Physical and Chemical Properties of Soybean Proteins

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ABSTRACT

Recent physical and chemical studies are reviewed for Bowman-Birk and Kunitz trypsin inhibitors, agglutinin, and 7S and 11S globulins of soybeans. Differences between gelation properties of crude 7S and 11S globulin fractions are also discussed.

INTRODUCTION

Edible soybean protein isolates and concentrates were introduced commercially in 1957 and 1959, respectively, and in the intervening years we have seen a steady increase in the use of these products as ingredients in a wide variety of fabricated or engineered foods. Annual production in the US in mid-1976 was estimated at 80 million lb for concentrates and 75 million lb for isolates (N.R. Lockmiller, personal communication). Widespread adoption of soybean proteins in foods has stimulated research on their physical and chemical properties in many laboratories. Consequently, the diversity and complexity of soybean proteins is gradually being unraveled and this review describes some of the most recent developments in this area. A summary of work up to about 1970 appears elsewhere (1).

TRYPSIN INHIBITORS

The number of trypsin inhibitors present in soybeans is still unknown, but two of them have been isolated and their primary structures are now known as a result of studies at Niigata University in Japan. Odani and Ikenaka (2) reported the complete amino acid sequence, including the disulfide bridges, for the Bowman-Birk inhibitor in 1973 (Fig. 1). This molecule consists of a single polypeptide chain containing 71 amino acid residues. With a mol wt of only 7861, the Bowman-Birk inhibitor is the smallest protein found in soybeans to date, but in aqueous solutions at concentrations of 0.1% and higher, the inhibitor associates into a dimer and possibly a trimer (3). This inhibitor has the unique ability to inhibit trypsin and chymotrypsin simultaneously. The trypsin inhibiting site is Lys^{16}-Ser^{17} whereas the chymotrypsin combining site is Leu^{43}-Ser^{44}. Another noteworthy feature of Bowman-Birk inhibitor is its high degree of symmetry. Each inhibitory site is located in a nine-membered peptide loop joined by a single disulfide bridge. Next is another nine-membered loop followed by a ten- (trypsin inhibitory portion) or eight-membered ring (chymotrypsin inhibitory portion). The seven disulfide crosslinks make the molecule fairly rigid and consequently it is remarkably stable to heat, acid, and proteolytic digestion. Bowman-Birk inhibitor may therefore be responsible for the residual trypsin inhibitor activity remaining in heated soybean protein products.

Primary structure of Kunitz trypsin inhibitor (Fig. 2) was determined by Koide and Ikenaka (4). With 181 amino acid residues, it has more than twice the molecular weight of the Bowman-Birk inhibitor, but the extent of cross-linking is much simpler because there are only two disulfide bonds. One disulfide bridge connects residues 39 and 86 while the other forms a much smaller loop connecting residues 136 and 145. Reduction of both disulfide bonds destroys the ability of the molecular to inhibit trypsin, but the bond at residues 136 and 145 lies in a very hydrophilic portion of the molecule and is readily reduced. Within the loop formed by the disulfide bridge one finds the sequence Glu^{141}-Asp^{142}-Asp^{143}, and eight residues from disulfide bond there is is the sequence Asp^{153}-Asp^{154}-Asp^{155}. Because of this high concentration of polar groups, the disulfide bond lies near the surface of the molecule and can be selectively reduced without any effect on inhibitory power (5).

The active center of the Kunitz inhibitor is the Arg^{63}-Ile^{64} bond found in the large loop formed by the disulfide bridge at residues 39 and 86. The Arg^{63}-Ile^{64} bond meets the substrate specificity requirements of trypsin and, indeed, catalytic amounts of trypsin hydrolyze this bond in the inhibitor (6). It is believed that the inhibitor interacts

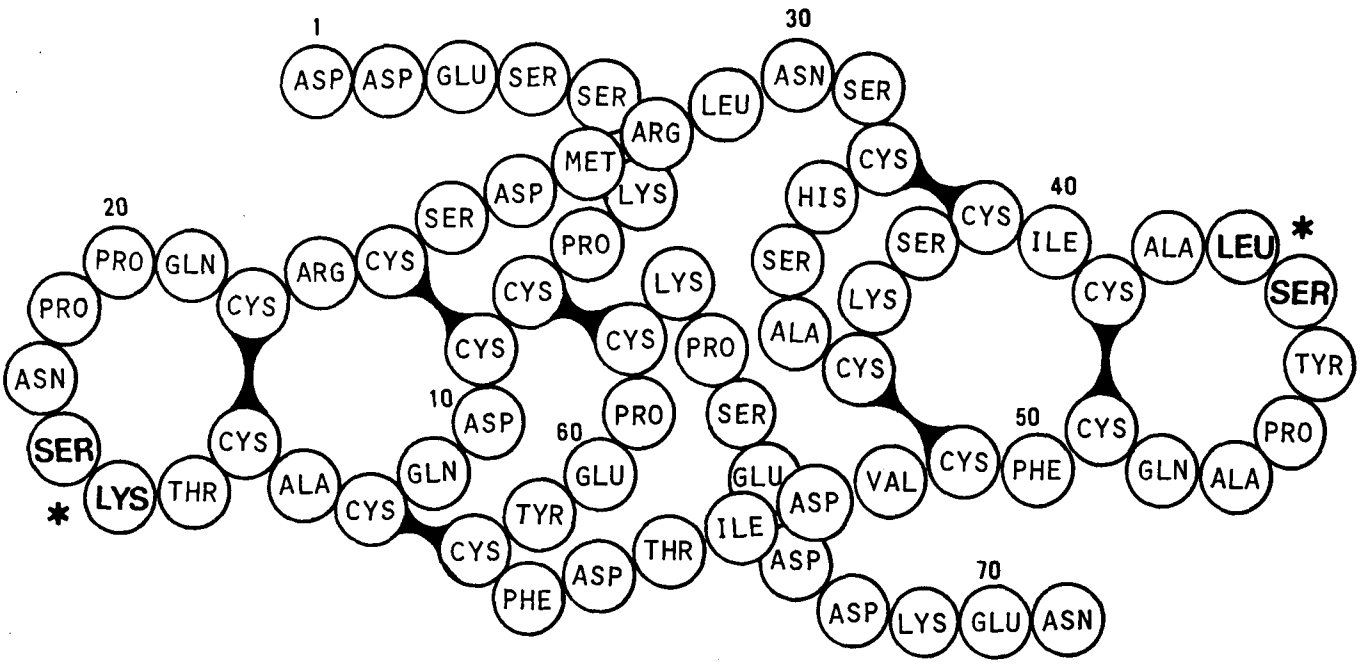

FIG. 1. Primary structure of Bowman-Birk soybean trypsin inhibitor according to Odani and Ikenaka (2). Residues are numbered beginning with N-terminal aspartic acid. Disulfide bridges are in black between half-cystine residues. Residues at the two reactive sites are marked in bold-faced type and have asterisks next to them. Reprinted with permission from Springer-Verlag.

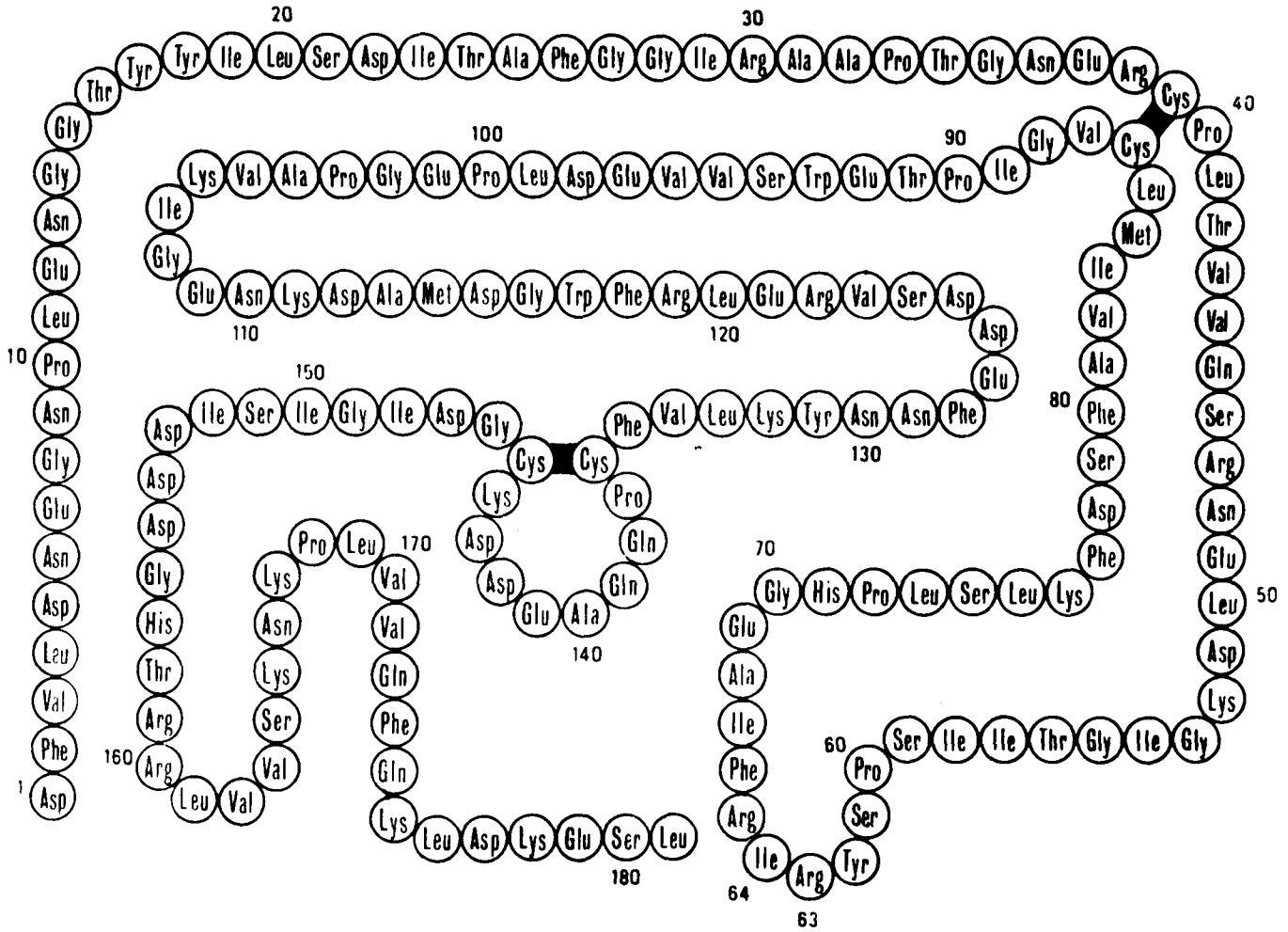

FIG. 2. Primary structure of Kunitz soybean trypsin inhibitor as determined by Koide and Ikenaka (4). Residues are numbered beginning with N-terminal aspartic acid. Disulfide bridges are in black between half-cystine residues. The active center is Arg63-Ile64. Reprinted with permission from European Journal of Biochemistry.

with trypsin because it mimics a good substrate for the enzyme.

The two-dimensional structure depicted by the amino acid sequence (Fig. 2) gives very little information about the three-dimensional structure of the inhibitor. Sweet et al. (7), however, have provided a great deal of detail about the secondary and tertiary structures of the Kunitz inhibitor by X-ray crystallography of the crystalline complex formed by the inhibitor with porcine trypsin. A model of the complex made from an electron density map at 5-Å resolution shows two globular structures attached to each other (Fig. 3). The inhibitor is approximately spherical with a diameter of about 35-Å. A surprising feature of the structure is the small region of contact between the two protein molecules that forms an extremely strong complex.

Studies at 2.6-Å resolution reveal that the inhibitor molecule consists of criss-crossing loops wrapped around a core of hydrophobic side chains (Fig. 4). No α-helical structure is present. Instead, most of the polypeptide chain is folded into an approximate β-pleated sheet structure. Only about 12 out of the 181 amino acid residues of the inhibitor make contact with trypsin in the complex. In this small region of contact, there are an estimated 300 interatomic contacts (pairs of atoms within 0.5-Å of the theoretical van der Waals' contact distance) of which about 18 are hydrogen bonds. No new or unforeseen types of interactions were discovered and the binding energy presumably arises from the sum of the many small energy terms involved.

AGGLUTININS

Soybeans contain up to four agglutinins which are proteins with the ability to cause red blood cells to clump together or agglutinate (8). A recent review summarizes work on these biologically active proteins found in soybeans and many other plants (9). Soybean agglutinin has been purified to a high degree of homogeneity by affinity chromatography and characterized in detail (10). Some of the properties of agglutinin are summarized in Table I. It is a glycoprotein containing D-mannose and N-acetyl-D-glucosamine and has a mol wt of 120,000 over a pH range of 2.2 to 10.8. In protein dissociating solvents such as 0.1% sodium dodecyl sulfate or 6M guanidine hydrochloride, the mol wt drops to 30,000 as a result of dissociation into four subunits. Each subunit appears to contain a carbohydrate side chain of 2000 daltons. Finding of four amino terminal alanines suggested that the four subunits were identical. Further studies now indicate that two types of subunits are present. Both have identical N-terminal sequences for the first 10 amino acid residues and appear to differ only in their contents of lysine, aspartic acid, and glutamic acid (11). Separation was effected by discontinuous polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate or 8M urea and by DEAE-cellulose chromatography at pH 7.3 in 8M urea.

For over 20 yr soybean agglutinin has been believed to contribute to the poor nutritive properties of raw soybeans. Turner and Liener (12) recently concluded that agglutinin contributes little, if anything, to the deleterious effects of unheated soybean products. They passed an aqueous extract of unheated soybean flour through a column of Sepharose-bound concanavalin A that selectively complexed and removed agglutinin activity from the extract. When the agglutinin-free extract was fed to rats, growth

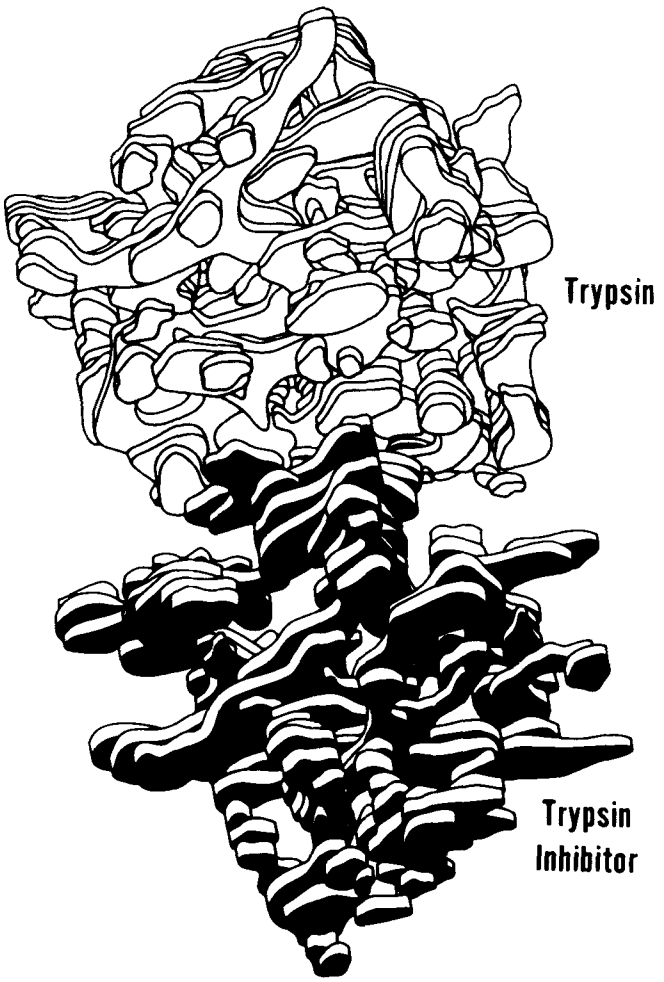

FIG. 3. Cystalline complex of porcine trypsin and Kunitz soybean inhibitor at 5 Å resolution as elucidated by Sweet and coworkers (7). Reprinted with permission from Biochemistry 13:4212 (1974). Copyright by the American Chemical Society.

rate and protein efficiency ratio were not significantly different from those obtained with the original extract from which agglutinin had not been removed.

The function of agglutinin in soybeans is still unknown, but Bohlool and Schmidt (13) observed that the agglutinin combined specifically with 22 out of 25 strains of *Rhizobium japonicum*, the soybean-nodulating bacterium. Binding of agglutinin did not occur with 23 other strains of rhizobia that do not form nodules on soybean roots. They suggested that the first step in nodule formation may be specific interaction of the polysaccharides on the surface of the appropriate Rhizobium cell with agglutinin on the soybean root surface. The presence of agglutinins in soybean roots apparently has not been reported, but this hypothesis is an intriguing alternative to some of the previous speculations about the biological role of agglutinins in soybeans.

7S GLOBULINS

When a water extract of defatted soybean meal is acidified to pH 4.5, ca. 80% of the total 7S protein fraction precipitates and is generally considered to be a mixture of globulins (14). Several research groups have fractionated the 7S globulins (Table II), but a review of these studies shows that they have resulted in contradictory conclusions.

The first 7S globulin preparation was reported by Roberts and Briggs (15) and was found to have a mol wt of 330,000 and underwent a reversible monomer-to-dimer conversion when the ionic strength was changed. The monomer (7S form) was observed at 0.5 ionic strength, whereas the dimer (9S form) was favored at 0.1 ionic strength. Carbohydrate content of their 7S globulin was 5.9%. Koshiyama (16) subsequently isolated a 7S protein with properties similar to those for the Roberts and Briggs preparation with one important exception. The mol wt of Koshiyama's 7S globulin was only 186,000.

Later, Catsimpoolas and Ekenstam (17) observed four major soybean globulins by immunoelectrophoresis. They designated them as alpha, beta, and gamma conclycinins plus glycinin (11S globulin). Examination of the previous

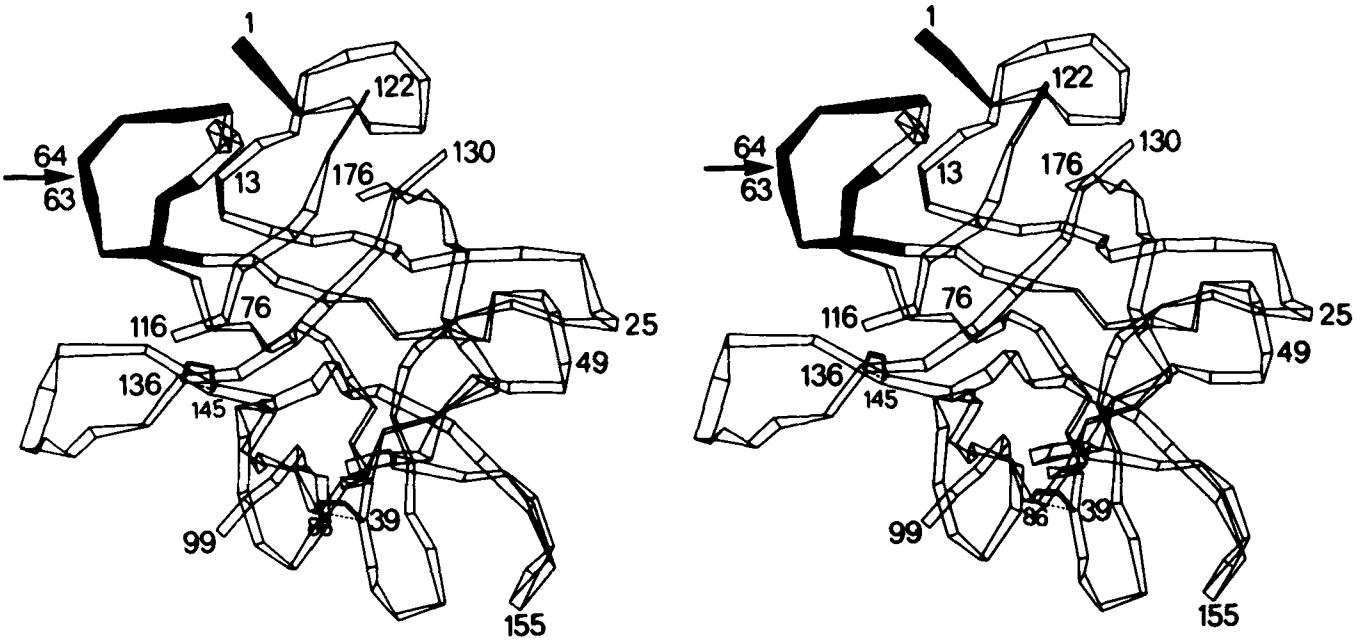

FIG. 4. Stereo pair showing chain folding of Kunitz soybean trypsin inhibitor (7). Arrow points to active site and residues in black make contact with trypsin in the trypsin-trypsin inhibitor complex. Sequences from 99-116, 122-130, and 176-181 are not shown because some of the residues in these portions of the structure could not be located in the electron density map. Reprinted with permission from Biochemistry 13:4212 (1974). Copyright by the American Chemical Society.

TABLE I

Physical and Chemical Properties of Soybean Agglutinin[a]

Property	Value
Nitrogen, %	15.5
D-Mannose, %	4.5
N-Acetyl-D-glucosamine, %	1.5
Isoelectric point	pH 5.81
Diffusion coefficient ($D^{\circ}_{20,w}$)	5.0 X 10^{-7} cm^2 sec^{-1}
Sedimentation coefficient ($s^{\circ}_{20,w}$)	6.0S (pH 4.5-8.9) 2.0S (in 0.1% SDS[b])
Molecular weight	120,000 (gel filtration in 0.9% NaCl) 30,000 (gel filtration in 0.1% SDS[b])
N-terminal residues	4 alanines

[a]From Lotan et al. (10).

[b]SDS, sodium dodecyl sulfate.

TABLE II

Comparison of 7S Globulin Preparations Reported in the Literature

Preparation	Properties[a]			Reference
	Molecular weight	7S ⇌ 9S Dimerization	Carbohydrate content (%)	
7S Component	330,000	+	5.9	Roberts and Briggs (15)
7S Protein	186,000	+	5.9	Koshiyama (16)
β- and γ-Conglycinin	·····	·····	·····	Catsimpoolas and Ekenstam (17)
7S Proteins (3)	·····	+	·····	Hill and Breidenbach (18)
7S Globulins (5)	·····	+	+	Thanh et al. (19)
β-Conglycinin	181,000	+	5.3	Koshiyama and Fukushima (20)
γ-Conglycinin	104,000	-	5.5	Koshiyama and Fukushima (21)

[a]Dotted line indicates that property was not reported.

7S preparations suggested that the Roberts and Briggs 7S protein was heterogeneous, but that beta conglycinin was a major protein present. Gamma conglycinin, in turn, was the major protein found in a partially purified 7S protein prepared by Koshiyama's procedure.

In 1974, Hill and Briedenbach (18) described studies on sucrose density gradient centrifugation of the pH 7.6 buffer-extractable proteins of defatted soybean meal. The resulting separation closely resembles that obtained by analytical ultracentrifugation by earlier workers, but it has the advantage that the separated fractions can be isolated and examined by other techniques. They also confirmed the conversion of 7S fraction into the dimer (9S) when centrifugation was carried out at 0.1 ionic strength. The dimer form was isolated and found to separate into three bands on polyacrylamide disc gel electrophoresis. Consequently, there is evidence for at least three proteins capable of undergoing the monomer-dimer transformation with changes in ionic strength.

Further complexity of the 7S globulin fraction is evident from the recent work of Thanh and coworkers (19). They first precipitated the 11S protein from the 7S and other proteins by adjusting the pH to 6.6 with tris-HCl buffer and cooling at 2-3 C. The 7S globulins were then precipitated at pH 4.8 and purified on a Sepharose 6B gel filtration column. The resulting purified 7S globulin fraction underwent complete dimerization at 0.1 ionic strength and when it was chromatographed on a DEAE-cellulose column, it yielded five fractions. Disc gel electrophoresis confirmed the presence of five proteins although some of them gave diffuse bands. All five proteins appear to be glycoproteins.

Recently, Koshiyama and Fukushima (20,21) reexamined the relationship between beta and gamma conglycinins [as defined by immunoelectrophoresis by Catsimpoolas and Ekenstam (17)] and the previously isolated 7S globulins. They found that beta conglycinin was the major protein in the 7S globulin of Roberts and Briggs (15) in agreement with Catsimpoolas and Ekenstam. They likewise reported that Koshiyama's 7S globulin was identical with beta conglycinin, but this is at variance with the conclusions of Catsimpoolas and Ekenstam who reported it to be mainly gamma conglycinin. Koshiyama and Fukushima found that the molecular weights for beta and gamma conglycinin differed significantly and that gamma conglycinin did not dimerize at 0.1 ionic strength. Gamma conglycinin appears to be a minor protein because it was estimated to represent about 3% of the total soybean globulins. In contrast, beta conglycinin made up 28% of the globulins. The sum of beta plus gamma conglycinin accounted for about 91% of the total 7S globulin. Some minor 7S globulins thus still appear unaccounted for.

From this summary of the studies on the 7S fraction, it is clear that further work is needed to more fully understand this portion of the globulin mixture that comprises about one-third of the total proteins found in commercial soy protein isolates.

11S GLOBULIN

Badley and coworkers (22) found that precipitation steps in previous methods for purifying 11S globulin (glycinin) caused partial modification of the protein, but that the modified protein is removed by gel filtration on Sepharose 6B in the presence of 1M KH_2PO_4. They characterized their 11S preparation in great detail as summarized in Table III.

N-terminal analysis of their 11S globulin indicated the presence in each molecule of six glycines, two phenylalanines, two leucines, and two isoleucines. Previous workers had reported four leucines or isoleucines because they were unable to distinguish between the two amino acid residues. The 12 N-terminal residues indicate the presence of 12 subunits per molecule and these have acidic and basic properties (23). The acidic subunits contain only N-terminal leucine, isoleucine, or phenylalanine whereas the basic subunits terminate only in glycine.

Badley et al. also determined molecular weights of the

TABLE III

Physicochemical Properties of 11S Globulin (Glycinin)[a]

Property	Value
General	
Nitrogen content, %	16.3
Extinction coefficient, $E^{1\%}_{1\,cm,280}$	8.1 ± 0.1
Partial specific volume, $\bar{V}$, 20 C	0.730 ± 0.001 ml g^{-1}
Sedimentation constant, $S^{\circ}_{20,w}$	12.35 ± 0.15
Diffusion constant, $D^{\circ}_{20,w}$	3.44 ± 0.1 X 10^{-7} cm^2 s^{-1}
Radius of gyration, Rg	44 Å
Stokes' radius, r	58.5 Å
Hydration, δ	0.36 g/g
Number of subunits	12
N-terminals	Leu, 2; Ile, 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	100 X 100 X 70 Å (as observed) 110 X 110 X 80 Å (allowing for hydrophobic region)
X-ray scattering	110 X 110 X 75 Å

[a]From Badley et al. (22).

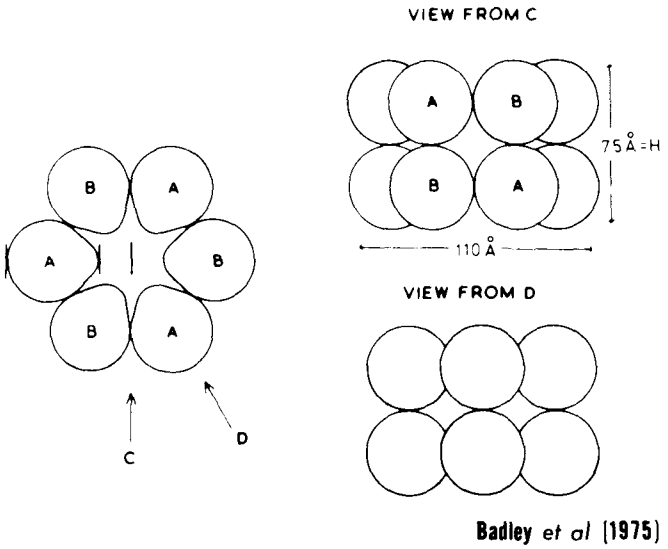

FIG. 5. Arrangement of subunits in the 11S molecule (22). Diagram at left is top view of hexagonal rings. Side views from positions C and D are shown at right. Acidic (A) and basic (B) subunits alternate as indicated. Reprinted with permission from Elsevier/North-Holland Biomedical Press.

subunits and found only two sizes. The basic subunits have mol wts of 19,600, but the acidic subunits are almost twice as large at 34,800.

Subunit structure and dimensions of the 11S molecule were investigated by electron microscopy and X-ray scattering. Results confirmed previous speculation of a structure consisting of two hexagonal rings stacked on top of each other (23). The resulting structure (Fig. 5) has a hole in the center when viewed from the top or bottom of one of the hexagonal rings. Dimensions of the hollow oblate cylinder are estimated to be 110 X 110 X 75 Å. Staining characteristics observed by electron microscopy suggest that the region between the two hexagonal rings is hydrophilic and, consequently, the interaction is speculated to involve electrostatic and/or hydrogen bonding. Interactions between subunits within a hexagonal ring structure are believed to be hydrophobic. The arrangement within a hexagonal ring is proposed to be alternating acidic (A) and basic (B) subunits (22,23). Alternation of acidic and basic subunits between the two layers of adjacent hexagonal ring structures is also considered likely (Fig. 5).

The intricate structure of the 11S molecule can be disrupted by various methods; one of these of special interest to the food industry is heat, which is widely used in processing. Previous studies showed that when the 11S protein is heated in neutral solution at 100 C, about one-half of it precipitates and the other half remains soluble (24). This phenomenon has now been clarified by Badley et al. (22); they observed that the basic subunits precipitate almost quantitatively on heating 11S protein in buffer at 100 C for 30 min.

Although Badley et al. (22) observed only three types of acidic subunits in the 11S protein, Kitamura and Shibasaki (25) found evidence for four subunits designated A_1, A_2, A_3, and A_4. N-terminal amino acids in A_1 and A_2 were phenylalanine whereas A_3 and A_4 terminated in leucine (isoleucine). The mol wt of A_4 was 45,000 in contrast to 37,000 for A_1, A_2, and A_3. The occurrence of subunit A_4 may indicate the presence of genetic variants of 11S protein. If variants do not exist, subunit A_4 requires modification of the model of 12 subunits per molecule or 6 subunits in two identical half-molecules consisting of three basic and three acidic subunits. One possibility is that the half-molecules are nonidentical; conceivably one may contain the A_3 subunit while the other contains the A_4 subunit.

ACID-SENSITIVE PROTEINS

In the preparation of soybean protein isolates, defatted flakes are extracted with water and the resulting extracts are then acidified to precipitate the globulins. After washing, neutralizing, and spray drying, the resulting isolate is no longer completely soluble in buffer at pH 7.6 because some of the proteins are sensitive to treatment with acid (pH 4.5). The acid-modified proteins are responsible for the turbidity of isolate dispersions in water.

Early studies (14) indicated that 2S and 7S proteins comprise the major part of the acid-sensitive fraction (ASF). Effects of pH and time of acid treatment on ASF have been studied (26). Anderson (27) extended these studies and observed that nonprotein material was bound more tightly to ASF than to the other proteins. He also found that the ASF contributed tan or brown color and increased turbidity of solutions of the proteins in propor-

TABLE IV

Flavor Scores and Acid-Sensitive Fraction (ASF) Content of Soybean Protein Preparations[a]

Preparation	% ASF	Flavor score[b]
Water-extractable proteins	23	5.3
Water-extractable proteins minus ASF	---	6.9**
Acid-precipitated proteins	31	5.6
Acid-precipitated proteins minus ASF	---	6.7*
0-28% $(NH_4)_2SO_4$ cut[c]	42	5.4
0-28% $(NH_4)_2SO_4$ cut[c] minus ASF	---	6.5**
60-100 $(NH_4)_2SO_4$ cut[c]	7	5.8
60-100 $(NH_4)_2SO_4$ cut[c] minus ASF	---	5.7

[a]From Anderson and Warner (28).

[b]*P<0.05; **P<0.01; 1 - strong, 10 - bland.

[c]Fraction precipitated in the specified range of $(NH_4)_2SO_4$ concentrations.

tion to its content. The ASF can be concentrated by fractionating the water-extractable proteins with ammonium sulfate. The fraction precipitating between 0-28% saturation had the highest content of ASF; concentration of ASF was about twice its content in the starting water extract.

A later study (28) showed that soy protein preparations containing ASF had lower flavor scores and higher intensities for grassy-beany flavors than the same fractions from which ASF had been removed (Table IV). This is the first demonstration that there is a selectivity of some of the proteins for binding of the compounds (probably oxidized lipids and derived compounds) responsible for the undesirable flavors characteristic of raw soybean protein products.

DIFFERENCES IN FUNCTIONAL PROPERTIES OF 7S AND 11S PROTEINS

Studies of functional properties of soybean proteins are generally made with isolates which are complex mixtures. Alteration of the functional properties of these mixtures is often done by heating or enzyme treatment or a combination of the two. Another approach is to fractionate the proteins and take advantage of their differences in properties.

Saio et al. (29) were the first to demonstrate that fractions of soybean proteins did not behave the same as the unfractionated mixture in tofu (bean curd). They prepared tofu from crude 7S fraction (freed of 11S fraction by cooling and treatment with calcium ion) and crude 11S fraction (cold-insoluble fraction). Tofu made from 7S fraction was soft and low in chewiness whereas tofu prepared from 11S protein was hard and chewy.

Saio and coworkers (30) conducted further studies in which protein dispersions were heated at 90 C, cooled to 70 C, and coagulated by adding calcium chloride. The precipitated gel was then molded and autoclaved in water or buffer. Autoclaving caused a gel expansion that varied with pH. Comparison of expanded gels from 7S-rich and 11S-rich fractions again showed significant differences. Gels made from 7S fractions were slightly expanded and dense in contrast to 11S gels that were greatly expanded, porous, and elastic. These results suggest that fractionation may be an alternative to chemical modification or enzymatic hydrolysis for altering functional properties of soybean proteins.

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