which α-globulin was

Table 11
AMINO ACID COMPOSITION OF α-GLOBULIN[301]

Amino acid	Residues per 100,000 g of protein
Aspartic acid	84
Threonine	41
Serine	59
Glutamic acid	155
Proline	21
Glycine	90
Alanine	71
Valine	46
Methionine	20
Half cystine	7
Isoleucine	32
Leucine	63
Tyrosine	24
Phenylalanine	34
Lysine	16
Histidine	20
Ammonia	—
Arginine	91
Tryptophan	11

Table 12
CHEMICAL AND PHYSICOCHEMICAL PROPERTIES OF TOTAL PROTEINS AND α-GLOBULIN OF SESAME SEED[301]

Property	Values	
	Total protein	α-globulin
Sedimentation constants ($S_{20,w}$)	2, 7, 11, and 16	11
Specific rotation $(\alpha)_{577}$	—	−40°
Extinction coefficient ($E^{1\%}_{1\,cm,\ 280\ nm}$)	13.0	10.8
Fluorescence emission maximum (nm)	327	328
Nitrogen content (%)	14.5	15.9
Amide content (%)	1.0	2.0
Phosphorus content (%)	0.09	0.04
Carbohydrate content (%)	4.3	0.8
Proteolytic activity	+ ve	Nil

obtained. Between pH 4.5 and 9.0, α-globulin appeared to be stable and indicated the presence of low concentrations of 19S and 23S components, depending upon the pH of the solution. The moving boundary electrophoresis of α-globulin indicated a single peak at pH 4.1 and 3.2; two partially resolved peaks were observed at pH 2.45, which may be due to dissociation of α-globulin at acid pH. In addition, these authors reported the absorption spectrum of α-globulin. The absorption maximum occurred between 278 to 280 nm, and $E^{1\%}_{1\,cm}$ at 280 nm was 10.8 (Table 12). Ventura and Lima[304] studied the sedimentation and diffusion characteristics of α-globulin. The value of frictional ratio was 1.5 and partial specific volume was 0.735 mℓ/g. The calculation of molecular weight by a combination of diffusion, sedimentation, and partial specific volume data yielded a value of 450,000 ± 30,000 daltons for the protein.

Prakash and Nandi[305] have reported that the anionic detergent, SDS, first causes dissociation of α-globulin and subsequently denaturates the protein. The detergent has been shown to bind to the protein with a binding constant of 3×10^3 ℓ/mol. The addition of SDS to the protein generates a UV-difference spectrum in the region 280 to 295 nm, and also causes quenching of protein fluorescence emission and shifts the emission maximum towards longer wavelengths. This has been shown to be due to the binding of detergent molecules near chromophoric groups. Such binding alters their electronic properties. At lower detergent concentration ($<5 \times 10^{-3}$ *M*), only tyrosine moieties are perturbed. At higher detergent concentration ($>5 \times 10^{-3}$ *M*), the tryptophan moieties are also enveloped by detergent molecules. The binding of the detergents in large numbers possibly increases the hydrodynamic volume even after denaturation is complete as evidenced from optical rotation and viscosity measurements. The following scheme has been suggested for the action of SDS on α-globulin:[305]

$$\underset{\alpha\text{-globulin}}{11S} \xrightarrow[\text{SDS}]{\leq 1 \times 10^{-3}\,M} 7S \xrightarrow[\text{SDS}]{>1.75 \times 10^{-3}\,M} 4S \xrightarrow[\text{SDS}]{>5 \times 10^{-3}\,M} 2S$$

(dissociation) (denaturation)

The effect of urea and GuHCl on α-globulin has been studied in detail by Prakash and Nandi[306] using physicochemical techniques. The protein undergoes dissociation, aggregation, and denaturation in the presence of urea or GuHCl. There is a critical concentration of each denaturant where aggregation is maximum. Both denaturation and aggregation are lower in buffers of high ionic strength. The above results were explained by considering two types of subunits in the protein molecule, one rich in hydrophobic amino acids and the other not so rich in hydrophobic amino acids. Based on the above results, Prakash and Nandi[306] have suggested the following scheme for the dissociation and denaturation of α-globulin in presence of urea and GuHCl:

$$11S \xrightarrow{<1\,M\text{ urea}} 7S \xrightarrow{>2\,M\text{ urea}} 4S \xrightarrow{>3\,M\text{ urea}} 2S \xrightarrow{6\,M\text{ urea}} \text{denatured } 2S$$

$$11S \xrightarrow{>1\,M\text{ urea}} 120S \xrightarrow{>2\,M\text{ urea}} 4S$$

The endothermic nature of the aggregation process has been considered to arise from hydrophobic interaction of aliphatic side chains of the relevant subunits. The protein exists in a more denatured state in GuHCl than in urea solution.

The effect of acid and alkali on α-globulin has been investigated by Prakash and Nandi.[307] Both acid (pH 4.2 to 3.0) and alkali (pH 7 to 12) induced dissociation and denaturation of the protein:

$$11S \xrightarrow{pH \geq 3.0 \text{ up to } 4.0} 2S \xrightarrow{pH \leq 3.0} 11S$$

$$11S \xrightarrow{pH > 8 \text{ up to } 10.0} 7S \xrightarrow{pH > 10} 2S$$

In stronger acid solutions, i.e., pH <3.0, reassociation of the dissociated fractions takes place by hydrophobic interaction. In alkaline solution, denaturation is preceded by an initial step of dissociation after pH 10. Denaturation in alkaline solution has been shown to be irreversible. The phenolic group has abnormal pK_{int} value of 10.6.

Prakash and Nandi[308] and Prakash[309] have shown by temperature effect on the association-dissociation of α-globulin that the subunits of α-globulin are associated predominantly by hydrophobic interactions. Dissociation of the proteins by various salts gave the following order of effectiveness, $SO_4^{-2} < Cl^- < Br^- < ClO_4^- < SCN^- \leq I^- < CCl_3COO^-$, the first two members being association-inducing ions. The order of effectiveness of cations in inducing association of the protein is $Cs^+ \sim K^+ \geq Na^+ > Li^+$. Both in the dissociation by anions and in the association by cations, no detectable change in protein conformation was observed. The dissociation has been explained by favorable energetics of interaction of the chaotropic ions with the amide dipole. On the other hand, it has been pointed out that the unfavorable positive free energy of interaction of nonpolar groups in Na_2SO_4 and NaCl solutions will dominate favorable interactions of ions with the amide dipole, which will stabilize the associated form of the protein.[310,311]

The behavior of α-globulin in the presence of a cationic detergent (CTAB) shows that, unlike in anionic detergent, the protein precipitates up to a concentration 5.5×10^{-4} *M* CTAB, above which redissolution of the protein is observed.[312] The higher aggregates observed in sedimentation velocity experiments are considered to be the soluble precursors of the insoluble aggregates. Other measurements indicate that there is a conformational change in the protein. The tryptophenyl groups that are in contact with aqueous phase are perturbed by the detergent.[312] The effect of nonionic detergents such as Triton® X-100 and Brij-36T on α-globulin has been studied in detail by Lakshmi and Nandi.[313] They have shown that at low concentrations these detergents induce dissociation and aggregation of the protein. From binding measurement, they have concluded that perhaps the micelles of the detergent predominantly bind to the protein, especially to the exposed hydrophobic surface on the protein subunits. However, no conformational changes were detected due to the interaction of these detergents with the protein.

Nishimura et al.[314] have studied the properties of α-globulin and have reported several physicochemical parameters such as molecular weight, sedimentation coefficient, viscosity, diffusion coefficient, partial specific volume, and amino acid composition. Okubo et al.[303] have separated the subunits of α-globulin into acidic and basic units on DEAE-Sephadex® A-50 gel-containing urea and 2-mercaptoethanol. They have suggested that the acidic and basic subunits are present in equimolar amounts (6:6). The molecular weights of the acidic and basic subunits were estimated by gel filtration and SDS-gel electrophoresis and found to be 30,500 to 33,500 and 20,000 to 24,500, respectively. There were significant differences in the amino acid compositions of the acidic and basic subunits. The N-terminal amino acids in the group of acidic subunits have been recognized as glycine and in the group of basic subunits as glycine and valine. Hasegawa et al.[315] have studied in detail the subunits of α-globulin. The molecular weights of basic and acidic subunits have been determined to be 21,000 and 28,600, respectively. The basic subunit contained glycine as the N-terminus and the acidic one contained leucine (or isoleucine). This is contrary to the results of Okubo et al.[303] The amino acid analysis indicated that the basic subunit was rich in methionine — 4.5%. The protein has been shown to undergo a temperature-dependent association-dissociation reaction and the results of these authors were similar to those of Prakash and Nandi.[308] Furthermore, they have shown that the generation of 7S component is favored by low-temperature freezing of the protein.

Prakash et al.[316] and Prakash and Nandi[317] have made a detailed study of the conformation of α-globulin under various solution conditions of SDS, acid, alkali, urea and GuHCl, Triton® X-100, Brij-36T, and CTAB. The protein in phosphate buffer pH 7.4, 0.2 *M* has about 5% α-helix, 25% β-structure, and the rest aperiodic or irregular structure. SDS has been shown to induce more α-helical structure in the protein (nearly 20% α-helix at 1×10^{-2} *M* SDS). As the pH is decreased from 7.4 to 3.2, there is a decrease in β-structure and a concomitant increase in aperiodic structure. At pH 1.60, there is regeneration of β-structure, the value being 20%, which is close to that of the control. On the alkaline side,

at pH 9.1, there is again regeneration of β-structure (34%), the rest being aperiodic. However, at extreme acid or alkaline pH, the protein has no α-helix, with β-structure content decreasing further. The protein attains 100% aperiodic structure in 6.6 *M* urea or in 6.0 *M* GuHCl solution. In the presence of CTAB, there is an increase in α-helix content, but it is much less than in SDS solution. In nonionic detergents, such as Brij-36T and Triton® X-100, specific β-structures like II-β and I-β were formed along with changes in α-helical and aperiodic structures. The above results indicate that the multimeric protein α-globulin has a fairly labile secondary structure with a fair degree of conformational freedom as revealed by its rapid response of conformational change to changes in environmental conditions, of which some are relatively mild.

Hybridization of the subunits of sesame and soybean 13S globulins has shown that several intermediates are formed when they are mixed under a reductively denatured condition and subjected to the reconstitution procedure.[318] It is concluded that the complexes are probably a hybrid intermediary subunit. The gelation and turbidity characteristics of sesame and soybean proteins have been investigated in detail.[319] The results indicate that upon hybridization of the 13S α-globulin and glycinin or 7S of soybean the critical concentration for gel formation increased with increase in sesame α-globulin concentration and decreased at pH 7.0. Also, precipitation of α-globulin was enhanced upon heating both in presence of NaCl and at higher pH (pH 10.0). Hasegawa et al.[151] have successfully prepared hybrid globulins from a combination of α-globulin acidic subunits and soybean legumin basic subunits. They have shown that the acidic and basic subunits are able to associate into a hybrid bicatenar monomer with reconstitution of disulfide linkages, having six acidic and six basic subunits. On the contrary, the reassociation of glycinin acidic subunits with sesame basic subunits only gives rise to monomers unable to polymerize.

Succinylated and maleylated α-globulins were prepared and studied in detail.[320,321] Both succinylation and maleylation were effective in solubilizing α-globulin in water. The molecular weight of the protein and the number of disulfide bonds remained intact on modification of the protein. However, the isoelectric point of the protein was shifted to pH 4.5 from pH 5.0. Both the succinylated and maleylated samples showed distinct differences in the titration curve of the protein in the pH range 7 to 10. Fujino et al.[322] have modified α-globulin by reductive alkylation using $NaBH_4$ and aqueous formaldehyde. The extent of modification was monitored by the trinitrobenzene sulfonic acid (TNBS) method. Reductive alkylation increased the solubility of the protein in water and low concentrations of NaCl. At higher NaCl concentration (>0.4 *M*), the solubility of the repeatedly alkylated α-globulin was lower than that of the unmodified protein. There was a slight decrease in the positive charge of the modified globulin as determined by disc electrophoresis. Reductive alkylation dissociated the 13S globulin to a 5S component. The modified protein increased the critical gelling concentration from 2 to 8% at pH 7 in 2% NaCl and from 2 to 4% at pH 10 in 5% NaCl.

The interaction of α-globulin with urea or GuHCl was investigated in detail by determining the apparent partial specific volume of the protein in these solvents both under isomolal and isopotential conditions.[323] The preferential interaction parameters were 0.08 and 0.1 g of urea and GuHCl, respectively, per gram of protein. In both cases, the interaction was not preferential with water. The total binding of urea and GuHCl to α-globulin was calculated and the correlation between the experimentally determined number of moles of denaturant bound per mole of the protein and the total number of peptide bonds and aromatic amino acids was found to be excellent. The values were 449 mol of urea and 507 mol of GuHCl per mole of protein, respectively. The volume changes upon transferring α-globulin from aqueous solution to that of urea or GuHCl have been determined to be -4700 ± 1600 and -5000 ± 1400 mℓ/mol for urea and GuHCl, respectively. Prakash[309] has studied in detail the hydrodynamic properties of α-globulin, both from the point of size and shape of the

molecule. The various hydrodynamic parameters of the protein are listed in Table 13. The protein has an $S_{20,w}$ of 12.8, $D_{20,w}$ of 4.9 $\times$ 10^{-7} cm^2/sec, and a partial specific volume of 0.725 mℓ/g. It is shown to be globular in shape since its intrinsic viscosity is 3.0 mℓ/g. The molecular weight of the protein as determined by analytical ultracentrifugation methods has been shown to vary between 2.6 to 2.74 $\times$ 10^5. The molecular weight from sedimentation equilibrium yielded a value of 2.7 $\times$ 10^5 in the native state and a value of 19,000 in the dissociated and denatured state in 6 *M* GuHCl. Evaluation of frictional ratios using Stokes radius and results from electron microscopy indicate the protein to be globular in shape. The protein consists of at least 12 subunits. The evaluation of the thermodynamic parameters and energetics of interaction of subunits indicate that the subunits of the protein are stabilized predominantly by entropically driven hydrophobic interactions with a negative free energy of interaction of 0.19 kcal/mol.

High concentration of sucrose (80%) has been shown to prevent heat coagulation of α-globulin.[324] The protein remains in solution when the sucrose concentration is decreased to 10%. It is shown that the protein dissociates in high concentrations of sucrose. Spectral studies indicate perturbation of aromatic side chains. Sucrose has been shown to induce dissociation of the protein, which reaggregates at higher concentration of sucrose solution. The observed stabilizing effect of sucrose on the prevention of heat coagulation of sesame α-globulin has been explained by favorable interaction of the sugar molecules with the polar amide groups of the protein, which overcomes the unfavorable interaction of the nonpolar groups in sugar solution leading to dissociation of the protein. Lakshmi et al.[325] have studied the effect of electrolytes such as Na_2SO_4, sodium potassium tartrate, sodium citrate, and NaCl on the prevention of heat coagulation of α-globulin. The degree of prevention of heat coagulation and the precipitation of protein at room temperature depended on the nature and concentration of the salt. The prevention of heat coagulation of α-globulin in the presence of certain polyanionic electrolytes has been explained as being due to dissociation of the protein to subunits, reduction in the overall charge of the protein molecule, and binding with the polar amide groups. This probably is achieved by electrostatic repulsion of the dissociated and denatured subunits of α-globulin.

C. Other Protein Fractions

Prakash[302] has isolated and characterized the low molecular weight, 2S, fraction of sesame seed. This component is homogeneous as tested at neutral pH by analytical ultracentrifugation and PAGE. However, in acid or alkaline pH, it gives three bands in PAGE. Possibly they may be the γ- and δ-globulins of Jones and Gersdorff.[296] The 2S fraction consists of proteins which have nearly 30% α-helix; this contrasts with high molecular weight protein fraction, α-globulin, which has 5% α-helix. Similarly, the proteolytic activity is also more in this fraction than in α-globulin. Further work is necessary for a proper understanding of the 2S fraction and its structure-function relationship. Similarly, more work is necessary with regard to β-globulin which is isolated in a crude form.[297,302]

A lectin has been isolated from *Sesamum indicum* by affinity chromatography on a chitin column.[326] The lectin is a single polypeptide chain of molecular weight ~56,000 as shown by SDS-gel electrophoresis. However, it could be dissociated into two polypeptide chains of ~30,000 and ~26,000 mol wt upon reduction with 2-mercaptoethanol. Specificity towards *N*-acetylglucosamine has been demonstrated. The lectin agglutinated human red blood cells without discrimination among blood groups as well as rabbit erythrocytes and mouse spleen cells. Treatment of erythrocytes with trypsin greatly increased agglutinability by the sesame lectin.

To conclude, α-globulin from sesame seed has been isolated in a homogeneous form. The sedimentation coefficient of the protein varies between 11.0 and 13.0, depending upon the ionic strength of the buffer. In the presence of anionic detergents, the protein undergoes

Table 13
HYDRODYNAMIC PARAMETERS OF α-GLOBULIN[309]

Parameters		Values
Hydrophobicity and related values		
Average hydrophobicity		872 cal/residue
NPS		0.26
P		1.36
Charge		0.362 units/residue
Sedimentation coefficient ($S_{20,w}$)		
Native		12.8S ± 0.1S
6 *M* GuHCl ($S_{20,w}$)		2.0S ± 0.2S
Diffusion coefficient ($D_{20,w}$)		4.9×10^{-7} cm²/sec
Intrinsic viscosity [n]		
Native		3.0 ± 0.2 mℓ/g
6 *M* GuHCl		38.5 ± 1.0 mℓ/g
Nonideality coefficient (g)		0.01 mℓ/mg
Partial specific volume		
Native		0.725 ± 0.002 mℓ/g
6 *M* GuHCl		0.684 ± 0.002 mℓ/g
Hydrated volume (Ve)		0.711 mℓ/g
Hydration factor (ϕ)		0.27 g of water per gram of protein
Molecular weight		
Approach to sedimentation equilibrium		2,50,000 ± 20,000
Sedimentation and diffusion measurement		2,36,000 ± 15,000
Sedimentation and intrinsic viscosity		2,52,000 ± 15,000
Sedimentation equilibrium		
Native	M_n	2,65,000 ± 12,000
	M_w	2,74,000 ± 14,000
6 *M* GuHCl	M_n	18,000 ± 1,000
	M_w	19,000 ± 1,500
Size		
Stokes radius		37 ± 3Å
From sedimentation measurements		47 ± 4Å
From diffusion measurements		43 ± 4Å
Radius of gyration		29 ± 3Å
Frictional ratios		
From radius of equivalent sphere		1.0 ± 0.05
From sedimentation and molecular weight		1.10 ± 1.0
From Stokes radius and hydrated volume		1.0 ± 0.15
Shape parameters		
β-Function		2.18×10^6
Perrin shape factor		1.50 ± 0.1
Simha shape factor		4.17 ± 0.5
Axial ratios		
Prolate ellipsoid of revolution		3.5
Oblate ellipsoid of revolution		3.0
Subunit number		
SDS-PAGE		6 × 2
6 *M* GuHCl		6 × 2
Subunit interactions		Predominantly hydrophobic

dissociation and denaturation. Both nonionic and cationic detergents have been shown to induce dissociation and aggregation of protein. The protein is rich in aspartic acid, glutamic acid, and methionine and is an acidic protein (Table 11). In the presence of low concentration of urea and/or GuHCl, the protein aggregates. At higher concentrations of urea or GuHCl, dissociation is followed by denaturation. Both acid (pH 4 to 3.0) and alkali (pH 7 to 12) induce dissociation and denaturation of the protein. In stronger acid solution of pH <3.0, reassociation of the dissociated fraction takes place by hydrophobic interaction. The subunits of α-globulin are held together predominantly by hydrophobic interactions. The association-dissociation of the protein in presence of various anions and cations does not follow the Hofmeister series. The acidic and basic subunits of α-globulin have been isolated and are shown to be present in equimolar amounts. Modification of α-globulin by succinylation, maleylation, or reductive alkylation has been shown to increase the solubility of the protein in water and low concentrations of NaCl. α-Globulin has been shown to bind nearly 450 mol of urea and 507 mol of GuHCl per mole of protein. The volume changes upon transferring α-globulin from aqueous solution to urea or GuHCl solution have been determined to be -4700 ± 1600 and -5000 ± 1400 mℓ/mol for urea and GuHCl, respectively. High concentrations of sucrose and polyelectrolytes have been shown to prevent heat coagulation of α-globulin and this has been explained by favorable interaction of the sugar molecules with polar amide groups of the protein as well as dissociation of the protein. Further work is needed on the separation of the subunits of α-globulin and evaluation of their chemical nature. Work on the regeneration of the parent molecule from the subunits is also indicated. Such studies might throw light on the structure-function relationship of α-globulin. Not much information is available on the low molecular weight proteins of sesame. More work on these is necessary to ultimately understand the role of each protein in the seed.

V. SUNFLOWER SEED PROTEINS

Sunflower seed (*Helianthus annus* L.) contains nearly 25% protein, and the defatted meal is rich in protein, containing about 55% protein. There are no known toxic and antinutritional factors in sunflower meal.[327] However, the hulls contribute to the high crude fiber content of the meal and the proteins become colored during alkaline extraction due to the presence of polyphenols.[328] Several methods are available to dehull the sunflower seed and to obtain flour low in crude fiber.[329-331] The polyphenols (total ~3%) include chlorogenic acid (as the major constitutent), caffeic, quinic, cinnamic, sinapic, coumaric, and ferulic acid derivatives.[322-324]

A. Nitrogen Solubility and Protein Fractions

Schwenke and Raab[344] have determined the nitrogen distribution in sunflower seeds. The values are 87 to 90% of protein nitrogen, 10 to 13% of peptide, amino acid, and other forms of nonprotein nitrogen, 46 to 50% of the protein nitrogen derived from globulins, and 25% from albumins. Jain et al.[330] have analyzed proteins from sunflower seed after dehulling, grinding, and extraction. The meal contained nearly 4.6% moisture, 50% protein, 6% crude fiber, 4.6% ash, 2.2% chlorogenic acid (CGA), 0.3% caffeic acid, and 0.25% quinic acid.

The nitrogen solubility of sunflower seed protein has been studied in detail by several workers.[328,329,335-338,342,348,350,351] All studies showed a broad minimum of solubility in the pH range 3 to 6. Most of the proteins could be extracted at pH 10.0. In the presence of salts even at pH 8.0, nearly 82% of the proteins could be extracted.[335] The solubility of the protein isolates obtained from untreated and acidic butanol-treated sunflower seed has been shown to be similar.[339,340] It has been shown by Kabirullah and Wills[338] that the nitrogen solubility increased with partial hydrolysis of the protein isolate by trypsin or pepsin.

The solubility of sunflower seed proteins has been studied as a function of several elec-

trolytes by Schwenke et al.[348] At low salt concentrations, the solubility curves of the proteins resembled those of salt-free solution with a minimum in the pH range 4 to 5 and high solubility at pH 3. At low $CaCl_2$ concentrations (0.01 to 0.15 *M*), however, a decrease in solubility occurred over a large pH range (4.5 to 10). Sunflower globulins showed a rapid increase in solubility in the isoelectric range (pH 5) in the range of ionic strength of 0.3 to 1.0. The extractability of nitrogen from the seed meal increased at pH 7 with the concentrations of the salts and this has been attributed to cation specificity rather than to a pure ionic strength effect. $CaCl_2$ precipitates almost all the precipitatable proteins from the aqueous extracts of the seed meal. Acid denaturation by incubation of the protein isolate at pH 2 to 3 decreases the solubility.

The salt-extractable proteins from embryos of *H. annus* have been separated into albumins (20%) and globulins (80%).[375] Schwenke and Rauschal[356] have reported that succinylation of sunflower seed protein isolates caused the broadening of the solubility minimum and a shift at its position from pH 4.5—5.5 to 2.5—4.8. Also, with progressive succinylation, up to 89% the protein dissociated into low molecular weight fractions and these were correlated with the functional properties of the protein.

Joubert[341] reported that the total proteins extracted in 10% NaCl contained four fractions with sedimentation coefficient of 18.1S, 11.9S (major fraction, 343,000 mol wt), 7.8S, and 1.7S (19,000 mol wt). Schwenke and Raab[344] and Schwenke et al.[345] reported that in addition to the 11S protein the 7S (9S) and 3S (4S) proteins as well as a high molecular weight (17S) protein occurred in sunflower flour. The 7S component could be a dissociation product of the 11S protein and 17S protein, an aggregate. The proteins extracted in phosphate buffer of pH 7.0 containing 1 *M* NaCl when subjected to concentration gradient of $(NH_4)_2SO_4$ indicated nearly nine components.[342] Sasek[343] has reported nearly 29 protein zones in the sunflower seed proteins by electrophoresis in starch gel medium. Sabir et al.[332] characterized the salt-soluble (2.5% NaCl) proteins of three varieties of sunflower by gel chromatography and disc electrophoresis. Five fractions were obtained on Sephadex® G-200. Their molecular weights ranged from 20,000 to 600,000. However, fraction V had a molecular weight of 5000. Disc electrophoresis of the fractions indicated that each fraction consisted of three to six bands. Salt-soluble proteins of sunflower seeds gave seven protein bands in disc gel electrophoresis.[351] Six to seven fractions were observed in DEAE and CM-Sephadex® chromatography.[352] Rahma and Narasinga Rao[353] have characterzied the total proteins of sunflower meal and CGA-free meal by disc electrophoresis, gel filtration, ion-exchange chromatography, and analytical ultracentrifugation. Nearly five to six bands were seen in electrophoresis. In sedimentation velocity experiments, three peaks of sedimentation constant 11S (70%), 7S (10%), and 2S (20%) were observed. In gel filtration and ion-exchange chromatography, three peaks were observed. The first and third fraction obtained in gel filtration contained considerable amounts of CGA. Proteins of sunflower seed soluble in 1 *M* NaCl were fractionated by Sephadex® G-200 gel chromatography, and four fractions — A, B, C, and D — were obtained.[360] Fraction B contained approximately 60% of proteins (1 *M* NaCl soluble) and represents the major protein fraction. Fraction D, on further fractionation on Sephadex® G-25 column, gave two fractions, I and II, with absorbance maximum at 260 and 320 nm, respectively. CGA has been shown to bind only to the low molecular weight components and not to the high molecular weight protein fractions. The salt-soluble sunflower proteins have been fractionated into four distinct peaks on Sephadex® G-50 gel.[350] The first two fractions were proteins and the last two consisted of low molecular weight components including CGA. Aqueous extracts of defatted sunflower seeds contained 12 protein components.[346] Saline extracts contained the proteins insoluble in water; their number was six or seven proteins.

Hydrophobic interaction chromatography has been used to fractionate saline-soluble sunflower proteins, and the fractions obtained were analyzed further by SDS-PAGE.[354] With 20% saturation of $(NH_4)_2SO_4$ in 0.02 *M* borate buffer of pH 7.4, containing 10% NaCl,

Table 14
AMINO ACID COMPOSITION OF TOTAL PROTEIN (MEAL), 11S GLOBULIN, AND 2S PROTEIN FRACTION OF SUNFLOWER SEED PROTEINS[345,358,379]

		No. of residues per 100,000 g of protein			
	Total protein[379]	11S globulin		2S fraction	
Amino acid	(g/16 g N)	Ref. 379	Ref. 358	Ref. 345	Ref. 379
Aspartic acid	8.70	105	107	71	64
Threonine	3.18	33	36	44	48
Serine	3.89	60	51	45	68
Glutamic acid	20.95	189	197	252	142
Proline	5.01	45	52	56	41
Glycine	5.05	88	85	96	248
Alanine	4.07	70	69	61	71
Valine	4.76	40	63	44	51
Methionine	1.91	20	19	22	22
Half cystine	1.82	9	11	42	64
Isoleucine	3.97	30	49	44	27
Leucine	6.13	60	68	67	51
Tyrosine	2.65	19	20	5	12
Phenylalanine	4.70	41	48	14	14
Lysine	3.77	17	19	46	41
Histidine	2.47	29	23	10	11
Ammonia	2.18	—	209	—	—
Arginine	8.91	64	66	71	33
Tryptophan	1.11	5	10	2	2

~98% of the salt-soluble proteins was bound to the maxtrix (Octyl-Sephadex® Cl4-B). Most of the CGA (98%) eluted unadsorbed. Seven protein fractions were obtained by this procedure, and the results of SDS-PAGE indicated that all seven fractions were composed partly of similar subunits and partly of different subunits.

Provansal et al.[347] observed that treatment of sunflower protein isolates with NaOH reduced their content of cystine, arginine, threonine, serine, isoleucine, and lysine. Unusual amino acids were formed during these treatments; alloisoleucine, ornithine, lysinoalanine, and lanthionine were identified. The presence of lysinoalanine and lanthionine indicated the formation of cross-links in the protein and this might explain the observed changes in the in vitro proteolytic digestibility. Drastic treatments with NaOH (~0.2 M, 80°C, 1 hr) also caused a marked degree of isomerization of the L-lysine to D-lysine as demonstrated by enzymic and microbial analyses.

The amino acid composition of sunflower seed protein isolate is given in Table 14.

From the above available literature, it is observed that sunflower total proteins are composed mainly of four fractions sedimenting as 18S, 11S, (~70%), 7S (10%), and 2S (20%) components. There are indications that the 7S component could be a dissociation product of the 11S protein. The 11S protein has been termed globulin, whereas the 2S protein has been termed albumin, obviously depending upon their solubility in salt solutions.

B. High Molecular Weight Protein Fraction

1. Isolation

Attempts were made as early as 1955 to isolate the 11S protein from sunflower seeds by $(NH_4)_2SO_4$ precipitation.[341] Schwenke et al.[358] have isolated the 11S globulin from sunflower seeds and have shown it to be fairly homogeneous. The procedure involved precipitating a 10% NaCl extract by dilution with water. This was repeatedly chromatographed on Seph-

Table 15
PHYSICOCHEMICAL PROPERTIES OF 11S GLOBULIN OF SUNFLOWER SEED[341,353,358,359,362,366,370,377,379]

Property	Values
Molecular weight	300,000—350,000
Intrinsic viscosity (n)	3.7 mℓ/g
Sedimentation coefficient ($S_{20,w}$)	11.2—12.8
Extinction coefficient ($E^{1\%}_{1cm,\ 280\ nm}$)	8.2
Fluorescence emission maximum (nm)	325
Carbohydrate (%)	0.31
Phosphorus	Nil
Chlorogenic acid (%)	0.31
SH groups	6.8 mmol/g protein
No. of subunits	6
Secondary structure (%)	
α-Helix	10
β-Pleated	30
Aperiodic	60
Stokes radius	50 Å
Shape	Oblate ellipsoid
Size	10.4—11.8 × 10.4—11.8 × 7.08—8.8 nm
Axial ratio	0.85
Hydration	0.25 g solvent/g protein
Frictional ratio	0.128 ± 0.05

adex® G-200. Schwenke et al.[348] have also isolated the 11S globulin of sunflower seeds by cryoprecipitation from solution of high ionic strength (0.5 to 1.8) with simultaneous reduction of the salt concentration (ionic strength 0.3) and repeated gel chromatography on Sephadex® G-200. By this procedure, the 7S component that occurs in small quantity is isolated too. Tyrurina and Klimenko[361] isolated the 11S globulin of sunflower seed by the salting out in cold of the salt-soluble fraction and using density gradient extraction with $(NH_4)_2SO_4$ on a column. The protein eluted between 26 to 28% $(NH_4)_2SO_4$ saturation; it had a sedimentation coefficient of 11.3S and contained all the essential amino acids. Rahma and Narasinga Rao[362] have isolated the major fraction of sunflower seeds in a homogeneous form using $(NH_4)_2SO_4$ precipitation.

2. Physicochemical Properties

The amino acid composition of the 11S protein is given in Table 14 and its physicochemical properties in Table 15.

Its $S_{20,w}$ value is 11.9 ± 0.02; $D_{20,w}$ value is 3.10×10^{-7} cm²/sec; partial specific volume is 0.730 mℓ/g; and molecular weight is 343,000. By sedimentation equilibrium technique, a molecular weight of 305,000 has been reported.[368] Rahma and Narasinga Rao[362] have reported a value of 11.6 for $S_{20,w}$ (at 0.6% protein concentration) and a molecular weight of 300,000. It had 0.31% carbohydrate and no phosphorus. The secondary structure of the protein consists of 10% α-helix, 30% β-structure, and nearly 60% aperiodic structure.[367] By electron microscopy,[374] the outer dimensions of the protein molecule have been estimated to be 10.4 × 10.4 nm and 8.8 nm. By small-angle X-ray scattering,[376] the molecule has been shown to have a spherical shape. However, it has also been reported from X-ray and quasielastic light-scattering techniques[370] that the molecular shape of the globulin is an oblate ellipsoid of revolution with the axial ratio (a/b) = 0.6. The frictional coefficient, assuming a solvation shell of thickness 0.5 nm, was 1.06 and has been shown to be typical of seed proteins.[370]

SDS-disc gel electrophoresis of the 11S protein revealed two major pairs of disulfide-

linked polypeptide subunits.[375] The molecular weight of one polypeptide chain was 59,000 and was composed of subunits of molecular weights, 37,000 and 22,000. The other polypeptide chain of molecular weight 51,000 was composed of subunits of molecular weights 29,000 and 22,000, respectively. The data suggest that these linked polypeptide pairs associate noncovalently in the ratio 2:3 to form the parent molecule.

Thus, the protein has subunit structure. The molecule consists of 6 subunits arranged as a trigonal antiprism with dihedral point group asymmetry of 32.[376] This model by Plietz et al.[376] is similar to that of Plietz et al.[370] and of Reichelt et al.[374] The subunits have a central channel parallel to the threefold axis of the molecule.[374]

The effect of pH,[365-367,377] urea,[364,525] GuHCl,[525] SDS,[367,525] heat,[525] and γ-radiation[363] on 11S protein is well documented. It dissociates to a 3S fraction through a 7S protein at extreme pH. However, in solutions of high NaCl concentration, dissociation progresses only up to 7S fraction.

Sripad[377] has reported the effect of acid and alkali on 11S globulin of sunflower seed. Data from viscosity, UV-difference spectra, fluorescence, and CD suggested denaturation of the 11S protein along with dissociation in the pH range 4.2 to 2.5. Below this pH, reaggregation and refolding of the protein have been observed, a property exhibited by many other seed proteins.[91,378] Based on this, a scheme is proposed:

$$11S \xrightarrow{\text{pH 4 to 8.0}} 2S \xrightarrow{\text{pH 2.5}} 12S$$

Sripad[377] has also reported the effect of pH on hydrolysis of 11S globulin by pepsin. It was observed that hydrolysis at pH 8.0 was poor compared to hydrolysis at pH 2.2. This has been attributed to the compact structure of the protein at pH 8.0.

The effect of alkali on the 11S protein is also reported.[377] Up to pH 9.0, the 11S protein dissociated to the 7S component, above which it dissociated into 2S component. At alkaline pH, [n] increased from 4.0 to 10.0 mℓ/g. Fluorescence quenching and red shift in fluorescence emission maximum were observed. This suggested denaturation of the protein. It is also indicated that the alkali-induced dissociation and denaturation of the protein was a multistep process. However, removal of CGA did not have any effect on the association-dissociation and denaturation of 11S protein under the above experimental conditions.

Urea[525] dissociated the 11S protein to 2S protein through an intermediate 7S protein. GuHCl[525] also exerted a similar effect. Urea[364] caused changes in viscosity, specific rotation, UV-absorption spectrum, and electrophoretic mobility. SDS[527] directly dissociated the protein to 2S protein. There was no change in the secondary structure of 11S protein at extreme pH and also in SDS solution.[367] The latter behavior is in contrast to that of other seed proteins.[316]

Heating a solution of the 11S protein at 90°C for 20 min dissociated the protein, but did not precipitate it.[525] Svestarova and Stefanora[363] have studied the effect of large doses of γ-rays (1×10^6, 6×10^6, and 20×10^6 rad) on the denaturation of two globulin preparations isolated from sunflower seeds. The solubility, viscosity, and UV-absorption spectra of native and irradiated preparations showed that doses as low as 1×10^6 rad denatured the protein. At a dosage of 20×10^6 rad, there was a change in the conformation of the protein.

In solutions of low ionic strength, the 11S protein dissociated to give a 7S component.[358] In aluminum-lactate buffer (pH 3.1), the 11S globulin also dissociated to 7S component and this was identical with the 7S component isolated by gel chromatography.[358] Thus, the 11S protein is an oligomeric protein that consists of subunits and can be dissociated into lower molecular weight fractions under a variety of conditions.

Schwenke et al.[348] have studied the effect of neutral salts on the solubility of 11S globulin in the pH range 2 to 9 using turbidimetric titration technique. The magnitude of the turbidity depended upon both the nature of the salt and its concentration. Maximum turbidity was

observed at ionic strength of 2.0, and extent of turbidity followed the order of cations (present as chlorides) and anions (present as Na or K salts), $Ca^{2+} > Mg^{2+} > Li^{+} > Na^{+} > K^{+}$; $SO_4^{2-} > Cl^{-}$; $SCN^{-} > ClO_4^{-}$, respectively. There was a hysteresis effect during back titration with alkali, which led to a shift of maximum turbidity. In the presence of rhodanide, no irreversible aggregation was observed; ClO_4^{-} showed this effect only at high (2 *M*) concentrations.

Turbidimetric titrations have been used to study the effect of sodium alginate on the solubility of the 11S protein in the pH range 2 to 8 at both low and high ionic strengths.[371] At low ionic strength of 0.1 and as the alginate concentration increased, the turbidity was shifted to lower pH; at the same time, the extent of turbidity also decreased. When the ionic strength was increased to 1.0, there was no shift in the turbidity maximum. This has been attributed to the electrostatic nature of the interaction between the protein and the polysaccharide. The reaction was irreversible in the absence of NaCl.

Sastry[379] has studied the binding of CGA by the 11S protein under various solution conditions and determined the energetics and nature of binding. It has been shown that CGA/CA binds to 11S with positive cooperativity. Effect of pH indicated that maximum binding occurred at pH 4.0. Decrease in pH increased the number of binding sites, whereas the binding constant was nearly constant. At pH 4.0, NaCl slightly decreased the binding, whereas Na_2SO_3 at 0.01 *M* completely abolished binding. Increase in temperature decreased the binding. At 55°C, no binding was observed. Among the solvents used, dioxane or ethanol at 4% abolished binding. However, methanol decreased the binding only to a slight extent. Succinylation of the protein reduced the affinity for CGA/CA, possibly due to increase in negative charge. Modification of SH group with NEM had no effect on the extent of binding of CGA/CA. Urea at 8 *M* was shown to completely abolish CGA binding to 11S globulin. The extent of hydrolysis of 11S protein by trypsin, α-chromotrypsin, and papain is affected by addition of CGA.[379] On the other hand, CA reduced the hydrolysis by trypsin, but not by the other two proteases. Binding of CGA/CA did not seem to affect the oligomeric structure, but affected the tertiary and secondary structures as shown by fluorescence, UV spectroscopy, and CD. Binding the polyphenols by the 11S protein followed the order CGA > CA > QA. This suggested that chain length of the molecule possibly determined the affinity for the protein.

Schwenke et al.[372] have modified the 11S globulin of sunflower seed using dialdehyde starches (DAS). It has been shown to react nonspecifically with the amino acid residues of the 11S protein. The modification of the protein caused a decrease in the content of each amino acid. Nearly 70% of the amino groups were blocked by reaction with DAS at pH 8.0. As a result of modification, there was a shift in the isoelectric point to pH 4.3, and small amounts of intermolecular cross-linked products with sedimentation coefficients of 17S and >17S were formed. By SDS-PAGE, dimers and trimers of the polypeptide chain were also detected.

The 11S globulin was succinylated with 1 *M* succinic anhydride in anhydrous Me_2CO.[373] Nearly 83% of the amino groups were blocked under these conditions. Succinylation decreased the isoelectric point, improved emulsifying capacity, and the protein dissociated into subunits.

C. Other Protein Fractions

The 7S protein constitutes nearly 10% of the total proteins.[353] At low ionic strength, the 11S protein dissociates to the 7S component and is shown to be in equilibrium with it. It appears that 7S protein is more negatively charged than the 11S component as judged by its mobility in PAGE.

The 2S protein, being predominantly water soluble, is termed albumin, and constitutes nearly 20% the total proteins.[353] Several methods for its isolation have been reported.[331,344,359]

Schwenke et al.[331] have used tannin for the isolation of albumins from sunflower seed proteins. The dissociation of the tannin-albumin complex was achieved with caffeine. It has also been shown that dextransulfate and polyphosphate could be used to precipitate the proteins. Albumins of the seeds were extracted in water and fractionated by gel chromatography on Sephadex® G-75 and CM-Sephadex.®[344] Two electrophoretically similar fractions were obtained. Both fractions had isoelectric points above pH 10.0. Schwenke and Simon[359] have been able to separate globulins and albumins from sunflower seeds by extraction with 10% NaCl and adjustment of the pH of the aqueous protein extract to 3.0 by HCl to give a sludge containing globulins. The supernatant was treated with 1 *N* NaOH to adjust the pH to 8.5 to 9.5. Centrifugation and heating the solution at 90.5°C precipitated the albumins. Globulins and albumins could also be separated simultaneously by adjusting the pH to 7.5 with 1 *M* NaOH, filtration, and adjusting the pH to 9.5 and heating at 90.5°C.

The amino acid composition of the 2S protein is shown in Table 14. It is unique by the high content of amide and cysteine which is different from that of the 11S globulin. The high cysteine content of the protein may have a role in providing sulfur reserves during germination of seed.[380]

The molecular weight determined from several methods was 14,000 to 16,000. The biological value of albumins was 54. Recently, a value of 60 was reported for the sunflower total proteins.[357]

Youle and Huang[380] have reported that in most species the nitrogen content of the 2S protein (albumin) is higher than that of the 7S and 11S proteins. Sripad[377] has observed that the α-helix content of the 2S protein is much higher (>30%) than that of the 11S globulin. Similar results have been reported by Schwenke et al.[366] with the albumins of rapeseed and 2S fraction of sesame.[302]

The formation of insoluble complexes of sunflower seed albumin with alginate or pectin was studied by means of turbidimetric titration and by determining the pH-dependent precipitability of protein.[369] The complex formation is based on electrostatic interaction and is a function of pH value and the protein polyanion ratio. It is affected by neutral salts.

D. Polyphenol-Protein Interaction

Several methods are available for the efficient removal of polyphenols (CGA) from sunflower seed.[349,355,377,379] Fan et al.[349] have shown that countercurrent extraction of sunflower flour with water, acid, or ethyl alcohol, and countercurrent diffusion of sunflower seed with acid were more efficient for CGA removal than batch extractions with fresh solvents. This process was capable of removing nearly 90% of the CGA from the meal or seed. The resulting protein concentrates contained ~70% protein and were light in color even at alkaline pH. Sripad et al.[355] have studied the effect of various solvents on the extractability of polyphenols of sunflower meal. The extractability of CGA increased with increase in pH, and at pH 8 in water nearly 70% CGA was removed in a single extraction. NaCl did not increase the extraction, however, $MgCl_2$ and $CaCl_2$ increased it, especially at higher concentrations. Methanol, ethanol, isopropanol, and acetone at 20% concentration in water extracted the maximum amount of CGA. The organic solvents were found to be poor solvents for extraction of polyphenols. Apart from these, there are a number of methods for the removal of polyphenols from sunflower seeds.[377,379] Most of the methods are based on the nature of interaction of CGA and the proteins of sunflower seed.

Several hypotheses have been put forward for the interaction between CGA and proteins of sunflower seed. Hydrogen bonding and also possibly salt or electrostatic linkages have been suggested in preference to other types of interaction.[379,381-383] Hydrogen bond has been shown to occur between phenolic hydroxyl hydrogen of CGA and oxygen of peptide bond.[384] Also, hydrogen bond could form between phenolic carboxyl oxygen of CGA and amide hydrogen of the protein. Ionic and salt linkages have been shown to involve ionized carboxyl

of hydrolyzable polyphenol and basic groups of the protein, in particular, arginine and lysine.[385] However, it is also shown that covalent interaction of polyphenols involves reaction of quinones (oxidation products of polyphenol) with proteins through amino, thiol, and active methylene groups.[386] Hence, it can be reasonably concluded that a favorable combination of hydrogen bonding and covalent linkages of quinones is involved in the interaction of polyphenols with the proteins of sunflower seeds. However, more definite generalizations can be drawn when the interactions of CGA/CA/QA with the 2S fraction are studied.

VI. MUSTARD SEED AND RAPESEED PROTEINS

Mustard and rapeseed belong to the *Brassica* species. They contain about 35 to 40% oil and 20 to 25% protein. The dehulled and solvent-extracted seed meal contains 35 to 45% protein.[387,388] The major proteins of mustard and rapeseed are storage proteins located in protein bodies called aleurone grains.[389] The crude protein contains nearly 5% nonprotein nitrogen.[390] The seeds also contain antinutritional factors such as thioglucosides which on hydrolysis yield goitrogenic substances; phytic acid as phytates; and low molecular weight ethanol-soluble component which has been shown to affect pregnant rats and cause anorexia.[389]

A. Nitrogen Solubility

The effect of various parameters on the extraction of nitrogen/protein from rapeseed meal was investigated by several workers.[391-404] Optimal conditions for extraction have been reported to be 1:10 meal to solvent and 0.1 to 0.2% NaOH with a shaking time of 20 to 40 min.[391] Pokorny and Sefr[392] obtained a protein isolate with a yield of 40 to 50% by precipitating the alkali extract at pH 3.5 using mineral acids. They extracted the rapeseed proteins with water, and 0.3% solutions of NaCl, Na_2HPO_4, Na_3PO_4, $NaHCO_3$, Na_2CO_3, or NaOH. In all cases, protein extractability increased with increase in the alkalinity of the solvent. The solubility of nitrogen from the defatted flour of both mustard and rapeseed has been investigated.[387] Of the total meal nitrogen, 44 to 52% was found to be water soluble, 20 to 25% in 5% NaCl, 3 to 5% in 70% ethanol, and 5 to 9% in 0.2% NaOH. The alkali-soluble proteins could be precipitated between pH of 4.4 to 4.6. However, the yield was less than 50%, which is low compared with protein isolates from other oilseeds. Bhatty et al.[393] observed that 10% NaCl extracted nearly 67% nitrogen as compared with 0.01 *M* sodium pyrophosphate buffer of pH 7.0 at 5°C from rapeseed meal. The effect of pH and temperature on the extraction of nitrogen from rapeseed flour was investigated by Korolczuk and Rutkowski using 0.1 *N* HCl or NaOH.[394] They observed that more than 80% of the total nitrogen was extracted at pH 9.5 to 10 (30 to 45°C), 20 to 25% at pH 6.5 to 8.0 (80 to 100°C), and 35% at pH 3.8 to 4.0 at 20°C. Girault[395] reported that whereas 0.1 *N* NaOH extracted nearly 89% of total meal nitrogen only 67% was extracted in 10% NaCl solution. At pH 3.0, 22% of the protein and at pH 6.5, 51% of the protein was precipitated from NaCl and NaOH extracts, respectively. Schwenke et al.[396] extracted proteins from defatted rapeseed flour using solvents such as water, 10% NaCl, 0.1% NaOH, and 0.1 *N* NaOH, and as a function of pH from 2 to 6. In water, the maximum extractability was 50% at any single pH value. On the other hand, complete extracton was achieved with salt and alkali with three successive extractions. Maximum extractability of about 75% was achieved at pH 6 in 3% NaCl.

The solubility of proteins from rapeseed as a function of pH and temperature shows that the percentage of nitrogen solubilized at each pH increased with increase in temperature. The point of minimum solubility also shifted to the alkaline side as the temperature increased, the values being pH 4.5, 4.8, 7.0, and 7.2 at 25, 35, 45, and 55°C, respectively. Quinn and Jones[398] reported the nitrogen solubility of rapeseed meal as a function of pH in water, 5% NaCl, and 5% $CaCl_2$ with a 1:20 flour-to-solvent ratio and a shaking time of 4 hr. The extractabilities were 65, 80, and 70% in water, 5% NaCl, and 5% $CaCl_2$, respectively. In

water, there were two points of solubility minimum, at pH values of 3.7 to 4.0 and 7.7 to 8.0. Thompson et al.[399] studied the effect of 2% sodium hexameta phosphate (SHMP) on the nitrogen solubility of rapeseed meal. Maximum extractability of 90% at pH 12.0 was observed with minimum solubility at pH 2.5. A second extraction with the same concentration of SHMP solution solubilized 97% of nitrogen. Finlayson et al.[400] have examined the extractability of nitrogen from the meal of *Brassica juncea* and *B. napus* in 0.1 *M* NaOH and Triton® X-100. In 0.1 *M* NaOH solution, nearly 83% of the total nitrogen of *B. napus* was extracted, whereas it was 91% in the case of *B. juncea*. The nonionic detergent, Triton® X-100, at concentrations of 1 and 5%, solubilized 80 to 84% of the meal nitrogen of the two species. In 0.01 *M* borate buffer of pH 8.0, nearly 65% of the meal nitrogen was extracted. Addition of 0.01 *M* β-mercaptoethanol did not affect the nitrogen extractability. However, extraction of the *B. napus* flour with 0.1 *M* β-mercaptoethanol alone solubilized nearly 35% of the meal nitrogen.[400] Gillberg and Tornell[401] studied the nitrogen solubility of the unheated and heat-treated defatted *B. napus* in water as a function of pH. In both samples, two points of minimum solubility were observed, at pH 4.0 and 8.0. The solubility was 40 and 60% and 25 and 35% for both the unheated and heated samples, respectively, at pH 4.0 and 8.0. Addition of sodium phytate enhanced the nitrogen solubility. El Nockrashy et al.[402] have developed a countercurrent procedure for the extraction of protein from defatted rapeseed meal. Using 0.02 *N* NaOH and a meal-to-solvent ratio of 1:25, as much as 94% of meal nitrogen was extracted. A two-step precipitation, first at pH 6.0 and then at pH 3.6, resulted in 69 and 24% of meal protein, respectively. On a dry weight basis, the pH 6.0 isolate contained nearly 93% protein and the pH 4.6 isolate contained nearly 99% protein. Gillberg[403] studied the influence of electrolytes on the solubility of rapeseed protein isolates at different pH values. The solubility profiles were different for the different salts and this has been explained by ion binding to the proteins and to other polymers present in the isolates. The higher the affinity of the proteins for the anions, the lower the nitrogen solubility of the protein isolate at pH 0.7. However, the same salts increased the nitrogen solubility at pH 5.4. Gururaj Rao et al.[404] have studied the extractability of protein from mustard flour in various aqueous solvents, namely water, 1 *M* NaCl, and 2% SHMP solution. The solubility-pH profile of mustard proteins in water and in 1 *M* NaCl solution showed two minima, at pH 3.8 to 4.0 and 7.8 to 8.0. Maximum solubility of 92% was observed at 11.0. However, the solubility profile in 2% SHMP showed two minima occurring at pH 3.0 and 10.0, different from that in water and 1 *M* NaCl solution.

The work on nitrogen solubility of mustard and rapeseed by various workers indicates that (1) most of the protein can be extracted under high alkaline pH conditions and in the presence of high concentration of salt; (2) the proteins show two minima in their solubility, one at pH ~4.0 and another at pH ~8.0; (3) the presence of various electrolytes had a profound effect on the extractability of proteins; and (4) the solubility of the proteins decreased with increase in temperature, possibly due to denaturation of the proteins.

B. Fractionation and Isolation of Proteins

There are several reports on the fractionation and isolation of the various components of mustard/rapeseed proteins. The earliest work on this has been by Bhatty et al.[393] They extracted the proteins of *B. napus* meal with low ionic strength sodium pyrophosphate buffer and were able to separate the proteins into four fractions by a combination of gel chromatography and ion-exchange chromatography. The 12S and 1.7S proteins were isolated. The 1.7S fraction was basic in nature. Lo and Hill[405] were able to separate by gel filtration the water extract of *B. napus* meal into four components: two protein fractions, a glucosinolate fraction, and a fraction rich in phenolic acids, chlorogenic acid, and caffeic acid. Lonnerdal and Janson[406] have separated two main fractions of the protein extract from *B. napus* by gel filtration and ion-exchange chromatography. One fraction contained the low molecular weight

Table 16
AMINO ACID COMPOSITION OF THE HIGH AND LOW MOLECULAR WEIGHT PROTEIN COMPONENTS OF MUSTARD SEED AND RAPESEED

	Residues per 100,000 g of protein					
	Mustard		Rapeseed			
Amino acid	12S[417]	1.3S[417]	12S[417]	12S[409]	1.3S[417]	2S[429]
Aspartic acid	53	26	71	83	14	20
Threonine	28	30	34	38	15	31
Serine	30	52	51	56	28	49
Glutamic acid	134	244	160	162	222	236
Proline	—	—	—	43	—	112
Glycine	75	79	90	81	54	81
Alanine	34	47	42	54	38	66
Valine	48	67	55	39	63	56
Methionine	22	45	23	14	30	17
Half cystine	—	—	—	—	—	67
Isoleucine	52	45	25	32	29	35
Leucine	9	67	59	66	49	73
Tyrosine	15	15	13	19	11	6
Phenylalanine	23	20	26	33	15	24
Lysine	17	58	24	23	45	61
Histidine	13	28	14	21	25	35
Ammonia	—	—	—	179	—	205
Arginine	—	—	38	39	—	50
Tryptophan	9	—	9	0	—	7

basic proteins and the other the unadsorbed neutral proteins. Each fraction was heterogeneous. MacKenzie and Blakely[407] have fractionated the NaCl extract of three species, *B. nigra*, *B. juncea*, and *B. hirta* meals, into three fractions by gel filtration. These fractions, upon further analysis with analytical ultracentrifugation, indicated a total of four components with sedimentation velocity coefficients of 1.8S, 7S, 12S, and 15S. The 12S fraction appeared to be more homogeneous compared to the others. The amino acid composition of the fractions was also determined. A similar study was made by Nagano and Okomato,[408] who fractionated the rapeseed proteins by gel filtration on Sephadex® G-200. They obtained three fractions designated α, β, and γ. The α and β fractions had sedimentation coefficients of 1 and 12, respectively. Gill and Tung[409] isolated the 12S fraction from commercial rapeseed (*B. campestris*) meal by gel filtration on Sephadex® G-100 and dialysis of the fraction that eluted at void volume. Some of the physicochemical properties of the protein were determined. The amino acid composition was determined and is given in Table 16. Lodh and Samantaray[410] extracted the water soluble proteins of mustard seed at neutral pH and subjected them to SDS-PAGE after exhaustive dialysis. From the electrophoretic pattern, it was concluded that the proteins consist of three fractions.

Simard et al.[411] fractionated the rapeseed proteins by $(NH_4)_2SO_4$ precipitation. They reported that at 25 to 30% $(NH_4)_2SO_4$ saturation the protein extract yielded a 12S protein fraction with more than 90% homogeneity. Higher saturation of $(NH_4)_2SO_4$ preferentially precipitated the 2S protein fraction. Gururaj Rao et al.[404] have isolated and characterized the total proteins and high and low molecular weight fractions from *B. juncea* and *B. campestris*. They obtained the high molecular weight protein fraction by 20% $(NH_4)_2SO_4$ precipitation of NaCl extract, and from the supernatant, the low molecular weight protein fraction was obtained by adjusting the pH to 3.8 with 0.5 *N* HCl. Schwenke and Raab[412] have isolated the 12S globulin from rapeseed and have determined its homogeneity by

ultracentrifugation and ion-exchange chromatography. The method consisted of the following steps: (1) extraction of the total proteins in 10% NaCl; (2) precipitation by dialysis; (3) repeated gel chromatographic purification on Sephadex® G-200; (4) ion-exchange chromatography on DEAE-Sephadex® A-50; and (5) isoelectric focusing to recover the protein at pH 7.25. The amino acid composition of the purified fraction was also reported.

C. Physicochemical Properties

From the literature it is evident that the proteins from *Brassica* species consist mainly of high and low molecular weight fractions. The sedimentation coefficient of high molecular weight fraction is 12S and that of low molecular weight fraction is 1.7S. The proteins have been isolated to a fair degree of homogeneity by more than one procedure and are characterized for properties such as viscosity, molecular weight, size, and shape, and also their behavior in urea, acid, and alkali. Their interactions with ligands such as phytates, glucosinolates, and isothiocyanates have also been studied.

1. High Molecular Weight Protein Fraction

Bhatty et al.[393] observed that the 12S mustard proteins dissociated into a 3S fraction upon decreasing the pH from neutral to pH 3.6. With further decrease in pH to 2.2, a part of the 3S component reaggregated to a 7S component. The dissociation was also observed by PAGE, where at least four components were observed, indicating that the molecule was an aggregate of subunits. It was also observed that a 1% solution of the 12S protein of *B. napus* dissociated into a 2.3S component in the presence of 6 *M* urea. Goding et al.[413] studied the dissociation of *B. napus* and *B. campestris* 12S globulin in acid pH by several physicochemical techniques. At pH 2.0 in the presence of 2 *M* urea, the globulin dissociated into four fractions. From this, a fraction having $S_{20,w}$ of 2.7 was isolated. It contained nearly 4.5% carbohydrate. The 2.7S fraction from both species was found to contain about 0.5% of their nitrogen as galactosamine, 4.5% reducing sugars such as glucose and arabinose, and had the same N-terminal amino acid. MacKenzie and Blakely[414] studied the effect of 6 *M* urea in the presence of 0.1 *M* mercaptoethanol on the dissociation-denaturation of 12S globulin from *B. juncea*. The resultant products were partially separated by preparative isoelectric focusing. At least 11 species, having isoelectric points ranging from 4.75 to 9.15, were detected. The results of PAGE and the amino acid composition of the fractions suggested that the isoelectric species were distinct subunits. At least five basic, five acidic, and one neutral protein component were detected. Gill and Tung[415] isolated the 12S fraction of rapeseed protein by precipitating the alkali-soluble protein from *B. campestris* at pH 9.2. The protein was found to be homogeneous, and a molecular weight of 129,200 was reported for the protein. It consisted of subunits as indicated by its behavior in 5 *M* urea solution. Electron microscopy of the protein revealed that it was highly agglomerated, probably due to charge effect. Heating aqueous dispersions at 1% protein concentration resulted in gelation. Blocking of free –SH groups with *p*-mercuribenzoate did not prevent gelation.

The effect of different extraction solvents on the molecular size of the *Brassica* proteins has been investigated in detail.[400] From the similarity of mobility in electrophoresis, it has been suggested that the rapeseed globulin had very little tendency to polymerize through the formation of intermolecular disulfide bonds, in contradiction to the suggestions of Bhatty et al.[393] Cysteine was shown to be present in all six fractions obtained upon alkylation of the reduced globulin. The 12S globulin of *B. campestris* has been characterized both chemically and ultrastructurally.[409] It was found to contain 12.9% (w/w) carbohydrate consisting of arabinose, galactose, glucose, inositol, glucosamine, and mannose. The observation that the rapeseed aleurone grains contain globoid bodies suggests the presence of phytic acid and could be related to the presence of inositol in the 12S component. The amino acid composition of the globulin indicates that it is high in acidic amino acids, glutamic, and

aspartic acids. By electron microscopy, the particle diameter was determined to be 120 Å.[409] The difference in the carbohydrate content of the 12S protein of the commercial meal and that obtained from seed could have resulted from nonenzymatic browning reactions. Gill and Tung[415] have also reported the electrophoretic and immunochemical properties of the 12S rapeseed protein. The 12S protein was separated into a major component by disc-gel electrophoresis, while SDS-gel electrophoresis resulted in the separation of subunits with apparent molecular weights of 12,200, 17,400, 30,100, 37,600, and 42,000. Electrophoretic patterns of nonreduced and reduced samples indicated the presence of intermolecular disulfide bonds, although the cystine content was low. The 12S protein self-associates to form aggregates of higher molecular weight (dimers), and the low molecular weight fragment containing the glycopeptide is located on the surface of the aggregate. The gelation of 12S rapeseed glycoprotein has been studied by scanning electron microscopy and rheological characterization.[416] It revealed a progression in three-dimensional ordering and a decrease in pore size as the pH was increased from 6 to 10. At pH 4.0, the gel was amorphous and readily reverted to a sol. Gel strength was affected by changes in pH and ionic strength. Urea and dithiothreitol had little effect on gelation. Selective reduction and alkylation prevented gelation, but the content of ϵ-amino groups was changed. Gill and Tung[416] have suggested the possibility of protein-carbohydrate interaction during gel formation since the 12S fraction contains nearly 13% carbohydrate.

The physicochemical properties and the effect of denaturants on the 12S fraction have been reported.[404,417-419] The protein had an intrinsic viscosity of 3.6 mℓ/g, an ultraviolet absorption maximum at 280 nm, maximum fluorescence emission intensity at 325 nm, a molecular weight of 230,000 by the Archibald approach to the sedimentation equilibrium method, and 8 subunits of molecular weight ranging from 11,000 to 70,000. The other properties are listed in Table 17. A comparative study of the high molecular weight fraction of mustard seed (*B. juncea*) and rapeseed (*B. campestris*) has been made.[417] The protein from the two species had nearly identical sedimentation coefficient, molecular weight, intrinsic viscosity, and fluorescence emission spectrum. However, differences in the amino acid composition were observed. In general, rapeseed contained higher amounts of most of the amino acids compared to mustard. Data on the rate of hydrolysis by proteolytic enzymes and CD suggested differences in the secondary structures and possibly amino acid sequences between the high molecular weight proteins from the two species. The amino acid compositions of the two proteins are tabulated in Table 16. Similarly, dissociation and denaturation of the high molecular weight protein fraction of mustard seed and rapeseed by urea and GuHCl were investigated.[418] Urea and GuHCl dissociate the 12S protein of mustard seed and rapeseed to 1.8S protein; the extent of dissociation depends upon the concentration of the denaturant. The process of dissociation/denaturation is complete around 8 and 5 *M* urea and GuHCl, respectively, as judged by analytical ultracentrifugation, viscosity, UV spectral, and fluorescence data. Analysis of the data shows that the perturbation of the aromatic amino acid residues takes place simultaneously with dissociation, and the final denaturation step is preceded by the exposure of most of the aromatic amino acid residues to the bulk solvent.[418] The effect of SDS on the association-dissociation of the high molecular weight protein fraction from both mustard seed and rapeseed indicates that at low concentration of SDS (<3.47 m*M*) mustard seed protein undergoes association, and, as the concentration of SDS is increased, it dissociates to a 1.8S component.[419] However, the rapeseed protein undergoes only dissociation at all concentrations of SDS. Changes in UV-difference spectra and fluorescence quenching are observed with both proteins in the presence of SDS. In the presence of high concentrations of SDS (~17 m*M*), it appears that mustard seed protein is denatured to a greater extent than rapeseed 12S fraction, as indicated by intrinsic viscosity results.[419]

Schwenke et al.[420] have reported the interaction of 12S globulin of rapeseed and phytic

Table 17
PHYSICOCHEMICAL PARAMETERS OF THE HIGH MOLECULAR WEIGHT PROTEIN FRACTION OF MUSTARD/ RAPESEED[404,409,417,421,423,431] PARAMETERS

Property	Mustard	Rapeseed
Sedimentation coefficient ($\times 10^{-13}$ sec)	12	12.7
Diffusion coefficient ($\times 10^{-7}$ cm²/sec)	11.7 (?)	3.8
Intrinsic viscosity (n) (mℓ/g)		
1 *M* NaCl	3.6	3.7
0.025 *M* Tris-glycine buffer (pH 8.3)	5.4	4.5
Stokes radius (Rs, nm)		
Quasielastic light scattering	—	5.7
Gel chromatography	—	5.5
Partial specific volume ($\bar{V}$, mℓ/g)	—	0.729
Molecular weight		
Archibald method	2.3×10^5	—
Sedimentation velocity and viscosity	2.4×10^5	—
Sedimentation velocity and diffusion	—	3.0×10^5
Sedimentation velocity and gel chromatography	—	2.94×10^5
Frictional ratio (f/f_o)	—	1.28
Carbohydrate content (%)	1.2	1.2—12
Phosphorus content (%)	0.025	—
$E^{1\%}_{1\text{ cm, }280\text{ nm}}$	9.9	—
Fluorescence emission maximum (nm)	325	—
No. of subunits	6	6
Secondary structure (%)		
α-Helix	9	11
β-Structure	27	31
Aperiodic	64	58
Hydration (gram of water per gram of protein)	0.3—0.8	
Myrosinase activity	+ve	+ve

acid using turbidimetric titration at 460 nm. It was observed that the turbidimetric titration of the salt-free 12S protein gave a sharp peak with a maximum turbidity at pH 6.2. In the presence of increasing concentrations of phytic acid, the pH of maximum turbidity shifted towards the acid side; also, the intensity of turbidity increased. The pH of maximum turbidity occurred at pH 5.6, 5.1, and 4.0 at phytic acid concentrations of 1.3, 2.7, and 12.8%, respectively. The hydrodynamic and quasielastic light-scattering properties of 12S globulin from rapeseed are reported by Schwenke et al.[421] They have reported an $S_{20,w}$ of 12.7; a $D_{20,w} = 3.8 \times 10^{-7}$ cm²/sec; and a molecular weight of nearly 300,000. The other properties such as radius and frictional coefficient reported by them are given in Table 17. The 12S rapeseed globulin has been isolated to homogeneity by the same group.[422] The isolated globulin showed an isoelectric point of pH 7.25 as determined by isoelectric focusing and an acidic to basic amino acid ratio of 1.0. As in other storage proteins, a high content of glutamic (19%) and aspartic (10%) acid and a low content of sulfur-containing amino acids were found. It contained nearly 7% arginine, contrary to literature reports. The sugar content of the globulin was low (0.5%). From the amino acid composition, the average hydrophobicity was calculated to be 1041 cal per residue. Schwenke et al.[423] have reviewed the structure of the 12S globulin from rapeseed. From the hydrodynamic properties as calculated from small-angle X-ray scattering and shape as seen in electron microscopy and by other studies, they have concluded that the 12S globulin of rapeseed is an oligomeric protein with

Table 18
STRUCTURAL PARAMETERS OF THE 12S GLOBULIN FROM RAPESEED DERIVED FROM SMALL-ANGLE X-RAY SCATTERING AND ELECTRON MICROSCOPIC INVESTIGATIONS[423,427,428]

Property	X-ray scattering	Electron microscopy
Molecular shape	Oblate ellipsoid of revolution	—
Axial ratio	0.80	0.81
Outer dimension (nm)	11.0 × 11.0 × 8.8	11.3 × 11.3 × 9.2
Solvation (gram solvent per gram of protein)	0.22	0.49
Radius of gyration (nm)	4.1	—
Volume (nm^3)	470	—
Surface (nm^2)	440	—
Maximum dimension (nm)	11	—
Quaternary structure	Trigonal antiprism	—
Number of subunits	—	6
Symmetry	Dihedral point group 32 (D_3)	—

a molecular weight of 300,000 and is composed of six subunits arranged in trigonal antiprism with point group symmetry of 32 (D_3). The protein consists of four polypeptide chains differing in their molecular weights as determined by SDS-PAGE, i.e., 18,500 ± 800, 21,000 ± 400, 26,800 ± 900, and 31,200 ± 1,600. From the behavior of the 12S globulin in various solution conditions such as ionic strength, pH, denaturants, etc., the following scheme has been proposed:

$$12S \underset{\text{high ionic strength}}{\overset{\text{low ionic strength}}{\rightleftharpoons}} 7S \underset{\text{urea}}{\overset{\text{extremely high or low pH}}{\rightleftharpoons}} 2S\text{—}3S$$

The various properties of the protein are tabulated in Table 18. The small-angle X-ray scattering studies have shown that the protein is an oblate ellipsoid of revolution with an axial ratio of 0.81. The secondary structure of the protein is characterized by a low content of α-helix (11%) and a relatively high content of β-conformation (31%), the rest being aperiodic. It has been shown that each of the four smaller polypeptide chains combine together to form one subunit of molecular weight 50,000, and six such subunits make the whole molecule.

The interaction of phytate with mustard 12S protein in the pH range 1.0 to 5.0 has been investigated by the techniques of precipitation, turbidimetry, electrophoresis, and electrometric titration.[424] At pH 3.0, maximum binding of 170 mol of phytate per mole of protein was observed. Electrometric titration studies indicated the possibility of the presence of soluble protein phytate complexes in the system. It is hypothesized that only one class of binding site is involved in the complex formation. It has also been reported that at pH 3.0, calcium inhibited the formation of protein-phytate complex. Kishore Kumar Murthy and Narasinga Rao[425] have shown that the 12S protein, upon interaction with allyliosthiocynate, does not undergo any major structural changes including its quaternary structure. The observed alteration in the UV and fluorescence spectrum and in vitro digestibility is attributed to the end product formed upon the interaction of 12S protein with allylisothiocyanate rather

than to any conformational change. They have also studied the effect of low pH on the association-dissociation and denaturation behavior of 12S globulin.[426] The results of ultracentrifugation and electrophoresis experiments indicated that the protein dissociated into lower molecular weight fraction as the pH was decreased from 5.0 to 3.0. The changes in the optical properties suggested increased unfolding of the protein molecule. They concluded that both dissociation and denaturation of the protein occurred in the pH range 5.0 to 3.0. Below this pH, refolding and/or reaggregation was indicated. Schematically, the reactions can be represented as

$$\underset{(\text{pH } 5.0)}{12\text{S}} \xrightarrow[H^+]{\text{up to pH } 3.0} \underset{\text{denaturation}}{\text{dissociation and}} \xrightarrow[H^+]{\text{below pH } 3.0} \underset{\text{reaggregation}}{\text{refolding and}}$$

The results discussed above indicate that mustard and rapeseed proteins are composed mainly of two fractions: the 2S and 12S proteins. The 7S and 15S components present are minor components. Most of the work reported is on the 12S globulin. The 12S protein is isolated by simple $(NH_4)_2SO_4$ fractionation. The protein appears to be very sensitive to changes in ionic strength and pH and dissociates to 7S. At high pH or in denaturant solutions, it dissociates ultimately to a 3S or 2S component. The protein appears to be rich in glutamic and aspartic acids and is an acidic protein. The protein binds nearly 170 mol of phytate per mole of protein, and calcium has been shown to inhibit the complex formation. The binding of allylisothiocyanate does not bring in any structural changes. Like other seed proteins, it exhibits dissociation and denaturation at low pH (pH <3.0), below which refolding and reaggregation are observed. Regarding the isoelectric point, there is a contradiction in the results, the value ranging from pH 5.0 to 7.25. This may be due to the fact that nearly 11 species having isoelectric points ranging from 4.75 to 9.15 are detected when the protein is dissociated in urea solution. Varietal differences and differences in the homogeneity of the proteins could also be the causes for this.

2. Low Molecular Weight Protein Fraction

Lonnerdal and Janson[406] have observed four proteins comprising the low molecular weight protein fraction of *B. napus*. They have shown that all four are basic in nature and their molecular weights range between 12,000 and 14,000. The proteins have similar amino acid composition and each protein consists of two chains held together by two disulfide bridges. Glycine is the terminal amino acid in all four proteins. The main chain, B_3, demonstrated microheterogeneity, consisting of 90 amino acids and 29-amino acids long polypeptide chains. They had proline and alanine, respectively, as their N-terminal amino acid. Schwenke et al.[429] isolated and characterized the rapeseed (*B. napus*) albumins. The molecular weight, as determined by various methods, was 14,400 ± 400. The amino acid composition was characterized by a relatively high content of cystine (6.9%) and lysine (9%). The values are given in Table 16. The proteins had an isoelectric point greater than pH 10. The partial specific volume of the protein was 0.727 mℓ/g. The secondary structure of the protein consisted of α-helix, 40 to 46%; β-structure, 11 to 16%; and aperiodic structure, 41 to 43%.

MacKenzie and Blakely[407] have isolated to homogeneity two globulins from the seeds of *B. juncea*, *B. nigra*, and *B. hirta*. The proteins, A and B, from the various species have similar physical properties such as size, shape, and molecular weight, but they differ significantly in the amino acid composition. Sedimentation velocity experiments with A fraction gave an $S_{20,w}$ value of 12.0S and that of B fraction gave 1.8S. A low molecular weight protein fraction was isolated which sedimented as a single peak in analytical ultracentrifugation with a sedimentation coefficient of 1.3. Both were found to be heterogeneous by other techniques such as ion-exchange chromatography on CM-Sephadex®. The presence

Table 19
PHYSICOCHEMICAL PARAMETERS OF THE LOW MOLECULAR WEIGHT PROTEIN FRACTION FROM MUSTARD AND RAPESEED[406,429-431]

Property	Values
Sedimentation coefficient ($S_{20,w}$)	1.3—1.8S
Intrinsic viscosity, [n] (mℓ/g)	5.4
Fluorescence emission maximum (nm)	325
Secondary structure (%)	
α-Helix	40—46
β-Structure	11—16
Aperiodic	41—43
Molecular weight	44,400 ± 400
Isoelectric pH	10.0
Carbohydrate content (%)	1.8
Trypsin inhibitor activity	9.8 TIU/mg protein

of chlorogenic acid was reported in these fractions. The intrinsic viscosity of this protein was 5.4 mℓ/g, a value suggesting it is more extended than the 12S protein. The other parameters such as absorption spectrum, fluorescence spectrum, and secondary structure are indicated in Table 19. Trypsin inhibitor activity (9.8 TIU/mg) is reported to be associated with this fraction.[430,431] Gururaj Rao et al.[404] have isolated and characterized the low molecular weight fraction of mustard seed. Some of the physicochemical properties reported by them are given in Table 19.

Compared to the 12S protein fraction, the 2S component appears to be a basic protein. It has high content of cysteine and lysine with an isoelectric point of pH 10.0. Also characteristic of this fraction is its high content of α-helix, nearly 45%, compared to 5% in 12S fraction.

D. Studies on Myrosinase

Myrosinases are a group of isoenzymes located in special cells (idioblasts) of rapeseed or mustard seed. Myrosinases hydrolyze the glucosinolates to isothiocyantes, glucose, and potassium bisulfate. One of the end products of hydrolysis, 5-vinyl-oxazolidine-2-thione, is shown to be a goitrin. However, it has been shown that the end products formed on hydrolysis of the glucosinolates by the endogenous enzyme in both mustard seed/rapeseed meal are much more complex compared to those observed in an isolated system.[431] Vaughan et al.[432] reported a method for the identification of myrosinase in *Brassica* and *Sinapsis* species after the separation of the protein by starch gel and immunoelectrophoresis. Lonnerdal and Janson[406] have isolated and characterized the myrosinase enzyme from *B. napus*. It is reported to be a glycoprotein containing 14% carbohydrate, with a molecular weight of 135,000, and consisting of two peptide chains with a molecular weight of 65,000 each. Isoelectric focusing showed that myrosinase existed in three different forms containing different amounts of carbohydrates and isoelectric pH 4.96, 4.99, and 5.06.

VII. COTTONSEED PROTEINS

Cottonseed *Gossypium* species is essentially a byproduct of the textile industry and is crushed for its oil. The utilization of the residual cake is limited because of the toxic component, gossypol. Although several methods are available for the efficient removal of

gossypol from the meal, none of them seem to be used on a commercial scale. Despite this limitation, a considerable amount of work has been done on the proteins of cottonseed.

A. Nitrogen Solubility

Several methods have been reported for the efficient extraction of proteins from cottonseed. Berardi et al.[433] have shown that a combination of enzymatic and chemical methods of extraction of cottonseed proteins has an advantage in that the method allows extraction of more protein, compared to a two-step process of extraction in water and dilute alkali; also, the ratio of flour to solvent can be cut down to 4:1, compared to 15:1 in the conventional treatments. Arzu et al.[434] and Rolz et al.[435] have used a number of proteolytic enzymes to hydrolyze the cottonseed proteins and then to extract them. Childs[436] has reported the extraction of proteins from the heated meal by treating it with proteolytic enzymes. Papain treatment did not markedly affect the amount of protein extracted, but trypsin treatment increased the efficiency of extraction. They obtained nearly 50% extraction of proteins from the meal held at 204°C for 30 min. Aripdzhanou et al.[437] have extracted the proteins from powdered cottonseed meal in 5% NH_4Cl solution (pH 5.7 to 6) and precipitated them with 5% HCl. The separated protein, after drying, contained 7% protein. Increasing the temperature to ~25°C increased the yield; it then decreased when heated to a temperature >70°C due to denaturation of the protein. Extraction of the material at high pH values imparted color and a characteristic odor to the protein. At pH ≥7, phytin was not extracted. Hanumantha Rao[438] has studied the extractability of cottonseed proteins in water, 0.5 and 1.0 *M* NaCl, and 0.5, 1.0, and 3% SHMP solution. In water and NaCl, minimum solubility occurred between pH 3 and 6 and at pH 8. However, in SHMP, the minimum solubility occurred at pH 2. Alkali peptization and isoelectric precipitation at pH 4 yielded an isolate that contained bound gossypol. However, extraction of the protein in 1 *M* NaCl solution and precipitation at pH 4 yielded an isolate that was practically free from gossypol. Addition of NaCl during alkali peptization also yielded an isolate low in gossypol.

The intact pigment glands are removed from ground cottonseed kernels by filtration in glycerol prior to protein extraction.[439] The proteins are then separated on a structural basis into storage and nonstorage isolates. Undenatured proteins are recovered with an average yield of 32% of which 67% are shown to be storage proteins. Cunningham et al.[440] have studied the hydrolysis of cottonseed storage protein with pepsin in an ultrafiltration cell and analyzed the hydrolysates by electrophoresis. Several new bands appeared in the peptic hydrolysates, but they were not as sharp as in the control; these are considered resistant peptides. The molecular weight of these peptides ranged from 7,800 to 23,000. Rizaev et al.[441] have successfully separated the free pigments, amino acids, and other impurities from cottonseed proteins by ultrafiltration in a cellulose acetate membrane. The protein retention in the membrane was 100%. El Tinay et al.[442] have reported the extractability of cottonseed protein and free gossypol as a function of pH and salt concentration. The results indicated that it was possible to obtain a protein isolate free from free-gossypol by alkaline extraction at pH values of 9 and 10. Electrolytes did not increase the extractability. However, they resulted in comparatively lower free-gossypol contents in the protein precipitate. Extraction in the presence of salts and alkali did not improve protein extractability and gave a protein isolate high in free-gossypol content. El-Allawy et al.[443] have reported an improved procedure for protein extraction from cottonseed meal. The procedure involves extraction treatment of the dried protein concentrate with Me_2CO at −10°C. This treatment increased the yield of total protein percentage from 86 to 93%. Succinylation of cottonseed protein isolates (40 and 50% modification of amino groups) increased the yield of protein.[444] The succinylated isolates were more water soluble, less heat coagulable in water, and lighter in color compared to conventional isolates, and also showed improved functional properties. Choi et al.[445] have shown that upon maleylation, succinylation and dimethyl glutarylation, and NA_2SO_3 treat-

ment of cottonseed flour more protein precipitated at pH 4.0 compared to the unmodified flour. The modified proteins did not coagulate upon heating. By the modifications, some of the functional properties of the proteins were also altered.

The above studies indicate that cottonseed proteins can be extracted at high alkaline pH and precipitated at isoelectric pH to obtain a protein isolate. The efficiency of protein extraction has been shown to increase markedly by treatment of the meal with proteolytic enyzmes. However, extraction of the material at high pH imparted color and odor to the protein. The protein exhibited two minima, one in the range of pH 4.0 and the other around pH 8.0. Addition of NaCl to the extraction solvent decreased the amount of gossypol in the isolate. However, other analytical procedures such as ultrafiltration have been used to remove pigments, etc. from the protein. Modification of cottonseed proteins increased the solubility of the protein in water and the protein was less heat coagulable.

B. Isolation and Characterization of Protein Fractions

The total proteins of cottonseed have been shown to consist of three fractions — 2S, 7S, and 12S — based on their sedimentation coefficients. The 7S component is the major fraction, constituting nearly 50% of the protein. Most of the work is concentrated on this fraction. However, of late, information is accumulating on the low molecular weight fraction also. The interaction of gossypol with these proteins and also allergens in cottonseed has been fairly well studied in the last decade.

Jones and Csonka[446] demonstrated for the first time the heterogeneity of cottonseed globulins. From the saline extract of cottonseed, they identified two globulins and also a pentose-containing protein. Karon et al.[447] have shown that cottonseed proteins consist of two major and two minor components. Purified fraction of the major protein could be obtained by fractional precipitation of the NaCl extract. The functional (minor components) and the storage proteins (major components) differed in their solubility characteristics at pH 4 and 7.0, respectively. Arthur and Saik[448] isolated a globulin fraction by dialysis of the 0.5 *M* NaCl extract. The protein is reported to contain 18.06% nitrogen. The intrinsic viscosity in urea solution at 25°C was reported to be 9.3 mℓ/g. This corresponds to an axial ratio of 9.4, assuming the protein to be an elongated macromelecule. Karon et al.[449] studied the effect of temperature on the extractability and electrophoretic behavior of cottonseed proteins. The proteins separated into five fractions and the relative concentrations were independent of the temperature of extraction. The effect of temperature in the range 0 to 20°C on the electrophoretic mobility indicated that in general the product of mobility and viscosity decreased with increasing temperature.

Naismith[450] reported four fractions having sedimentation values of 18S, 13S, 8S, and 2S in cottonseed total proteins. He was able to fractionate the 13S and 8S components using changes in NaCl concentration according to the procedure of Karon et al.[447] The purified proteins did not dissociate/associate with changes in ionic strength or pH in the range 8 to 10. Rossi-Fanelli et al.[451] isolated a fraction called acalin A from a 2-*M* NaCl extract of Acala cottonseed meal. The protein was found to be homogeneous and its solubility was markedly affected by temperature. The protein was soluble in salt solutions ($\mu > 0.2$) and insoluble in water. Mondovi et al.[452] extracted the proteins of cottonseed in saline and separated them into three fractions on Sephadex® G-100. Two out of these three protein fractions were found to be glycoproteins. Yuldashev et al.[453] separated the albumin and globulin fractions of cottonseed. By electrophoresis and DEAE-cellulose chromatography, the total proteins extracted in water could be separated into 14 components (in electrophoresis, 2 proteins moved to cathode and 12 proteins moved to anode).

The 7S globulin fraction from cottonseed total proteins was isolated to homogeneity by DEAE-Sephadex® A-50 chromatography.[454] The protein had a sedimentation coefficient of 7S and a molecular weight of 140,000. The N-terminal amino acid was histidine and the

Table 20
PHYSICOCHEMICAL PROPERTIES OF 7S (OR 6S) PROTEIN FROM COTTONSEED[438,470]

Property	Values
Sedimentation coefficient ($S_{20,w}$)	6S
Extinction coefficient ($E^{1\%}_{1\ cm,\ 280\ nm}$)	6.0
Molecular weight	140,000
N-terminal amino acid	Histidine
C-terminal amino acid	Leucine
Fluorescence emission maximum (nm)	325
Phosphorus content	Nil
Carbohydrate (%)	1.0
Secondary structure (%)	
α-Helix	—
β-Structure	16
Aperiodic	84
Subunits	7 (minimum)

C-terminal amino acid sequence was reported to be leucine, valine, alanine, and threonine. Karavaeva et al.[455] observed a total of nine components in electrophoretic pattern of the globulin fraction of cottonseed. They were able to isolate one of them to homogeneity and designated it globulin A. They concluded that the remaining components of this fraction were albumins. The effect of pH on the association-dissociation of 7S in the presence and absence of 8 *M* urea was investigated.[456] A method is also reported for the preparative isolation of globulin subunits by PAGE. A homogeneous subunit with a sedimentation coefficient of 2S and a molecular weight of 22,000 was isolated. The amino acid composition of this subunit was determined and histidine was found to be the N-terminal amino acid. Asatov et al.[457] have isolated two protein components from the total globulin fraction of cottonseed, one rich in arginine and the other in histidine. The histidine-rich component was further separated into three fractions of molecular weights 28,000, 24,000, and 120,000, and their amino acid composition determined. A homogeneous 7S fraction of cottonseed protein was isolated by 45% $(NH_4)_2SO_4$ precipitation of the total protein.[438] The protein was characterized for various physicochemical properties which are listed in Table 20. The effect of SDS on the 7S fraction was also studied and it indicated that SDS was effective only above 5×10^{-3} *M* in bringing about dissociation of the protein to a 3S protein. At higher SDS concentrations, there was perturbation of the chromophores and denaturation as observed in difference spectra and viscosity measurements, respectively. Redina et al.[458] have reported that cleavage of 7S globulin with trypsin and subsequent reduction and carboxymethylation yielded 40 unique peptides and these were isolated. The amino acid composition and N-terminal amino acid of each peptide were determined. Only one peptide, which was identified as the N-terminal peptide of subunit *II*, contained carbohydrate (mannose and hexosamine, in a 3:5 ratio). OvChinnikova et al.[459] have isolated nearly 65 unique peptides by hydrolysis of 7S globulin by chymotrypsin and subsequent reduction and carboxymethylation. The amino acid composition and N-terminal amino acid determinations indicate that 38 of the peptides contain arginine and lysine as the N-terminal residues, very similar to the tryptic peptides reported earlier by Redina et al.[458] Cunningham et al.[460] have reported the molecular weight of the cottonseed major storage protein by SDS-PAGE. At least 11 polypeptide chains were detectable and these ranged in molecular weight from 57,660 to 6100. Cunningham et al.[461] have shown that cottonseed storage protein hydrolyzed with pepsin did not give sharp bands in electrophoresis. The molecular weights of these peptides were determined and fall in the range of 6000 to 60,000. Asatov et al.[462] have reported the complete primary

structure of the subunit C of 11S globulin. It contains nearly 136 amino acid residues. They have shown that the carbohydrate moiety is attached to Asx(21).

The subunits of 11S globulin of cottonseed of two varieties, Tashkent I and 108-F, are isolated and compared.[463] The 11S protein is made up of three types of subunits, and the primary structure of these subunits varied in both varieties. Youle and Huang[464] have carried out detailed studies on the various protein fractions of cottonseed total proteins. They have reported that cottonseed contains three major types of proteins having sedimentation values of 2S, 5S, and 9S existing in equal amounts. The 5S and 9S proteins are typical globulin storage proteins and have similar amino acid composition. The 2S proteins are albumins and are also storage proteins as judged by their amino acid composition, developmental properties, and high amount in the seed. However, the 2S proteins are distinct from the 5S and 9S proteins in solubility, amino acid composition, sedimentation values, and SDS-PAGE patterns. The 2S storage albumin proteins are shown by SDS-gel electrophoresis, immunocross-reactivity, and amino acid composition to be identical with the cottonseed allergens. The authors have suggested that the nomenclature "storage" and "nonstorage" protein must be revised since all of them (2S, 5S, and 9S) are shown to be storage proteins. The native 11S and 7S globulins gave a two-step wave in the polarographic pattern.[465] The relative proportion of the two halves of the polarographic pattern changed as the 11S globulin denatured above 50°C. However, similar results were not obtained in urea denaturation and did not contain any steps in the polarographic pattern. Denaturation of the 11S globulin by either heat or urea was reversible. Shadrina et al.[466] have shown that in the isolated cottonseed globulins the ratio of arginine to histidine (1:3) did not change even upon repeated precipitation of the protein. The yield of basic globulin fraction was 17% of the defatted meal. Long-term storage decreased the globulin yield, but did not change the ratio of arginine to histidine. Youle and Huang[467] have studied by sucrose density centrifugation the proteins from nearly 11 families comprising sunflower, mustard, linseed, almond, lupin, peanut, cucumber, Brazil nut, yucca, castor bean, and cottonseed. The 2S proteins, being water soluble, were termed albumins and were comprised of 20 to 60% of the total seed proteins; the rest were comprised of faster-moving globulins. The amino acid composition of the 2S proteins was different from that of the classical globulin storage proteins in that it had high amide and cysteine content. It has been suggested that the 2S protein plays an additional and unique role as a sulfur reserve for germination.

Kuchenkova et al.[468] have isolated a glycopeptide containing aspartic acid, glucosamine, and mannose in the ratio 1:2:5 from the hydrolyzate of 7S globulin. The mannose chain was shown to have a branched structure. Yunusov and Yunusova[469] have isolated and characterized the 7S and 11S globulins of cottonseed. The 7S protein was termed arginine-globulin and the 11S protein was termed histidine-globulin. Both gave a single peak in the analytical ultracentrifuge. However, upon chromatography, the 11S globulin showed three peaks of molecular weight: >400,000, ~260,000, and ~130,000. Electron microscopy of the 11S protein showed a single type of protein particle and looked like two doughnuts with a diameter of 100 Å stacked on top of one another, giving an overall height of 60 Å. Reddy et al.[470] have reported isolation and characterization of one of the globulins, 7S, from cottonseed proteins. It has a sedimentation coefficient of 6 and contains seven nonidentical subunits. The protein consisted predominantly of β- and aperiodic structures. Some of the other properties reported by these authors are listed in Table 20. Reddy and Narasinga Rao[471] have isolated and characterized the high molecular weight fraction of cottonseed which is free from gossypol (both bound and free) and nucleic acid impurities. The protein has an $S_{20,w}$ value of 12.5 and an intrinsic viscosity of 3.4 mℓ/g and consists of at least five nonidentical subunits. The secondary structure of the protein has been shown to consist predominantly of β- and aperiodic structures, similar to other high molecular weight seed proteins.[378] The 11S globulin has been investigated to obtain information regarding its primary

structure by subjecting it to hydrolysis by trypsin.[472-475] The various peptides obtained are separated by both gel filtration and ion-exchange chromatography. The amino acid sequences of the peptides are partly determined.[472-475] At present, the complete amino acid sequence of 11S globulin of cottonseed is not available. The denaturation of cottonseed proteins by heat is reviewed by Cherry and Berardi[476] for product formulation. The texturized products were comparable to those formed by the spinning and thermoplastic extrusion processes. Succinylation and acetylation have been shown to improve the texturization. These results indicate the sensitivity of cottonseed proteins to temperature, the effect of which brings about an irreversible change in the properties of the protein.

Even though work on the fractionation and isolation of cottonseed protein started as early as 1925, there appears to be uncertainty about the number of proteins and their S values. Only the low molecular weight fraction has consistently been labeled 2S fraction. The sedimentation coefficient of the high molecular weight component varies anywhere between 9S to 13S. Similarly, the sedimentation coefficient of the major fraction varies between 5S to 8S. There can be three probable reasons for this anamoly: (1) varietal difference; (2) maturity and harvesting time of the seed; and (3) association-dissociation of the cottonseed proteins as a function of pH and/or ionic strength and protein concentration. One has to proceed cautiously in interpreting the various results reported.

The 7S protein has been isolated to a fair degree of homogeneity and its amino acid composition and some of the physicochemical properties have been well documented (Table 20). On the other hand, not much work has been done on the high molecular weight component (11S); it needs to be studied. Unlike other seed proteins, the 2S protein of cottonseed is also classified as a storage protein based upon its amino acid composition, the amount present in the seed, and its rapid degradation during germination. Table 21 tabulates the amino acid composition of 2S, 5S, and 9S fractions.

VIII. SAFFLOWER SEED PROTEINS

Safflower (*Carthamus tinctorius*) seed is one of the oldest cultivated oilseeds. The production, processing and utilization of safflower seeds have been fairly well reviewed.[477-480] The safflower seed consists of 45 to 50% kernel and nearly 50% hull or pericarp, which consists mainly of fiber. Some of the newer varieties contain 35 to 40% oil, 13 to 17% protein, and 35 to 45% hull.[480,481] One of the major benefits of concentrating or isolating safflower seed protein is the removal of fiber. The seed also contains a bitter and cathartic principle. The bitter taste of safflower seed is well recognized, and this inhibits its use for food and feed purposes.[481-483]

A. Nitrogen Extractability and Total Proteins

One of the earliest studies on the total proteins of safflower seeds was by Van Etten et al.[484] They studied the amino acid composition of safflower seed kernels, proteins from kernel, hulls, and solubility of kernel nitrogen in water at various pH values. The nitrogen solubility vs. pH curve of safflower kernel indicated a minimum solubility at pH 4.0, and nearly 90% of the nitrogen was soluble at pH 1.5 and 8.0. The kernel contained nearly 60% crude protein and the hull 3%. Comparison of the amino acid composition of the kernel protein with that of other oilseed kernel proteins showed that the safflower proteins contain arginine and valine in high proportion, and the lysine content is low. Hydroxyproline was reported in the hull. The protein isolate was obtained by precipitation at pH 4.0 of alkali-soluble protein and its amino acid composition determined. The composition and amino acid content of a number of varieties of safflower seed have been investigated.[485] A new factor (5.43) for converting nitrogen to protein for safflower seed protein has also been calculated. The effect of various factors such as pH, extraction temperature, time of extraction, etc. on

Table 21
AMINO ACID COMPOSITION OF 2S (LOW MOLECULAR WEIGHT), 5S (MEDIUM MOLECULAR WEIGHT), AND 9S (HIGH MOLECULAR WEIGHT) PROTEIN FRACTIONS FROM COTTONSEED[464]

	Residues per 100,000 g of protein		
Amino acid	**9S**	**5S**	**2S**
Aspartic acid	92	83	73
Threonine	34	45	28
Serine	61	83	47
Glutamic acid	143	130	211
Proline	36	39	30
Glycine	158	147	152
Alanine	94	79	62
Valine	58	69	17
Methionine	15	14	14
Half cystine	31	18	75
Isoleucine	31	33	14
Leucine	58	60	23
Tyrosine	11	20	18
Phenylalanine	37	12	11
Lysine	22	31	58
Histidine	15	21	16
Ammonia	—	—	—
Arginine	58	47	66
Tryptophan	3	Traces	9

the nitrogen extractability of safflower meal has been studied.[486] The pH minimum of extractability spreads from pH 2.0 to 6, which is different from that observed by Van Etten et al.[484] As the temperature was increased from 10 to 70°C, the nitrogen extractability increased from 70 to nearly 90%. Also, the time of shaking (up to 2 hr) did not have any marked effect on the extractability of nitrogen. The precipitation of extracted nitrogen from various safflower meals indicated that nearly 80% of the protein precipitated between pH 4.0 and 6.0. Safflower protein isolates containing nearly 95% protein have been prepared by alkali peptization and acid precipitation, and lysine was found to be the limiting amino acid. The functional properties of the protein isolate were determined by Betschart et al.[487] The properties included nitrogen solubility, foaming capacity and stability, emulsification stability, and water- and fat-binding capacities. It was observed that the safflower protein isolate precipitated at pH 6.0 was more soluble and performed more favorably at acidic pH values than that precipitated at pH 5. A protein product isolated from safflower cake contained nearly 90% protein.[487] The functional properties of this isolate were investigated. The viscosity and pH index of the product were close to those of soybean protein isolate. Latha and Prakash[488] studied the composition of safflower seed and also the total proteins. The total protein in the seed was extracted in low ionic strength buffers at pH 7.5 in the presence of NaCl and also at different pH values. The extracted total protein consisted predominatly of one major fraction of sedimentation value 12S, and three other components of sedimentation values 2S, 7S, and 17S. The various physicochemical properties of the safflower seed

Table 22
CHEMICAL AND PHYSICOCHEMICAL PROPERTIES OF SAFFLOWER SEED TOTAL PROTEINS[488]

Property	Total protein
Sedimentation coefficient ($S_{20,w}$)	2, 7, 12, and 17
Extinction coefficient ($E^{1\%}_{1\,cm,\ 280\ nm}$)	17.3
Absorption maximum (nm)	279—280
Fluorescence emission maximum (nm)	325
Nitrogen content (%)	14.2
Protein content (N × 6.25) (%)	88.8
Phosphorus content (%)	0.23
Carbohydrate content (%)	4.0
Proteolytic activity	22 Kunitz units

Table 23
AMINO ACID COMPOSITION OF THE TOTAL PROTEIN AND THE HIGH MOLECULAR WEIGHT PROTEIN FRACTION (CARMIN) OF SAFFLOWER SEED[491]

Amino acid	Total protein (g/16 g N)	Residues per 100,000 g of protein
Aspartic acid	12.3	110
Threonine	3.1	27
Serine	4.0	62
Glutamic acid	26.3	181
Proline	—	46
Glycine	6.8	92
Alanine	3.7	67
Valine	7.1	38
Methionine	3.5	10
Half cystine	—	10
Isoleucine	3.4	23
Leucine	6.8	57
Tyrosine	3.0	32
Phenylalanine	4.7	21
Lysine	3.0	17
Histidine	3.0	17
Ammonia	—	82
Arginine	—	64
Tryptophan	—	11

total protein are listed in Table 22. The amino acid composition (Table 23) shows that the protein is rich in glutamic and aspartic acid, as well as phenylalanine and leucine. The color extracted along with the protein could be detected mostly with the low molecular weight protein as monitored by gel filtration and ion-exchange chromatography. The methanol extract of the safflower flour had two absorption maxima, one at 270 nm and the other at 320 to 325 nm. By a detailed ultracentrifugal analysis, it is shown that the presence or absence of NaCl during extraction has a profound effect on the extractability of various components of the total protein. Glycoproteins are isolated from *C. tinctorius* and are used as interferon inducers since they are less toxic than presently available inducers.[490] The isolation involves filtration through UK-200 ultrafiltration membrane and chromatography on Sephadex® G-200 and DEAE-Sephadex® A-50 columns. This protein was found to induce interferons in rabbits.

Table 24
CHEMICAL AND PHYSICOCHEMICAL PROPERTIES OF CARMIN[491]

Property	Values
Extinction coefficient ($E^{1\%}_{1\text{ cm. }280\text{ nm}}$)	11.2
Absorption maximum (nm)	279—280
Fluorescence emission maximum (nm)	323
Nitrogen content (g/g protein)	15.5
Phosphorus content	Not detectable
Carbohydrate content (%)	1.0
Proteolytic activity	Not detectable
Phytic acid	Not detectable
Polyphenols as chlorogenic acid	Not detectable
Hemagglutinin activity	Nil
Urease activity	Nil
Secondary structure (%)	
α-Helix	3 ± 2
β-Pleated	15 ± 3
Aperiodic	82 ± 5
Hydrophobicity and related parameters	
Average hydrophobicity	824 cal/residue
NPS	0.26
P	1.69
Charge	0.61 units/residue

B. High Molecular Weight Fraction

1. Isolation and Characterization

Latha and Prakash[491] have shown that safflower seed proteins contain four components with sedimentation values of 2S, 7S, 11S, and 17S. The 11S component is the major fraction and constitutes nearly 60% of the total protein. This fraction has been isolated and purified and is termed carmin.[491] The procedure consists of repeated precipitation of the total protein by 10.4% ammonium sulfate and dialysis of the precipitate vs. water, lyophilization, and dialysis against buffer required. The protein has been characterized for its chemical, physicochemical, and hydrodynamic properties, and these are listed in Tables 24 and 25. The protein has an $S_{20,w}$ of 12.7; a $D_{20,w}$ of 5.5×10^{-7} cm²/sec; and a partial specific volume of 0.735 mℓ/g. The intrinsic viscosity of the protein is 3.7 mℓ/g, indicating it to be globular in shape. The molecular weight of the protein as determined by various approaches in analytical ultracentrifugation varies from 240,000 to 290,000. The molecular weight from sedimentation equilibrium yields a value of 260,000. The evaluation of frictional ratios using Stokes radius and other parameters confirms the conclusion that the protein is globular in shape (Table 25). It has been shown to consist of at least six major subunits and is rich in aperiodic structure. The amino acid composition indicates the protein to be rich in aspartic and glutamic acids as well as hydrophobic amino acids (Table 23). The protein has no detectable phosphorus or phytic acid, proteolytic, hemagglutinin, or urease activity. The secondary structure consists of 3 ± 2% α-helix, 15 ± 3% β-pleated, and nearly 82 ± 5% aperiodic structure. In 6 *M* GuHCl, the protein has an $S_{20,w}$ value of 1.6 ± 0.2; and intrinsic viscosity of 24. 0 ± 0.5 mℓ/g; and a partial specific volume of 0.680 ± 0.002 mℓ/g. The protein has a Stokes radius of 40 ± 3 Å and a radius of gyration of 31 ± 3 Å. The frictional ratio of 1.06 indicates the protein to be globular in shape and confirms this conclusion from viscosity data.

Latha and Prakash[492] have determined the hydrogen ion-titration behavior of carmin. The pK_{int} of carboxyl, imidazole, and ε-amino groups are fairly normal and that of tyrosine is abnormal. The titration curve could be fitted with the number of various groups determined

Table 25
HYDRODYNAMIC PARAMETERS OF CARMIN[491]

Parameters	Values
Sedimentation coefficient ($S_{20,w}$)	
Native	12.7 ± 0.1
6 *M* GuHCl	1.6 ± 0.2
Diffusion coefficient ($D_{20,w}$)	5.5×10^{-7} cm²/sec
Intrinsic viscosity [n]	
Native	3.7 ± 0.2 mℓ/g
6 *M* GuHCl	24.0 ± 0.5 mℓ/g
Nonideality coefficient (g)	0.01 mℓ/mg
Partial specific volume	
Native	0.735 ± 0.002 mℓ/g
6 *M* GuHCl	0.680 ± 0.002 mℓ/g
Hydrated volume (V_s)	0.743 mℓ/g
Hydration factor (ø)	0.1 g of water/gram of protein
Molecular weight from	
Approach to sedimentation equilibrium	2,50,000 ± 10,000
Sedimentation and intrinsic viscosity	
Native	2,90,000 ± 20,000
6 *M* GuHCl	45,000 ± 5,000
Sedimentation and diffusion measurement	2,40,000 ± 15,000
Sedimentation equilibrium	2,60,000 ± 10,000
Size	
Stokes radius	40 ± 3 Å
From sedimentation measurements	38 ± 4 Å
From diffusion measurements	46 ± 4 Å
Radius of gyration	31 ± 3 Å
Frictional ratios	
From radius of equivalent sphere	1.05 ± 0.05
From sedimentation and molecular weight	1.10 ± 0.1
From Stokes radius and hydrated volume	1.02 ± 0.15
Shape parameters	
β-Function	$2.41 \pm 0.1 \times 10^6$
Perrin shape factor	1.50 ± 0.1
Simha shape factor	13.6 ± 0.5
Axial ratios	
Prolate ellipsoid of revolution	10
Obtate ellipsoid of revolution	12.5
Subunit composition	
SDS-PAGE	6
6 *M* GuHCl	6

by amino acid composition data. The titration in the presence of 6 *M* GuHCl normalizes all the pK_{int} values. This indicates that most of the tyrosine groups are hidden in the interior of the protein molecule or are present in hydrophobic pockets.

C. Toxic and Minor Constituents of Safflower Seed

It is fairly well recognized that the protein isolate from safflower seed is bitter to the taste and is colored. This presents a serious problem in utilizing ths isolate in various food systems. Even though the bitterness has been identified as being due to the presence of glucosides, not much work has been done in the identification of these glucosides. Similarly, even though the cathartic principles are identified, it is not clear whether only one principle or a group of them is responsible for the above cathartic activity. Only limited literature is

available on these aspects and it is reviewed here. A lignan glucoside, L-matairesinol-mono-D-glucoside, is recognized as a bitter principle in safflower meal.[493] Another lignan in the safflower seed meal which is tasteless but exhibits cathartic activity when tested on experimental rats was also isolated and has been identified as 2-hydroxyarctin with a molecular formula of $C_{27}H_{34}O_{12}$. Lyon et al.[494] have reported the removal of deleterious glucosides or modification of the same by extraction of the kernel with water at the isoelectric point, or with methanol or enzymatic treatment with β-glucosidase or by dialysis. They have also quantitated the two phenolic glucosides, namely 2-hydroxyarctin and matairesinol monoglucoside, which are associated with cathartic activity and bitterness, respectively, in the meal. Sakamura et al.[495] have isolated four new serotonins and phenolic constituents in the safflower seed and have identified them as *N*-feruloylserotonin, *N*-(*p*-caumaroyl) serotonin, *N*-(*p*-coumaroyl) serotonin mono-β-D-glucopyranoside, and acacetin. Along with these, they have also isolated and identified 2-hydroxyarctin and matairesinol mono-β-glucoside. In order to obtain a meal high in protein, low in fiber, and nonbitter, several attempts have been made and most of the processes are patented.[477]

IX. IS THERE A "HOMOLOGY" AMONG OILSEED PROTEINS?[526]

The literature on the physicochemical properties of some oilseed proteins such as soybean and groundnut is fairly extensive. An examination of the available data suggests that there are similarities in many properties of these proteins. The term "homology" has been used to describe similarity in amino acid sequence of proteins. We would like to use it in a more general sense to include similarity in physicochemical properties also. For the purpose of the discussion, we categorize the proteins of oilseeds into three groups, namely total proteins, high molecular weight protein fraction, and low molecular weight protein fraction. Since more data are available on the total proteins and the high molecular weight proteins, the discussion is confined to these two classes of proteins.

A. Total Proteins

1. Nitrogen Solubility

The solubility of the total proteins generally varies with pH. It is high at low and high pH. The solubility curve generally passes through a minimum in the pH range 4 to 6 for most proteins. This is characteristic of groundnut, soybean, and sunflower seed proteins. On the other hand, other oilseeds such as safflower, cottonseed, mustard, rapeseed, and sesame show two minima: one around pH 8 and another at pH 4. The two minima could possibly be due to two types of proteins, namely acidic and basic. Perhaps the two minima represent the isoelectric region of these two classes of proteins. It is known that in rapeseed the low molecular weight proteins are basic proteins and the high molecular weight protein is an acidic protein. This seems to be the case with safflower proteins also. Thus, nitrogen extractability vs. pH profile of the total proteins may provide some idea about the nature of the proteins. The absence of two minima may not mean that all the proteins belong to one type, acidic or basic. Conceivably, if the proportion of one type is very low, its contribution to the solubility profile may not be seen.

It is also observed in most of the oilseed flours that the protein extractability decreases with increase in temperature. This is possibly due to heat denaturation of the proteins leading to decrease in solubility. Protein-protein interactions at elevated temperature leading to aggregates and polymers may also decrease the solubility.

2. Number of Protein Fractions

The number of protein fractions in the total proteins is generally recognized from the sedimentation velocity pattern. In fact, it has become the standard method to designate the

Table 26
SEDIMENTATION COEFFICIENT ($S_{20,w}$) VALUES OF THE VARIOUS PROTEIN COMPONENTS FROM VARIOUS OILSEEDS

	Protein fractions[a]				
Oilseed	LMW	MMW	HMW	Polymer	Ref.
Soybean	2	7	11	15	25
Groundnut	2	7	11	18	206
Sesame seed	2	7	11	15	301
Sunflower seed	2	7	12	16	377, 379
Mustard seed	2	7	12	—	430
Rapeseed	2	7	12	—	430
Cottonseed	2	7	11	18	450
Safflower seed	2	7	12	17	488

[a] LMW, low molecular weight; MMW, medium molecular weight; HMW, high molecular weight.

protein fractions in the total proteins or isolates by their sedimentation coefficient, such as 11S, 7S, 2S protein, etc. If one were to examine the reported data on protein fractions of various oilseeds, there appears to be a great similarity in (1) the number of fractions and (2) their sedimentation coefficient. Table 26 summarizes the available data. All oilseeds, except mustard and rapeseed, consist predominantly of four protein fractions. However, the proportion of each protein fraction appears to vary from oilseed to oilseed. For example, soybean has more or less equal amounts of 7S and 11S fractions, whereas in mustard and rapeseed, the proportion of the 2S and 11S fractions is high and that of 7S fraction is very low. Based on $S_{20,w}$ values of the various fractions, one can tentatively conclude that, in general, the total proteins of oilseeds are made up of four fractions whose $S_{20,w}$ values are approximately 2S, 7S, 11S, and 15S. These can be designated for the purpose of the discussion as 2S (low molecular weight protein fraction), 7S (medium molecular weight protein fraction — corresponds to vicillin of legumes), 11S (high molecular weight protein fraction — corresponds to legumin of legumes), and 15S ("polymer" — resulting from possible aggregation of 2S, 7S, or 11S, or may be inherently present in the seed). It is not certain if the polymer is an aggregate of other proteins present in cytoplasm. It is observed that substances such as trypsin inhibitors, hemagglutinins, polyphenols, glucosinolates, color, bitter principles, etc., which are inherent constituents of some of the oilseeds, are generally present in the 2S fraction.[353,431,488,497] This may have some relation to the conservation of other protein fractions for use during germination.

It should be emphasized that generalization from $S_{20,w}$ values is an oversimplification (for the various protein fractions), since the $S_{20,w}$ values of all the proteins have not been determined under identical conditions of pH, solvent composition, temperature, etc. In some cases, the values are possibly S_{app} values uncorrected for solvent viscosity, density, and temperature. Generally, $S_{20,w}$ values have been determined at a single protein concentration (~1%) and have not been determined at several protein concentrations and extrapolated to zero protein concentration. The $S_{20,w}$ varies with protein concentration. Furthermore, the high molecular weight proteins are oligomeric and consist of subunits. These dissociate into subunits and the dissociation reaction is markedly dependent on temperature, pH, concentration, and the nature of the salt. The measured $S_{20,w}$ value depends on the solvent used. Thus, the $S_{20,w}$ value of the same protein can show considerable variation depending on the experimental conditions used.

A redetermination of the $S_{20,w}$ values of the proteins of oilseeds under identical conditions

Table 27
HIGH MOLECULAR WEIGHT PROTEIN FRACTION FROM VARIOUS OILSEEDS

Oilseed	Botanical name	Existing name	Suggested name	$S_{20,w}$ value	Total proteins (%)
Soybean	*Glycine max.*	Glycinin	Glycinin	11	30
Groundnut	*Arachis hypogea*	Arachin	Arachin	11	55
Sesame seed	*Sesamum indicum*	α-Globulin	α-Globulin (sesamin)	11	65
Sunflower seed	*Helianthus annus*	12S Protein (Helianthin)	Helianthin	12	60
Mustard seed	*Brassica* species	12S Protein	Brassin (M)	12	25
Rapeseed	*Brassica* species	12S Protein	Brassin (R)	12	25
Cottonseed	*Gossypium* species	11S Protein	Gossypin	11	20
Safflower seed	*Carthamus tinctorius*	Carmin	Carmin	12	65
Poppy seed	*Papaver somniferum*	10S Protein	Poppyverin	10	60
Linseed	*Linum usitatissium*	12S Protein	Linin	12	65

of pH, salt concentration (possibly same salt), and temperature is clearly indicated. Such measurements should preferably be made at least at three pH values (acid, netural, and alkali) and at different salt and protein concentrations. Only such rigorous measurements can decide whether the fractions from different oilseeds have close enough $S_{20,w}$ values.

B. High Molecular Weight Protein

The 10 to 12S protein fraction, called high molecular weight protein fraction, is the major fraction in groundnut, sesame seed, sunflower seed, safflower seed, poppy seed,[496] and linseed.[497] On the other hand, in mustard seed, rapeseed, cottonseed, and soybean, it is present to the extent of only 20 to 30%. However, in these seeds it also forms a significant proportion. A considerable amount of work has been reported on these fractions as shown previously. An analysis of their physicochemical properties is attempted here. In the literature, these proteins are usually referred to as 11S or 12S proteins. To avoid confusion, names based on botanical species to which they belong have been suggested (Table 27). New names have been suggested for the proteins of sunflower seed (partially in use), sesame seed (partially in use), safflower seed, poppy seed, linseed, mustard seed, rapeseed, and cottonseed.

The homology in properties such as amino acid composition, size and shape, viscosity, molecular weight, secondary structure, subunit composition, association-dissociation at high and low pH, effect of denaturants, hydrolysis, and quaternary structure will be reviewed here.

1. Amino Acid Composition

The content of a few selected amino acids of the high molecular weight proteins from various oilseeds is given in Table 28. For comparison, the amino acid composition of bovine serum albumin, an animal protein, is also shown. All the high molecular weight proteins are rich in acidic amino acids, especially glutamic acid, and are low in lysine. They also appear to be fairly rich in aromatic amino acids. Mosse and Pernollet[19] have reviewed the intraspecific and phenotypic variation of amino acid composition of legume seeds.

2. Hydrophobicity and Related Parameters

The structural parameters of proteins that depend only on the amino acid composition of the molecule have been shown to be Waugh's[498] NPS or frequency of nonpolar side chains;

Table 28
CONTENT OF SELECTED AMINO ACIDS (NO. OF RESIDUES PER 100,000 g OF PROTEIN) OF THE HIGH MOLECULAR WEIGHT PROTEIN FROM VARIOUS OILSEEDS (DATA ON BOVINE SERUM ALBUMIN ARE ALSO INCLUDED FOR COMPARISON)

Protein	Aspartic acid	Glutamic acid	Lysine	Tryptophan	Tyrosine	Phenylalanine	Ref.
Bovine serum albumin	64	91	89	3	29	41	378
Glycinin	106	169	33	7	24	34	378
Arachin	95	133	17	3	25	36	378
α-Globulin	84	155	16	11	24	34	301
Helianthin	105	189	17	5	19	41	379
Brassin (M)	53	134	17	9	16	23	378
Brassin (R)	71	160	24	9	14	26	378
Gossypin	92	143	22	3	11	37	464
Carmin	110	181	17	11	33	210	491
Poppyverin	78	204	19	4	24	29	496
Linin	98	153	24	7	14	40	497

Fisher's[499] P or the ratio of the volume occupied by polar residues to that occupied by nonpolar residues; and Bigelow's[500] average hydrophobicity, based on Tanford's[501] free energies of transfer of amino acid side chains from an organic environment to an aqueous environment. In Table 29, the average hydrophobicity, NPS, and P values of high molecular weight are listed. Also included in Table 29 are data on ovalbumin, ribonuclease, and fibroin. These three are typical animal proteins, two of them are globular and fibroin has an extended structure.

Two interesting points emerge from this analysis. First, the values of these parameters are nearly the same for all the high molecular weight proteins except for gossypin, whose values of average hydrophobicity and NPS are slightly lower than the average value. The values of average hydrophobicity and NPS and P are 860 cal per residue and 0.28 and 1.31, respectively. Except for the values of P, which range from 1.00 to 1.73, all other values of high molecular weight proteins are remarkably close to each other. Second, compared to ovalbumin and ribonuclease, the average hydrophobicity value of high molecular weight proteins is lower. Bigelow[500] has reported that except for fibrous proteins, which have very low hydrophobicity (400 to 800 cal per residue), all other proteins have values in the range of 810 to 1310 cal per residue. Based on these values, high molecular weight proteins seem to form an intermediate class between globular proteins such as ovalbumin and fibrous proteins such as fibroin. This may have some relation to the low α-helical content and high content of β- and aperiodic structure in the high molecular weight proteins. Also, the values of high molecular weight proteins do not fit any portion of the Bigelow[500] plot of average hydrophobicity vs. molecular weight. Similarly, the NPS values of high molecular weight proteins are higher than those reported for other proteins in the literature or are at least in the same range of 0.28 to 0.40.

In the case of high molecular weight proteins, on an average there are 870 amino acid residues per molecule of molecular weight 100,000. Except for arachin and brassin (M), this value is fairly close in most of the high molecular weight proteins, also indicating a certain degree of homology in the length of the polypeptide chain. The variation one sees in the last column of Table 29 may be due to the heterogeneity of the high molecular weight proteins, the method of determining the amino acids, acid hydrolysis values not being extrapolated to zero time, and many other experimental variations. Amino acid analysis under identical conditions of highly purified high molecular weight proteins alone can

Table 29
HYDROPHOBICITY AND RELATED PARAMETERS OF THE HIGH MOLECULAR WEIGHT PROTEINS OF VARIOUS OILSEEDS (VALUES OF OVALBUMIN, RIBONUCLEASE, AND FIBROIN ARE INCLUDED FOR COMPARISON)

Protein	HQ[a]	NPS[b]	P[c]	Total number of amino acids per 100,000 g of protein
Glycinin	782	0.30	1.28	874
Arachin	860	0.29	1.73	804
α-Globulin	872	0.26	1.36	885
Helianthin	832	0.26	1.25	924
Brassin (M)	962	0.31	1.03	563[d]
Brassin (R)	900	0.30	1.00	735
Gossypin	804	0.24	1.00	957
Carmin	824	0.26	1.69	885
Poppyverin	878	0.28	1.49	876
Linin	881	0.27	1.22	871
Ovalbumin	1110	0.34	0.92	—
Ribonuclease	880	0.23	1.73	—
Fibroin (silk)	440	0.02	0.07	—
Average ± SD	860 ± 50	0.28 ± 0.02	1.31 ± 0.25	868 ± 61

[a] Average hydrophobicity.[500]
[b] Frequency of nonpolar side chains.[498,500]
[c] Ratio of the volume occupied by polar residues to that occupied by nonpolar residues.[499,500]
[d] Not included in the calculation of average values.

determine if there is homology in the polypeptide chain length. This may have a relation to the nucleotides in the probable common ancestral genes.

Bigelow[500] has indicated that various proteins including some of the plant proteins such as pumpkin globulin (HQ_{ave} = 980 cal per residue) and hemp edestin (HQ_{ave} = 950 cal per residue) have quite low hydrophobicities and this may be related to their solubility in salt solutions. Bigelow[500] has shown that a large majority of the globular proteins have HQ_{ave} values in the range 1000 to 1200 cal per residue. He has plotted the average hydrophobicity values against Waugh's NPS values and found good correlation between the two parameters. If we assume that the HQ_{ave} of high molecular weight proteins is 860 cal per residue, from the Bigelow[500] plot we obtain a value of 0.22 for NPS, which is less than the calculated value of 0.28 ± 0.02. This indicates that high molecular weight proteins (Table 29 and 30) probably are more compact than other proteins of the same volume because of higher content of hydrophobic amino acids. It is important to note that the hydrophobic bond is not the only force that stabilizes proteins; other forces which contribute to protein stability have been ignored in calculating the above parameters.[502] Also, it is assumed that most of the hydrophobic groups are buried inside the protein molecule.[502] It is our opinion that quantities such as average hydrophobicity (HQ), NPS, and P provide a better basis for comparison of proteins than just the amino acid composition data. The high molecular weight proteins considered here have close values of these parameters.

Nakai et al.[503] have obtained a fairly linear relationship between surface tension or emul-

Table 30
INTRINSIC VISCOSITY AND SECONDARY STRUCTURE OF THE HIGH MOLECULAR WEIGHT PROTEINS FROM VARIOUS OILSEEDS (VALUES OF INTRINSIC VISCOSITY FOR RIBONUCLEASE AND COLLAGEN AND VALUES OF SECONDARY STRUCTURE FOR RIBONUCLEASE AND ELASTASE ARE INCLUDED FOR COMPARISON)

		Secondary structure (%)			
Protein	[η] mℓ/g	α-Helix	β-Structure	Aperiodic	Ref.
Glycinin	4.9	5	20	75	378
Arachin	4.7	5	20	75	378
α-Globulin	3.0	5	25	70	378
Helianthin	3.6	2	28	70	378
Brassin (M)	3.6	9	28	63	378
Brassin (R)	3.7	9	28	63	378
Gossypin	4.0	5	20	75	463, 471
Carmin	3.7	3	15	82	491
Poppyverin	3.5	5	20	75	496
Linin	3.1	3	17	80	497
Ribonuclease	3.3	40	13	24	504, 524
Collagen	1150	—	—	—	504
Elastase	—	7	52	26	524

sifying activity with hydrophobicity for some of the plant proteins such as sunflower seed protein, rapeseed, and soybean proteins. The theoretical values of the hydrophobicity parameters calculated here may help in predicting some of the functional properties of the seed proteins, especially surface properties.

3. Intrinsic Viscosity

The intrinsic viscosity of the high molecular weight protein from various oilseeds is given in Table 30. They all have an intrinsic viscosity value of 3 to 5 mℓ/g. Tanford[504] has shown from a study of synthetic polypeptides that polymers having an intrinsic viscosity of 3.0 mℓ/g are generally globular in shape. Based on this criterion, all the high molecular weight proteins appear to be compact and globular in shape. For comparison, the values of ribonuclease, which is a globular protein, and of collagen, a highly asymmetric protein molecule, are shown.

4. Secondary Structure

The high molecular weight proteins give a characteristic cotton effect in the ORD and far UV-CD spectra. The CD spectrum of these high molecular weight proteins is generally characterized by a minimum of around 208 to 212 nm with a shoulder of around 224 to 228 nm.[378] This suggests that the high molecular weight proteins have low content of α-helix and are rich in β- and aperiodic structures. Table 30 summarizes the secondary structural data of all the high molecular weight proteins. They all have less than 10% α-helix, 20 to 30% β-structure, and the rest aperiodic structure. Thus, the proteins have more disordered than ordered structure. However, attempts have not been made to calculate from the CD data the number of β-turns. Levitt and Chothia[505] and Blake and Johnson[506] have classified all the proteins into five classes. These are defined in terms of the content and arrangement of α-helices and β-sheets. The various classes are (1) all α-proteins; (2) all β-proteins; (3) α- + β-proteins; (4) α/β-proteins; and (5) "coil proteins". The high molecular weight protein fractions do not seem to fit into any of these classes since they are rich in β-sheet

Table 31
MOLECULAR WEIGHT AND NUMBER OF SUBUNITS OF THE HIGH MOLECULAR WEIGHT PROTEINS FROM VARIOUS OILSEEDS

Protein	Mol wt (× 100,000)	No. of subunits	Ref.
Glycinin	3.0—3.5	6	378
Arachin	3.0—3.5	6	378
α-Globulin	2.3—2.7	6	309, 378
Helianthin	3.0—3.5	6	378
Brassin (M)	2.3—2.4	6 (8)	378
Brassin (R)	2.9—3.0	6	378
Gossypin	2.2—2.5	6 (5)	450, 466
Carmin	2.4—2.9	6	491
Poppyverin	2.0—2.3	6	496
Linin	2.5—3.0	6	497

structure and aperiodic or coil structure. Maybe a sixth class, termed "β + coil proteins", may be appropriate to describe these proteins. Since these are storage proteins, it may not be necessary for them to have more ordered structure as they do not have any reported biological activity.

5. Molecular Weight

The molecular weight of the high molecular weight proteins is given in Table 31. The molecular weight ranges from 2.0×10^5 to 3.5×10^5. Thus, they all have high molecular weight. This large molecular weight of the protein is probably essential for the maximum number of amino acids to be released during germination from a minimal volume occupied by these globular proteins. Even though the values vary over a wide range, the same order of magnitude of the values suggests certain similarity between these proteins.

It should be pointed out that different workers have used different techniques to calculate the molecular weight of the high molecular weight proteins. Further uncertainty arises from the facts that (1) all protein preparations may not be homogeneous; (2) the buffers used were not of identical composition, especially with regard to ionic strength; (3) rigorous methods to obtain $S_{20,w}$ and $D_{20,w}$ have not been used; and (4) the value of partial specific volume has been assumed in most cases. Murthy[507] has determined the $S_{20,w}$, $D_{20,w}$, and partial specific volume values of arachin, α-globulin, sunflower 11S protein, and mustard 12S protein in the same buffer and finds considerable differences in their molecular weight. However, they are in the range 190,000 to 330,000. The estimation of molecular weight of high molecular weight proteins by a thermodynamically sound method such as sedimentation equilibrium is clearly indicated.

6. Subunit Composition

These are high molecular weight proteins with molecular weight of 250,000. From an energetics point of view, as well as the minimization of genetic errors in the synthesis of such long polypeptide chains, it should be expected that the proteins would have quaternary structure. This is substantiated by the oligomeric nature of the proteins as shown in Table 31. Most of the high molecular weight proteins contain between 6 to 8 subunits per mole of the protein. The subunit molecular weights range from 7000 to 80,000 indicating a wide range of length of the polypeptide chain as determined mostly by SDS-PAGE. However, these values are not confirmed by other techniques.

Pernollet and Mosse[17] have proposed a general model for the legumin quaternary structure. The molecule consists of a pair of acidic and basic subunits interlinked through S–S bonds; three such pairs exist in a molecule. These are stabilized mostly by noncovalent interactions such as hydrogen, hydrophobic and other weak interactions, and form a ring. Two such rings are sandwiched one above the other such that once again the acidic and basic subunits are in contact with each other and form a small hollow cylinder as shown in Figure 1. It is quite possible that such a model could also exist with four acidic and three basic subunits.

7. Fluorescence

The fluorescence emission maximum of the high molecular weight proteins is generally around 320 to 330 nm.[378] This is characteristic of tryptophan emission, the residues being embedded in the interior of the protein molecule. The high molecular weight proteins contain a fair amount of tryptophan (Table 28). The fluorescence emission spectrum suggests that in the high molecular weight proteins the tryptophan residues are embedded in the interior of the protein.[508,509] This is compatible with the globular shape of the protein in highly compact form. However, all the high molecular weight proteins contain a fair amount of tyrosine as shown in Table 28. (On average, nearly 5 to 10 tryptophans and 15 to 30 tyrosines are present per mole of each protein.) However, tyrosine emission is not observed. Shifrin et al.[509] and Teale[508] have shown that proteins having both tryptophan and tyrosine give only tryptophan fluorescence emission spectrum, i.e., the tryptophenyl fluorescence dominates over tyrosinyl fluorescence.

The results of fluorescence measurements suggest that there is similarity in the microenvironment and location of nonpolar groups in the interior of the protein, in spite of the fact that these proteins do not possess α-helical structure. It has been shown that the aromatic amino acids of α-globulin are in the subunit contact areas stabilizing the quaternary structure.[316,324,325]

8. Hydrolysis

The susceptibility to hydrolysis of the high molecular weight fractions by proteolytic enzymes such as trypsin, chymotrypsin, pepsin, and papain has been well documented.[55,217,377-379,430] Compared to casein, these proteins are hydrolyzed to a lesser extent. Furthermore, the susceptibility to proteolysis varies among the high molecular weight proteins. For example, if the hydrolysis of casein by α-chymotrypsin is taken as 100, the hydrolysis of arachin is 20%, brassin (M or R) 6%, and glycinin 50%. Of course, the values will depend on whether or not the high molecular weight protein is totally free from protease inhibitors since most of the oilseeds are reported to contain protease inhibitors. Even in those cases where it has been shown that these are absent, the extent of hydrolysis of high molecular weight protein is low. Perhaps these proteins have a "hard core" amino acid sequence which the proteases are unable to hydrolyze. Another possible explanation is that these high molecular weight proteins may be highly specific to only one or two proteases. This would facilitate conserving the high molecular weight proteins in the seed until such time as germination occurs, when possibly the specific enzymes are liberated and transported for hydrolyzing the proteins and releasing the needed free amino acids and peptides for synthesis of new proteins. The presence of protein-specific proteases in oilseeds needs to be investigated.

Sharon and Lis[510] and Sharon[511] have reported that the carbohydrate units of proteins (glycoproteins) protect them against hydrolysis by proteolytic enzymes. Most of the high molecular weight proteins are reported to contain carbohydrates (Table 32).

9. Carbohydrate Content (Glycoproteins?)

Except for glycinin, most of the high molecular weight proteins have a carbohydrate

Table 32
CARBOHYDRATE CONTENT (% DRY WEIGHT BASIS) OF THE HIGH MOLECULAR WEIGHT PROTEIN FRACTIONS IN VARIOUS OILSEEDS

Protein	Carbohydrate (%)	Ref.
Arachin	0.3	217
α-Globulin	0.8	301
Helianthin	0.4	377, 379
Brassin (M)	1.0	430
Brassin (R)	1.0	430
Gossypin	0.5	464
Carmin	1.0	491
Poppyverin	1.2	496
Linin	0.2	497

content of ~1% (Table 32). The carbohydrate moiety may possibly have a role in the biochemistry of these proteins. The question that arises is, are the high molecular weight proteins glycoproteins and, if so, what part does the carbohydrate play?

Sharon and Lis[510] and Sharon[511] have reviewed the work on glycoproteins from various sources, their role in nature, biosynthesis, and medicinal applications, etc. It is evident that carbohydrates in glycoproteins do perform a variety of functions. Also, different glycoproteins have different requirements of carbohydrates. The role played by carbohydrates in modifying the physicochemical properties of glycoproteins is well documented.[514] Sufficient data have accumulated to show that the carbohydrate moieties are needed for folding, acquiring, and stabilizing the correct conformation of certain proteins. It is also known that the carbohydrate moieties participate in subunit interaction. In many of the glycoproteins, the carbohydrates have been shown to protect the protein against intracellular proteolysis during biosynthesis and transport and are therefore required for membrane insertion and secretion. Some of the other properties of proteins that may be modified by carbohydrates are change of hydrophobicity, electrical charge, mass, and size. All the high molecular weight proteins are oligomeric proteins, and they are also storage proteins. Possibly the carbohydrate moieties of the high molecular weight proteins have a role in subunit interactions, folding of the protein molecule into a compact structure, and also offering resistance to proteolysis.

10. Association-Dissociation

Many proteins show the property of association and dissociation under different solution conditions. The literature on this is quite voluminous.[515] All the high molecular weight proteins exhibit the property of association and dissociation. The reaction depends on (1) ionic strength, (2) pH, (3) protein concentration, and (4) temperature. It seems to also depend on other factors such as nature of the buffer ion, cation, and anion, and the presence of other protein fractions acting as nucleus for the association-dissociation reaction, etc.

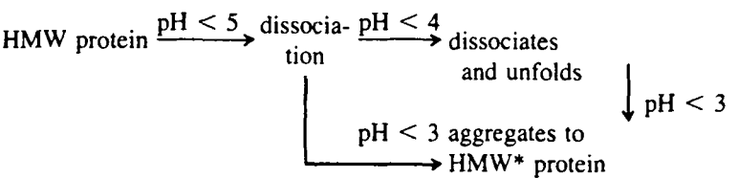
Glycinin (11S) is partially associated into faster-sedimenting components at 0.1 ionic strength. This reaction is reversed by increasing the ionic strength.[25] Furthermore, dissociation into subunits occurs at very low ionic strength of 0.001, or high or low pH. A similar observation has been made with arachin,[221] where the 14S molecule dissociates to a 9S molecule. The equilibrium position depends on pH, salt concentration, and the nature of

salt. Similar results have been reported by Naismith and Kelley.[226] Prakash[309] has shown that in α-globulin, the association-dissociation equilibrium exists between 11S, 13S, and 7S components. Here again, the equilibrium is dependent on pH, buffer ion, and temperature. High temperatures favor the 11S component, whereas lower temperatures favor the 7S component. The large variations in S value of the cottonseed protein, gossypin, have been shown to be due to the variation in ionic strength and pH of the medium.[456,464] Dissociation of high molecular weight proteins like helianthinin,[365] carmin,[512] brassin[419] (M), and brassin (R)[423] has been reported. Based on the available experimental data, a general scheme for the association-dissociation of high molecular weight proteins may be proposed as follows:

$$\text{polymer} \underset{\text{protein concentration}}{\overset{\text{ionic strength, pH, temperature}}{\rightleftharpoons}} \text{HMW} \underset{\text{protein concentration}}{\overset{\text{ionic strength, pH, temperature}}{\rightleftharpoons}} 7\text{S} \underset{}{\overset{\text{high or low pH}}{\rightleftharpoons}} \begin{matrix}4\text{S or}\\3\text{S or}\\2\text{S}\end{matrix}$$

11. Dissociation-Reassociation

Another interesting but anomalous property of the high molecular weight proteins of oilseeds is their unusual behavior at low pH values. The effect of low pH in the range of 5 to 1 on the oligomeric structure, spectral properties, and conformation of α-globulin,[307] arachin,[513] brassin (M),[426] poppyverin,[496] and helianthin[377] has been reported. In the pH range 5 to 3, the proteins dissociate; below pH 3, they reaggregate. The proteins have also been shown to unfold between pH 5 and 3 and refold below pH 3. These proteins are made up of acidic and basic proteins (subunits) and it is possible that below pH 3, the acidic and basic subunits reassociate (possibly to a different polymer) because of charge effects. It may also be due to the entropically driven hydrophobic interaction as has been shown with α-globulin.[307] The dissociation and reassociation in acid pH of high molecular weight protein can be represented schematically as follows:



This newly associated polymer (HMW*) may be entirely different from the original HMW protein, even though it has been shown to have a sedimentation coefficient of 11S. These results suggest that the forces which hold the subunits together in α-globulin, arachin, brassin (M), poppyverin, and helianthin are possibly the same. Also, the mechanism of reassociation must be very similar since all of them reassociate to a 11S component.

12. Dissociation-Denaturation

The high molecular weight proteins behave very similarly when they are treated with classical denaturants such as urea, GuHCl, and SDS. Table 33 summarizes the various components detected in the presence of urea, GuHCl, or SDS and the intrinsic viscosity value in 6 *M* GuHCl. Although various intermediates are produced in the course of dissociation, the end product appears to be a denatured 2S protein. This occurs in 8 *M* urea, 6 *M* GuHCl, or 1×10^{-2} *M* SDS. The dissociation and denaturation of the high molecular weight proteins involve more than a single step as suggested by the intermediates produced at different denaturant concentrations. All of them seem to have the same (final) conformation, namely random coil, since the value of intrinsic viscosity in 6 *M* GuHCl is close

Table 33
INTERMEDIATE COMPONENTS ($S_{20,w}$ VALUE) AND THE INTRINSIC VISCOSITY OF HIGH MOLECULAR WEIGHT PROTEIN COMPONENTS FROM VARIOUS OILSEEDS IN UREA, GuHCl, AND SDS (COMPILATION OF THE RESULTS REPORTED IN EARLIER PAGES)

Protein	Components detected in various concentrations of urea GuHCl, and SDS ($S_{20,w}$) value	[n] mℓ/g in 6 *M* GuHCl (protein concentration = 1%)
Glycinin	11, 7, 4, 3, 2, 1, 2	28
Arachin	14, 12, 9, 7, 4, 3, 2	22
α-Globulin	11, 7, 4, 2	38
Helianthin	11, 7, 2	—
Brassin (M)	12, 4, 1.6	22
Gossypin	11, 7, 2	24
Carmin	11, 7, 4, 2	24

to that of random coil proteins[504] and no ordered structures are present in these conditions.[91,316] However, α-globulin appears to be more asymmetric than other high molecular weight proteins in 6 *M* GuHCl.[306] These results indicate a certain similarity in the oligomeric high molecular weight proteins.

13. Quaternary Structure

These data on association-dissociation, dissociation-reassociation, and dissociation and denaturation support the modified model of the "hexamer sandwich"[17] as shown in Figure 1. It shows the heterodecamer model for the oilseed proteins that could be adopted for explaining the physiochemical properties of the high molecular weight proteins.

On the basis of this model, some features of association-dissociation and denaturation of high molecular weight proteins can be explained. According to this model, the 12S protein is made up of two hexamers. This structure is not stabilized by covalent bonding. Probably the stabilizng force is noncovalent interaction, especially entropically driven hydrophobic interaction similar to that in nucleic acid stacking.[516] As a consequence of this, minor variations in the solution composition such as pH, ionic strength, nature of the salt, etc. can disrupt these forces driving the reaction in the direction of the 7S trimer or vice versa. Since temperature has a marked effect on noncovalent and, in particular, hydrophobic interactions, it may be expected that higher temperatures (~50°C) associate the protein. Above this temperature (~50°C), dissociation may occur. The reaction may not proceed to unfolding of the subunits because the basic and acidic subunits are held together by a much stronger covalent bond, i.e., S–S bond. The formation of 7S trimer could also be facilitated by low concentration of urea, GuHCl, or SDS, which destabilizes the hydrophobic interactions between the monomers. This results in a situation where the noncovalent interactions between the trimers in the hexamer molecule are also destabilized. In fact, sufficient experimental evidence exists to show that in α-globulin,[306] arachin,[221] helianthinin,[365] etc., before the 12S → 7S reaction goes to completion, a 3S or a 4S component is also observed in the mixture. However, in other high molecular weight proteins such as glycinin,[81] brassin (M),[418,419] brassin (R),[418,419] etc., the 12S molecule appears to dissociate directly to the 2S molecule. In these cases, either the intermediate components are not formed or their concentrations are very low, or the half-life of 7S or 4S molecules is so short that they are not

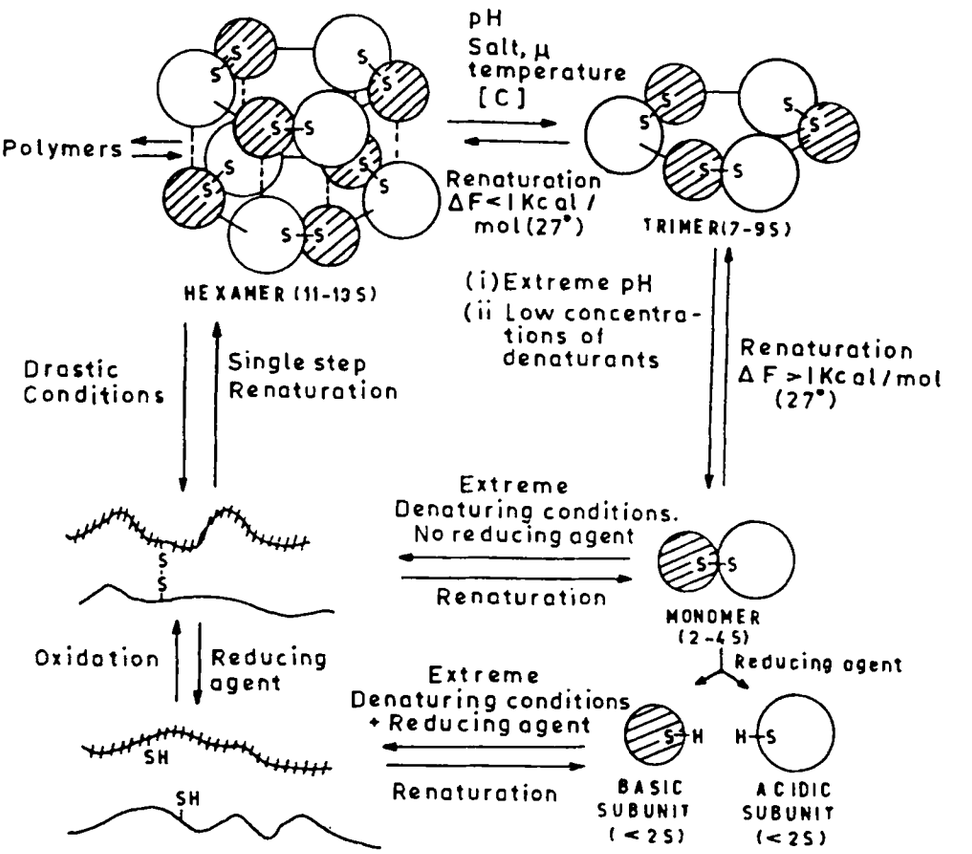


FIGURE 1. Schematic model for the high molecular weight oilseed protein quaternary structure. Part of the model is similar to the one proposed by Pernollet and Mosse.[17] Dashed lines, weak noncovalent bonds; solid lines, strong noncovalent bonds and S–S, disulfide bridge.

detected in sedimentation velocity runs. It could also mean that the nature of noncovalent interaction between the trimers and the hexamers could be different in these proteins (Figure 1, dashed line and solid line). At 8 or 6 *M* urea and/or GuHCl, respectively, or 1×10^{-2} *M* SDS, the monomer or the 2S form is stabilized since all noncovalent interactions cease to exist at these concentrations of the denaturants.[517] If a reducing agent is present, these monomers could further dissociate into acidic and basic subunits (Figure 1) and would exist as completely unfolded polypeptide chains (Figure 1). On the other hand, if there is no reducing agent, then the monomer would denature to a single polypeptide chain linked by a S–S bond increasing the asymmetry of the uncoiled molecule (Figure 1). This may be the reason for the high value of reduced viscosity of α-globulin in 8 *M* urea or 6 *M* GuHCl without any reducing agent.[306]

Neutral salts[308] or alkanes such as 2-nanone[518] have a profound effect on the dissociation of the hexamer to trimer. Some salts such as CCl_3COO^- or $NaClO_4$ dissociate the trimer to monomer (4S component) through intermediates. Only such a stepwise dissociation could explain the great variety of sizes of intermediates reported in the dissociation of various high molecular weight proteins. Intermediates with S values of 1.5, 1.6, 2, 2.2, 2.6, 3, 3.7, 4, 4.5, 5, 7, 7.5, and 9 to 16 have been reported (Table 33).

Based on this hexamer sandwich model, one can possibly explain the unusual effect of acidic pH on the dissociation and reassociation of the high molecular weight proteins. It is

conceivable that as the pH is lowered from pH 7.0 to 4.0 hydrophobic interactions are weakened due to the increase in the concentration of H^+.[310,311,522,523] Hence, the molecule dissociates to a 2S component. From the spectral data, it is known that at this pH the aromatic groups are exposed, probably being the contact groups between the subunits.[316,324,325] At still higher concentrations of H^+, i.e., pH 3.0, the 2S monomer probably unfolds as indicated by the increase in reduced viscosity and shift in the fluorescence emission maximum towards 345 nm. Since there is no reducing agent, the acidic and basic subunits still probably remain as one single polypeptide chain (Figure 1). At pH values below 3 or 2.5, a reassociation of these dissociated and denatured molecules is observed. It has been shown that at extreme pH of 2.5 and below the hydrophobic interactions have more energy of interaction compared to other pH values.[522,523] This increased interaction energy may lead to the reassociation of these polypeptide chains rather uniquely since the hexamer is stabilized by noncovalent interactions. Hasegawa et al.[151] have shown that the acidic subunits of sesame α-globulin and the basic subunits of soybean glycinin can be hybridized to the monomer, which further trimerizes finally to a hexamer.

To summarize, the proposed model has four main features. These are (1) the area of association between acidic and basic subunits probably originates from the same precursor polypeptide chain (i.e., the disulfide linkage area); (2) the monomers form trimers by noncovalent association where each acidic (or basic) subunit associates with two basic (or acidic) subunits; (3) the trimer noncovalently associates with another trimer to form the hexamer with 12 subunits; and (4) the inter- and intra-noncovalent interactions of the trimers appear to be different from each other.

X. DISCUSSION

Thus, in many of their properties such as amino acid composition, hydrophobicity, intrinsic viscosity, secondary structure, molecular weight, subunit composition, fluorescence spectrum, susceptibility to hydrolysis, association-dissociation, dissociation-reassociation, and dissociation-denaturation, high molecular weight proteins of oilseeds resemble one another closely. It would be interesting to speculate if there is any relationship between the physicochemical properties of high molecular weight proteins and their biological role.

The function of the high molecular weight proteins in the oilseeds is to mainly serve as storage proteins, even though lately they have also been speculated to be secretory proteins.[19] These proteins are hydrolyzed to amino acids during germination for resynthesis into new proteins and as a quick nitrogenous source to facilitate growth of the plant. It is conceivable that the subunits are held together by weak forces ($\Delta F < 1$ kcal/mol at 27°C) which facilitate the breakdown of the oligomeric structure without much energy during germination.[309]

Globular shape and high molecular weight would facilitate dense packing of these proteins in the cellular structures. This is based on the simple principle that a sphere occupies minimum volume; so, for a given volume of a cell, more protein could be packed with globular and high molecular weight proteins. Another school of thought has postulated that the folding of storage proteins has been maintained during evolution and conserved in order to fit peculiar endoproteases which hydrolyze seed proteins during germination.[17] Pernollet and Mosse[17] have put forward the "maximal packing" hypothesis for the storage proteins. According to them, the maximal packing can be achieved in two degrees: (1) compactness is increased by the formation of a quaternary structure and (2) folding of polypeptide chain may be in favor of a maximal packing of amino acids within the protein molecule. Also, they have concluded that it is not by chance that these reserve proteins are able to associate into this kind of quaternary structure. This is supported by the facts that (1) there is sequence homology of basic polypeptide chains of soybean, broad bean, pea, and pumpkin globulin (not strictly in oilseeds); (2) the globulin subunits of plant species like *Glycine max* and *Sesamum indicum*,

even though distant, can associate in vitro between themselves and form molecular hybrids;[149,151] and (3) there appears to be conservatism of predicted secondary structures which are hardly altered by point mutations. Other experimental evidence in support of the maximal packing of amino acids within a minimum molecular volume is the repetitive sequence in w-gliadin due to fixed dihedral angle of proline and the formation of the β-turn hydrogen bond; a plausible structure would be successive planes joined by glutamyl residues.[17] This may be one reason why the high molecular weight proteins are rich in glutamic acid residues. This structure would allow compact packing of amino acid residues. The current X-ray diffraction work on vicillins may generate data to test this model.[17]

The poor digestibility of high molecular weight proteins by proteases might provide protection against insects that may otherwise easily attack the seed material and digest it. Also, undesirable constituents such as trypsin inhibitors in soybean; phytic acid in sesame seed, mustard seed and rapeseed; chlorogenic acid in sunflower seed; glucosinolates in mustard seed and rapeseed; gossypol in cottonseed; glucosides in safflower seed; and linatin in linseed may also act as protectants to protect the oilseed materials against infestation.

Thus, there seems to be considerable homology in the high molecular weight proteins of oilseeds. Unfortunately, there is no such detailed information available on the physicochemical and other properties of the other protein fractions of oilseeds. Generation of such data is essential in order to gain insight into the physical, chemical, and biological role of proteins in oilseed.

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