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Proteins of Soybean Seeds

I. ISOLATION AND CHARACTERIZATION OF THE MAJOR COMPONENTS[1]

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ABSTRACT

Soybean (*Glycine max*) storage proteins were characterized by sedimentation and by polyacrylamide gel electrophoresis under dissociating (8 M urea) and nondissociating conditions. Three sedimenting classes of proteins were found, with sedimentation coefficients of 2.2S, 7.5S, and 11.8S. The coefficients were related to the bands obtained by electrophoretic separation. The results support the idea that relatively few proteins make up the bulk of the seed protein.

A large proportion of the dry weight of the mature seeds of many legumes is protein. Isolation and characterization of these proteins is essential to physiological studies of their accumulation during development, or of their utilization during germination.

Osborne (15) first classified the proteins of soybeans and other legumes on the basis of differential solubility. His major soybean protein, named glycinin, was later shown to be heterogeneous (8). More recently these proteins have been further characterized by studies of Wolf and co-workers (6, 18–22) and Catsimpoolas *et al.* (1–4). The number of these proteins and their properties are still not well understood, largely because the methods of separation and characterization used are different and unrelated to each other. In fact, no acceptable nomenclature has been established as a standard for the soybean proteins (17). They are referred to most commonly by sedimentation coefficients, and the trivial name glycinin is now used only for the 11S sedimenting fraction (2).

This report describes isolation of the soybean proteins by sucrose density gradient sedimentation and subsequent analysis on polyacrylamide discontinuous gels. These techniques were combined in an attempt to clarify questions of the numbers and relative amounts of these proteins.

MATERIALS AND METHODS

Plant Material and Protein Extraction. Seeds of an early maturing soybean (*Glycine max* var. Portage) were obtained from University of Minnesota seed stocks. The seeds were cracked in a Waring Blendor, passed over an air stream to remove the seed coats, and ground to a fine powder in a Wiley mill. A portion of this meal was saved, and the remainder was hexane extracted in a Soxhlet apparatus and air-dried. Proteins were extracted from both defatted and undefatted meals into 10 volumes of a homogenizing buffer (0.035 M phosphate; 0.4 M NaCl; 0.01 M β-mercaptoethanol; pH 7.6; $\mu = 0.5$) described by Wolf and Briggs (20). The NaCl was omitted from the extraction buffer and the sucrose gradients for studies at low ionic strength. The meals were initially homogenized with buffer in a VirTis blender at high speed for two 30-sec intervals. The brei was filtered through cheesecloth and centrifuged at 31,000*g* for 20 min. Protein in the filtered brei and the supernatant was determined by the method of Lowry (12). The clarified supernatant was placed either directly on sucrose gradients or dialyzed against an appropriate buffer.

Sucrose Density Gradient Centrifugation. Linear sucrose gradients, 34 ml in volume and 10 to 30% (w/w) in concentration, were prepared with a laboratory-constructed dual cam-driven syringe pump. Aliquots of the supernatant obtained at 31,000*g* (about 20 mg protein) were layered on the gradient along with an overlay of homogenizing buffer. The gradients were centrifuged 24 hrs at 105,000*g* in a Spinco L-2 65B preparative ultracentrifuge. Standards used for determination of sedimentation coefficients were bovine liver catalase (Sigma, Lot 18B-8040) and soybean trypsin inhibitor (Calbiochem). The gradients were divided into 1-ml fractions, and protein was assayed by the method of Lowry (12). The fractions corresponding to the peak protein regions were pooled and dialyzed against the buffer for later analysis by polyacrylamide discontinuous gel electrophoresis.

Polyacrylamide Discontinuous Gel Electrophoresis. Two discontinuous polyacrylamide gel systems were used: the procedure of Davis with 5% (w/v) acrylamide in the running gel at pH 8.9 (tris-glycine) (5); and a β-alanine, plus 8 M urea dissociating system described by Wu and Bruening (23) and modified as described below. Protein was included in the stacking gel, and the levels were adjusted to give clear banding patterns (usually 150 μg protein per gel for supernatant protein and 50 μg protein per gel of the pooled gradient fractions). The current applied was 2 mA per gel until the bromphenol-blue tracking dye cleared the stacking gel, and was then increased to 4.5 mA per gel.

In the dissociating system, the running gel contained 10% (w/v) acrylamide. The gel buffers were modified from the system of Wu and Bruening to 0.74 M *n*-butylamine in the large pore (Solution A) and 0.66 M tris-HCl in the small pore (Solution B). Both contained 0.48 M HCl. Protein was dialyzed overnight against the running buffer (β-alanine, 0.06 M; *n*-butylamine, 0.045 M; β-mercaptoethanol, 0.01 M; in 8 M urea) and was electrophoresed by a method similar to that described above except that electrophoresis was continued for 45 min

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after the tracking dye had moved beyond the end of the gel. Runs for shorter periods had previously shown that no major protein components were lost by this procedure. The gels from both systems were stained with 0.05% (w/v) naphthol blue-black in 7% (v/v) acetic acid and destained electrically.

Gel Scanning. The destained gels were scanned in a Gilford linear transport attachment to a Beckman DU monochromator.

RESULTS

Proteins were extracted from both defatted and undefatted meals prepared from mature seeds. Table I shows that our method of extraction solubilized nearly 90% of the total protein from both types of meal. The total amount of protein per gram of meal recovered in the supernatant did not differ between hexane-extracted and unextracted meals. Furthermore, distribution of the protein components by the criteria applied was similar in the presence or absence of lipid.

Figure 1 shows the distribution of the major protein components of defatted soybean meal on sucrose gradients prepared in standard buffer. Three prominent peaks were found, with maxima in fractions 10, 17, and 22 (Fig. 1). The approximate sedimentation constants for these peaks were determined by the method of Martin and Ames (13) to be 2.2S, 7.5S, and 11.8S relative to catalase (11.3S) and commercial soybean trypsin inhibitor (2.3S). The relative amounts and positions of the proteins on the gradient were identical when undefatted meal was sedimented, although some turbidity due to lipid was then visible in the upper portion of the gradient.

Omitting 0.4 M NaCl from the standard extraction buffer and from the sucrose gradient solutions resulted in a different protein distribution. Figure 2 shows that the 7.5S protein, seen as a peak in fraction 17 at high ionic strength, was considerably diminished at low ionic strength. Also, at conditions of low ionic strength a new peak appeared as a shoulder on the more slowly sedimenting side of the 11.8S component. Although all of the proteins sedimented more rapidly in gradients of lower ionic strength, the change in sedimentation rate is greater for the 7.5S protein than for the other components.

Total seed proteins, as well as the proteins separated first by sedimentation on sucrose gradients, were characterized by discontinuous electrophoresis on polyacrylamide gels by the method described by Davis (5). Total seed proteins were separated into five bands, numbered consecutively in order of increasing relative mobility in Figure 3a.

The electrophoretically distinguishable proteins were then related to the proteins separated by sedimentation. Figure 3b shows that the soybean proteins with the highest relative mobility in this electrophoretic system are composed largely if not completely of proteins with a sedimentation coefficient of about 2.2S. The 2.2S protein fraction is not electrophoretically homogeneous, however. When the cross-linking of the gel is increased by increasing the acrylamide concentration to 7.5%, at least one additional band is resolved. One of the bands at this higher gel concentration, constituting more than half of the resolved 2.2S protein coincides with the major component of commercially obtained soybean trypsin inhibitor (Fig. 4b).

Table I. *Recovery of Protein from Hexane-defatted and Undefatted Soybean Meal*

Sample	Total Protein in Meal	Protein Recovered in 31,000g Supernatant of Meal	
	mg/g	mg/g	% of total
Defatted	394	347 (285)[1]	88
Undefatted	324	288	89

[1] Estimate of the protein with the meal weight adjusted to that of undefatted meal, assuming 18% lipid.

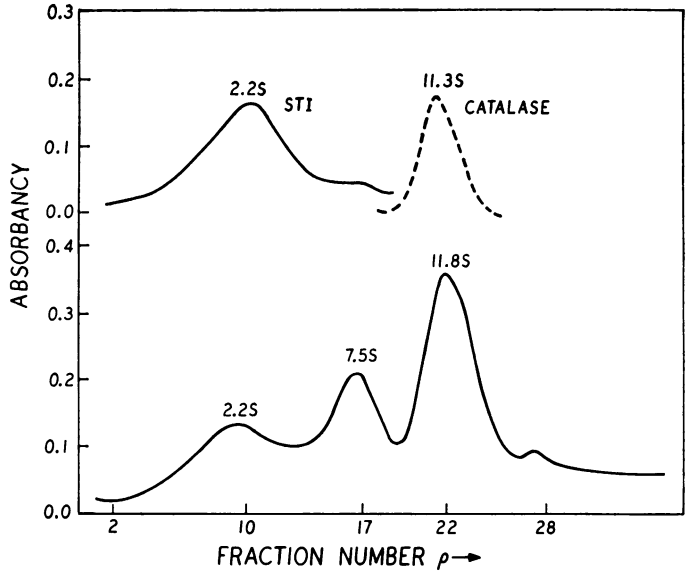

FIG. 1. Separation of soybean storage proteins and commercially prepared soybean trypsin inhibitor (STI) and catalase according to their sedimentation velocity on sucrose gradients. Gradients 10 to 30% sucrose containing standard buffer ($\mu = 0.5$).

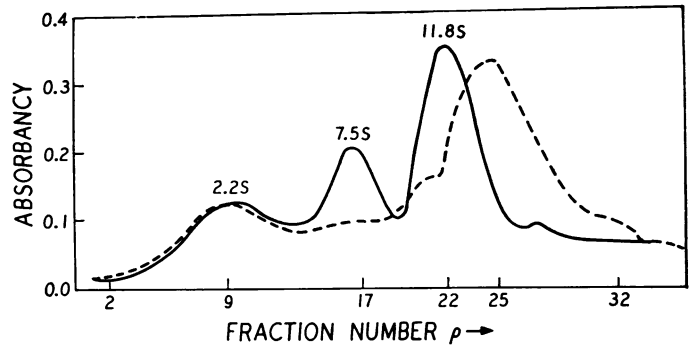

FIG. 2. Separation of soybean storage proteins according to their sedimentation velocity on sucrose gradients. Gradients 10 to 30% sucrose containing standard buffer (——) and standard buffer without NaCl (-----, $\mu = 0.1$).

Figure 3c shows that the 7.5S protein separates into three bands with electrophoretic mobilities indistinguishable from those of bands 1, 2, and 3 obtained by electrophoresis of the total protein extract.

In contrast to the 7.5S protein, the 11.8S protein gave a single diffuse band in the nondissociating buffer system (Fig. 3d). Its electrophoretic mobility on gels was greater than that of the 7.5S protein in spite of its higher sedimentation constant. In contrast to the 2.2S protein, the 11.8S protein remained as a single band when the acrylamide concentration was increased.

Neither the 7.5S nor the 11.8S sedimenting component showed any detectable cross contamination upon electrophoresis in the nondissociating buffer system. A small fraction of the 2.2S protein had an electrophoretic mobility similar, though not identical, to the mobility of the three electrophoretic band (bands 1, 2, and 3) from the 7.5S protein.

The relatively large shift in the 7.5S peak upon sedimentation in a medium of low ionic strength (Fig. 2) allowed further examination of any relation between electrophoretic bands 1,

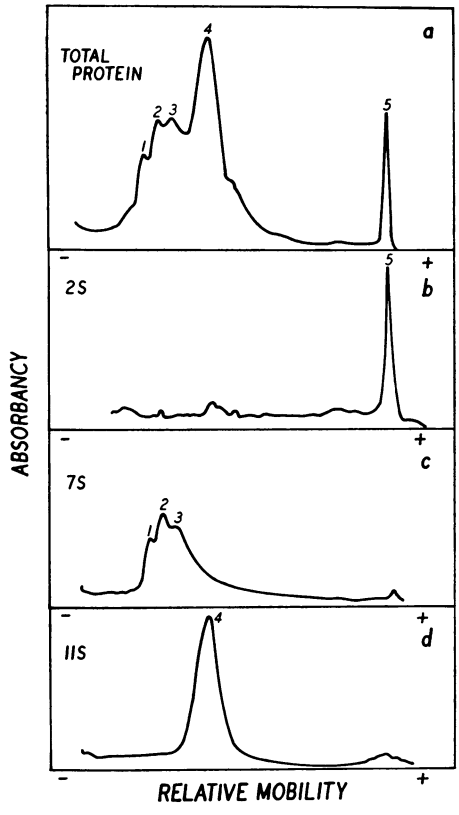

FIG. 3. Electrophoretic patterns of soluble storage proteins and the three sedimenting groups on polyacrylamide gels under nondissociating conditions. a: Total protein; b: 2.2S protein; c: 7.5S protein; d: 11.8S protein.

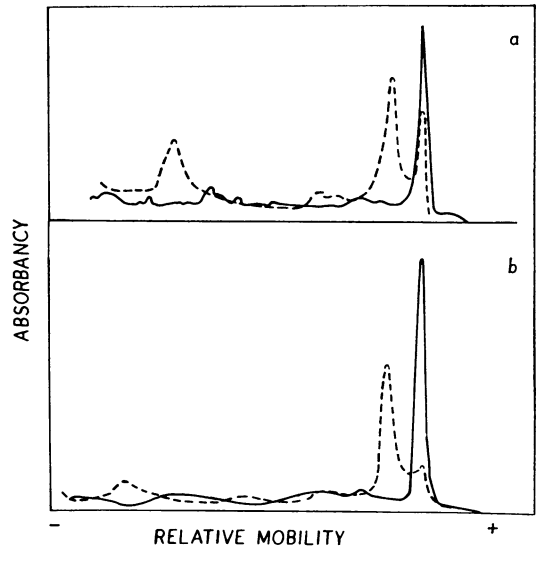

FIG. 4. Comparison of the 2.2S fraction with soybean trypsin inhibitor (STI) on 5% and 7.5% (——) (-----) polyacrylamide gels under nondissociating conditions. a: 2.2S protein; b: soybean trypsin inhibitor (STI).

2, and 3. Figure 5 shows that the more rapidly sedimenting new shoulder at about 9S gave bands with electrophoretic mobilities identical to those of bands 1, 2, and 3 from total protein extracts. The small amount of protein remaining in the 7S region of the sucrose gradients of low ionic strength gave no discernible electrophoretic bands at the protein concentration obtained by pooling the fractions from this region.

Total soluble protein from whole meal and proteins isolated by sedimentation were dissociated in a β-alanine plus 8 M urea buffer system (23) and subjected to discontinuous polyacrylamide gel electrophoresis in this buffer. Figure 6a shows at least 14 bands in the dissociated soluble protein. These bands are labeled with letters to avoid confusion with the numbered

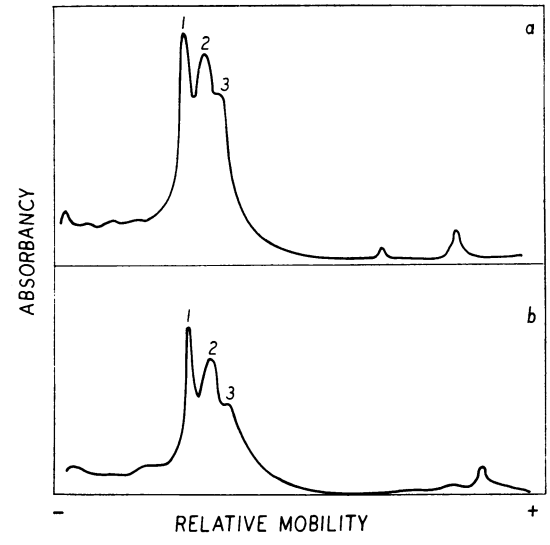

FIG. 5. Electrophoretic patterns obtained with 7.5S protein isolated on sucrose gradients in (a) Standard buffer containing 0.4 M NaCl ($\mu = 0.5$); compared with (b) the 9S shoulder obtained by isolation on gradients without NaCl ($\mu = 0.1$).

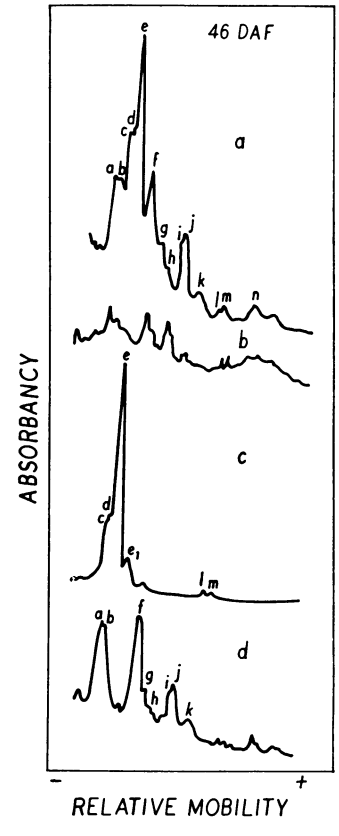

FIG. 6. Electrophoretic patterns of soluble storage proteins and the three isolated sedimenting species on polyacrylamide gels under dissociating conditions. a: Total protein; b: 2.2S protein; c: 7.5S protein; d: 11.8S protein.

electrophoretic bands described earlier. The pattern and relative distribution of these bands were consistent as long as β-mercaptoethanol was present in all of the buffers used. Without a reducing agent, a relatively diffuse large band appeared near the origin, suggesting that disulfide-linked polymers were formed.

When proteins in the 2S region of the sucrose gradient were electrophoresed in the dissociating medium, a number of bands were observed, all at low concentrations (Fig. 6b). None of these protein bands appeared to contribute significantly to the bands obtained with the total protein. Figure 6c illustrates that the bands observed when the 7.5S sedimenting proteins were electrophoresed in the dissociating buffer, correspond to the major components, *c, d,* and *e,* and the minor components, *l* and *m,* found in the whole-meal protein. Also present in significant amounts is an additional band, e_1, which was not resolved from *f* in the whole-meal extracts. Thus, the 7S protein can be resolved into six components by the dissociating conditions. Three of these, however, constituted the bulk of the protein.

Figure 6d shows that the 11.8S component, a single band in the nondissociating electrophoretic buffer, was dissociated into several bands by the 8 M urea plus β-alanine system. Seven bands are easily seen that correspond to *a, b, f, g, i, j, k* in the whole-meal protein. Lesser bands (such as *h*) were also discernible, but their extremely low concentration makes them difficult to relate to bands in the total protein gels. The five or six bands comprising the major components are clearly related to bands obtained from the whole-meal extracts. It should also be noted that the banding pattern of the 11.8S proteins is distinct from that obtained from the 7.5S sedimenting species, again indicating very little cross contamination between them.

DISCUSSION

Despite considerable study, much remains unknown about the physical complexity, biological function, and biochemical regulation of the reserve proteins of soybeans and other leguminous seeds. One element of confusion is that the various methods used in purifying and characterizing these proteins have not always been related to each other. This study therefore relates their sedimentation properties with their electrophoretic properties in two very different buffer systems in order to establish the number of protein species present in major amounts, together with their relative concentrations.

Our study supports the notion that only a limited number of proteins, perhaps serving only as storage materials, make up the bulk of the protein in mature seeds. Of the three distinct sedimenting protein fractions (*i.e.,* 2.2S, 7.5S, and 11.8S), the 7.5S and 11.8S proteins account for up to 70% of the total seed protein. The possibility is not excluded that the major proteins possess specific enzymatic activities, although that seems unlikely. A number of enzymes have been reported in mature soybean seeds (19), and their concentration relative to the total protein must be too small to be detected except by catalytic, immunologic, or other similarly sensitive and specific methods.

The sedimentation coefficients for the three major sedimenting fractions agree with those reported by Naismith (14) and determined in extensive studies of Wolf and co-workers, recently reviewed by Wolf (18, 19). Those studies used the analytical ultracentrifuge directly as a determinant of composition and purity, not as a method of isolating the sedimenting components. Since sedimentation alone is an insufficient criterion for characterizations, we analyzed the three groups further after separating them by ultracentrifugation.

Our results strongly suggest that the 11.8S component may be largely a single protein as indicated by sedimentation and subsequent electrophoresis (Figs 1 and 3). The electrophoretic behavior of the 11.8S protein under dissociating conditions indicates that six or seven subunits could be present in significant amounts (Fig. 6). Under nondissociating conditions the somewhat diffuse single band observed suggested some diversity in mass-charge ratio. This behavior is characteristic of glycoproteins, as Ericson and Chrispeels (7) found in *Pisum aureus* and attributed to variations in glucosamine content.

Other workers (9, 11, 16) have isolated major individual components of soybean protein by ammonium sulfate fractionation and gel permeation chromatography. Among these are proteins that exhibited 7S sedimentation coefficients as determined by analytical ultracentrifugation. However, the entire 7S sedimenting fraction has not previously been isolated directly by sedimentation from total soluble protein preparations. Although a single symmetrical 7.5S peak was obtained on sucrose gradients, three major electrophoretic bands were seen under both nondissociating and dissociating conditions.

Proteins with 7S sedimentation coefficients isolated by other workers have been shown to undergo a shift in sedimentation properties at low ionic strength (10, 16). All three of the bands found in the 7.5S fraction by electrophoresis under nondissociating conditions were seen in the 9S fraction, indicating that all three components were similarly shifted by low ionic strength. The shift at low ionic strength was not due to a decrease in protein solubility, because the amounts of total protein extracted in buffer of either high or low ionic strength differed nonsignificantly. These changes in sedimentation properties must reflect aggregation of the 7.5S proteins, because the sedimentation of the 11.8S and 2.2S proteins is unaffected and the shift is too large to be accounted for by changes in the shape or density of the 7.5 species.

The other group of proteins at 2.2S is defined less clearly. However, these represent a smaller proportion of the total and bear some resemblance to soybean trypsin inhibitor preparations.

It can be concluded that if the bulk of the protein comprises only a few protein species, this system presents important opportunities for studying plant protein synthesis and its regulation inasmuch as a single 300-mg seed can produce 45 mg of protein or more in 6 to 10 days. The system becomes even more useful if, as reported in a subsequent paper, the synthesis of these proteins shows a temporal difference over this short period.

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LITERATURE CITED

1. Catsimpoolas, N. 1969. Isolation of glycinin subunits by isoelectric focusing in urea-mercaptoethanol. FEBS Lett. 4: 259–261.
2. Catsimpoolas, N. 1969. A note on the proposal of an immunochemical system of reference and nomenclature for the major soybean globulins. Cereal Chem. 46: 369–372.
3. Catsimpoolas, N., C. Ekenstam, D. A. Rogers, and E. W. Meyer. 1968. Protein subunits in dormant and germinating soybean seeds. Biochim. Biophys. Acta 168: 122–131.
4. Catsimpoolas, N. and E. W. Meyer. 1968. Immunochemical study of soybean proteins. Agr. Food Chem. 16: 128–131.
5. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121: 404–427.
6. Eldridge, A. C., R. L. Anderson, and W. J. Wolf. 1966. Polyacrylamide gel electrophoresis of soybean whey proteins and trypsin inhibitors. Arch. Biochem. Biophys. 115: 495–504.
7. Ericson, M. and M. Chrispeels. 1973. Isolation and characterization of glucosamine-containing storage glycoproteins from the cotyledons of *Phaseolus aureus*. Plant Physiol. 52: 98–104.
8. Jones, D. B. and F. A. Csonka. 1932. Precipitation of soybean proteins at various concentrations of ammonium sulfate. Proc. Amer. Soc. Biol. Chem. 26: 29.

9. KOSHIYAMA, I. 1965. Purification of the 7S component of soybean proteins. Agr. Biol. Chem. 29: 885–887.
10. KOSHIYAMA, I. 1968. Factors influencing conformation changes in a 7S protein of soybean globulins by ultracentrifugal investigations. Agr. Biol. Chem. 32: 879–887.
11. KOSHIYAMA, I. 1968. Chromatographic and sedimentation behavior of a purified 7S protein in soybean globulins. Cereal Chem. 45: 405–412.
12. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
13. MARTIN, R. G. AND B. N. AMES. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 236: 1372–1379.
14. NAISMITH, W. E. F. 1955. Ultracentrifuge studies of soya bean protein. Biochim. Biophys. Acta 16: 203–210.
15. OSBORNE, T. B. 1924. The Vegetable Proteins. Longmans, Green and Co. London.
16. ROBERTS, R. C. AND D. R. BRIGGS. 1965. Isolation and characterizations of the 7S component of soybean globulins. Cereal Chem. 42: 71–85.
17. WOLF, W. J. 1969. Soybean protein nomenclature: a progress report. Cereal Sci. Today 14: 75.
18. WOLF, W. J. 1969. Chemical and physical properties of soybean proteins. Baker's Dig., October. pp. 30–37.
19. WOLF, W. J. 1970. Soybean proteins: their functional, chemical, and physical properties. Agr. Food Chem. 18: 969–976.
20. WOLF, W. J. AND D. R. BRIGGS. 1956. Ultracentrifugal investigation of the effect of neutral salts on the extraction of soybean proteins. Arch. Biochem. Biophys. 63: 40–49.
21. WOLF, W. J. AND D. A. SLY. 1965. Chromatography of soybean proteins on hydroxylapatite. Arch. Biochem. Biophys. 110: 47–56.
22. WOLF. W. J. AND D. A. SLY. 1967. Cryoprecipitation of soybean 11S protein. Cereal Chem. 44: 653–668.
23. WU, G-J., AND G. BRUENING. 1971. Two proteins from cowpea mosaic virus Virology 46: 596–612.