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# Varietal Influence on the Quantity of Glycinin in Soybeans

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Glycinin content (11S protein) was determined in 10 soybean varieties (all grown in a uniform environment) by analysis of eluted Coomassie blue dye from proteins separated on $NaDodSO_4$-gradient polyacrylamide gels. Significant differences in glycinin content between varieties were identified. Total protein content for the 10 varieties (Coles, Corsoy, Hodgson, Kitamusume, Tokachi-nagaha, Toyosuzu, Vinton, Wase-Kogane, Weber, and Yuuzuru) ranged from 39.4 to 44.1%. The content of glycinin per total protein was observed between 31.4 and 38.3%. The percent glycinin per total weight was found between 13.5 and 17.8%. High concentrations of total protein did not necessarily correlate with high glycinin concentration although on a total glycinin per seed basis, this correlation was much closer. Glycinin has previously been reported to possess gelatination properties vital for the production of some soy foods. The confirmation that varietal variation in glycinin content does exist and application of a method to quantitate these differences might aid in soybean selection for soy food manufacture.

Differences in the quantity of individual soy proteins (namely, glycinin, β-conglycinin, and the 2S fraction) between soybean varieties were reported by Wolf et al. (1961). The source of these differences, genetic or environmental, has not been previously investigated. Recently, several groups have reported that there was heterogeneity in the glycinin fraction both among soybean varieties (Kitamura et al., 1980; Mori et al., 1981) and within single varieties (Utsumi et al., 1981). However, these reports have focused on qualitative data in terms of glycinin. We are interested in the potential quantitative difference in glycinin concentration that might be observed in soybean varieties. In this study, all soybean varieties were exposed to equivalent environmental influences so that the importance of variety alone could be evaluated. Glycinin was selected for study in these soybean varieties because it has been reported to be responsible for most of the hardness, cohesiveness, and springiness in tofu, a gelatinous soy food (Saio et al., 1969). Tofu producers in the United States and Japan have observed varietal differences in the suitability of soybeans for tofu making (Leviton, 1979; Smith et al., 1960).

Polyacrylamide gel electrophoresis is a highly sensitive technique making possible identification of micrograms of protein. It produces better separation of protein mixtures than do other available techniques (Fishbein, 1972). These characteristics make utilization of this technique desirable for quantitation as well as identification of proteins. The method of Fenner et al. (1975), with some modification to accommodate soy protein, has been utilized in the present study.

## MATERIALS AND METHODS

**Plant Materials.** Ten varieties of soybeans, five of Japanese lineage (Kitamusume, Toyosuzu, Yuuzuru, Tokachi-nagaha, and Wase-Kogane) and five of American lineage (Hodgson, Corsoy, Cole, Vinton, and Weber), were used for comparison in this study. The varieties were all grown in Ames, IA, during the summer of 1980. The 10 soybean varieties each were analyzed for protein by a micro-Kjeldahl technique (AOAC, 1970, Method 38.012). The crude lipids were measured by hexane extraction (AACC, 1969). Protein in solution, after extraction from

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polyacrylamide gel electrophoresis analysis was measured by using the biuret method (AOAC, 1970, Method 2.066). All chemicals were analytical reagent grade.

**Purification of Glycinin.** Purified glycinin was used to prepare the standard curve required for quantitation. This material was isolated from Vinton variety soybeans. The beans were ground in a Cyclone sample mill (Boulder, CO) and extracted with hexane, in a Laboratory Construction Co. (Kansas City, MO) Goldfisch apparatus. The ground beans were extracted in 0.03 M tris(hydroxymethyl)aminomethane (pH 8.0) (Sigma, St. Louis, MO)–0.01 M mercaptoethanol (Sigma, St. Louis, MO). The pH was reduced to 6.4 and the precipitate collected (Thanh and Shibasaki, 1976). The crude glycinin precipitate was dispersed in phosphate buffer (0.0026 M $KH_2PO_4$, 0.0325 M $K_2HPO_4$, 0.4 M NaCl, and 0.01 M mercaptoethanol, pH 7.6, $\mu = 0.5$) and separated on a concanavalin A–Sepharose column (Sigma, St. Louis, MO) and a Sepharose 6B-CL column (Sigma, St. Louis, MO) (Kitamura et al., 1974). The concanavalin A–Sepharose column was 2.5 cm × 28 cm, with a flow rate of 9 mL/h, and the Sepharose 6B-CL column was 3 cm × 76 cm, with a flow rate of 5 mL/h. Phosphate buffer was used as the eluent in both instances.

A large peak without affinity for concanavalin A, including glycinin, was observed eluting from the concanavalin A–Sepharose column first. A solution of methyl α-D-mannopyranoside (0.1 M) eluted a small peak of material with affinity, presumably β-conglycinin. Base-line resolution of aggregated material, glycinin, and β-conglycinin was observed on the Sepharose 6B-CL column.

Two techniques were used to confirm the purity of the glycinin produced; sodium dodecyl sulfate–gradient polyacrylamide gel electrophoresis and immunodiffusion. The $NaDodSO_4$–gradient polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) with acrylamide and bis(acrylamide) concentrations of 10–15%. Antibodies to whole soy water extract were produced in a young white rabbit (Hurn and Chantler, 1980) and concentrated by the method of Tumer et al. (1981). The immunodiffusion test was performed on agar immunofilms (Sebia Co., Paris, France). The immunofilms were equilibrated in a buffer from Catsimpoolas and Meyer (1968).

**Quantitation of Glycinin.** The basic and acid polypeptide bands of glycinin were cut out of Coomassie blue R250 (Miles Laboratories, Elkhart, IN) dyed, and destained 10–15% $NaDodSO_4$–gradient polyacrylamide gels. Blanks were prepared by cutting out gel sections without protein samples on them. These pieces of gel were submerged in 25% pyridine (Mallinckrodt, Inc., Paris, KY) in $H_2O$. The Coomassie blue dye was released from the polyacrylamide by shaking in the pyridine overnight. The amount of dye dispersed was determined by measuring the absorbance at 605 nm on a Gilford 250 spectrophotometer (Oberlin, OH). A standard curve of purified protein was used to relate absorbance to protein concentration (Fenner et al., 1975).

This method is based on the acidic properties of Coomassie blue dye. In acid media, the dye is electrostatically attracted by Van der Waals forces to the ammonium groups of the protein. In basic media, such as 25% pyridine solution, the dye–protein complex will disassociate. The protein stays trapped in the polyacrylamide gel matrix, and the dye disperses into the solution (Fazekas De St. Groth et al., 1963).

**Statistics.** The correlation coefficient of the standard curves used in the biuret and dye–elution methods were calculated as in Ott (1977). The $F$ test and Fisher's least significant difference test also were performed according to Ott (1977).

Table I. Crude Lipid and Total Protein Results

| | % crude lipid[a] | | % total protein[a] | |
|---|---|---|---|---|
| | average | SD | average | SD |
| Kitamusume | 27.5[a] | 0.45 | 40.4[cd] | 0.42 |
| Toyosuzu | 25.1[cd] | 0.11 | 42.8[a] | 0.21 |
| Yuuzuru | 25.2[cd] | 0.61 | 40.6[bc] | 1.08 |
| Tokachi-nagaha | 23.7[ef] | 0.84 | 41.6[b] | 0.06 |
| Wase-kogane | 23.1[f] | 0.57 | 44.1 | 0.45 |
| Hodgson | 26.5[ab] | 0.57 | 40.4[bc] | 0.35 |
| Corsoy | 25.2[cd] | 0.61 | 39.4[d] | 0.45 |
| Coles | 24.6[de] | 0.65 | 40.1[cd] | 0.03 |
| Vinton | 24.6[de] | 0.10 | 42.9[a] | 0.32 |
| Weber | 26.0[bc] | 0.41 | 40.9[bc] | 0.12 |

[a] Values sharing common superscripts were not significantly different by Fisher's least significant difference test, $a = \alpha/2 = 0.025$.

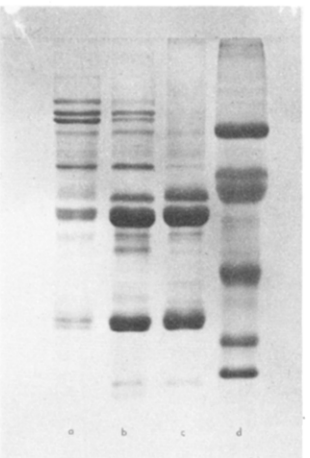

**Figure 1.** Glycinin with increasing purity on a 10–15% $NaDodSO_4$–gradient polyacrylamide gel. Left to right: lane A, whole soy extract (17.8 μg of protein); lane B, isoelectrically precipitated glycinin (21.8 μg of protein); lane C, column-purified glycinin (16.5 μg of protein); lane D, molecular weight marker proteins 66K, 45K, 24K, 18K, and 14.3K (50 μg of protein).

## RESULTS AND DISCUSSION

The results of Kjeldahl protein and crude lipid analysis of the soybean varieties are presented in Table I. An analysis of variance was run on both the protein and crude lipid analyses to determine if a significant difference existed between Japanese and American varieties in this study. Percentage total protein was significantly higher in Japanese soybeans than in the American varieties in this study but only at the 90% confidence limit. The crude lipid analysis showed a trend toward higher lipid content in American soybeans than in Japanese soybeans, but no statistically significant difference was found. These protein and lipid content relationships between Japanese and American varieties are in agreement with Smith et al. (1960). The earlier workers found larger differences, which may be at least partially explained by environmental differences in the Smith et al. (1960) soybean samples.

The $NaDodSO_4$–gradient electrophoretic analysis (Figure 1) of the purified glycinin resulted in the major acidic subunits migrating between $M_r$ 35000 and 45000 while the basic subunits migrated to the 19000 molecular weight area of the gel. This is in agreement with Moreira et al. (1979). These estimated molecular weights were determined by plotting the electrophoretic mobilities of the bands of in-

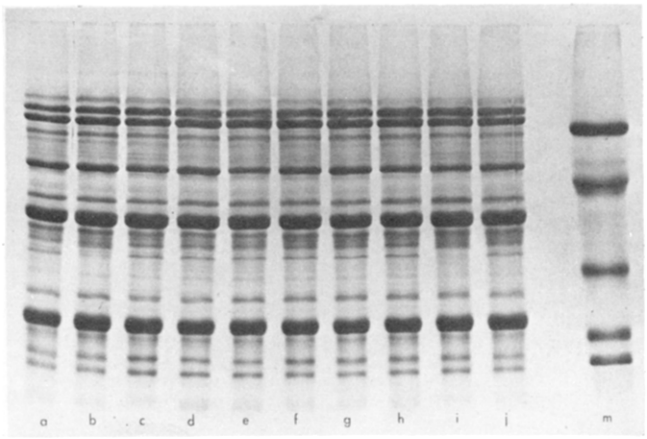


**Figure 2.** Soybean varieties on a $NaDodSO_4$–gradient polyacrylamide gel: (A) Kitamusume (28.4 μg of protein); (B) Toyosuzu (30.3 μg); (C) Yuuzuru (30.4 μg); (D) Tokachi-nagaha (30.0 μg); (E) Wase-kogane (27.4 μg); (F) Hodgson (29.4 μg); (G) Corsoy (29.2 μg); (H) Coles (29.8 μg); (I) Vinton (30.0 μg); (J) Weber (28.2 μg); (M) molecular weight marker proteins 66K, 45K, 24K, 18K, and 14.3K.

terest vs. the logarithm of known molecular weight marker proteins (Sigma, St Louis, MO), with the resulting correlation coefficient of 0.999. No bands were observed at $M_r$ 54 000, the size of the α and α′ polypeptides of β-conglycinin (Thanh and Shibasaki, 1978) in the purified glycinin solution.

Double immunodiffusion was performed to further verify the purity of the glycinin solution. Antibodies to whole soy protein extract were allowed to diffuse against different protein concentrations of a whole soy extract or the purified glycinin solution. The whole soy extract antibody gels produced a sharp band and a broad band, indicating a heterogeneous group of proteins (Fritz and Schenk, 1979). When the antibodies to whole soy were allowed to diffuse against the purified glycinin, only a sharp thin band was produced, indicating that only a single protein was present.

The purified glycinin was used to prepare a standard curve by utilizing the dye–elution method to relate absorbance to protein concentration ($r$ = 0.996). Protein concentrations of 0–17 μg/mL were used. This standard curve was used to calculate unknown concentrations from absorbances obtained by dye–elution analysis.

Extracts of the 10 soybean varieties of differing protein concentrations were electrophoresed simultaneously on a 10–15% $NaDodSO_4$–gradient polyacrylamide gel, as shown in Figure 2. The dye–elution technique was used to determine the amount of glycinin per total protein for each variety. The analysis was repeated 3 times. Statistical analysis was performed on the results.

Significant differences in amount of glycinin per total protein between varieties did exist at the 98% confidence limit ($F_{9,20}$ = 2.99), but a significant difference between the Japanese soybeans as a group and the American soybeans as a group was not found. The results are presented in Table II. Vinton variety had the greatest amount of glycinin/total protein. However, it was not significantly greater than that of Toyosuzu or Hodgson according to the Fisher's least significant difference test (Ott, 1977). Many of the varieties midway in percentage protein as glycinin were not significantly different from each other. Wase-Kogane has significantly less glycinin per total protein than did Tokachi-nagaha, Hodgson, Yuuzuru, and Kitamusume. It is of interest that Vinton, the variety highest in percentage protein as glycinin, had high total protein while, in contrast, Wase-Kogane also was high in total protein while quite low in percentage protein as glycinin. This indicates that high total protein does not necessarily indicate large amounts of protein as glycinin.

Table II. Glycinin Concentrations in Terms of Total Protein and Total Soy Constituents

| | % protein[a] as glycinin | | % soy (dry basis)[a] as glycinin | |
|---|---|---|---|---|
| | average | SD | average | SD |
| Kitamusume | $34.2^{b}$ | 1.21 | $14.1^{d}$ | 0.50 |
| Toyosuzu | $35.7^{bc}$ | 1.14 | $16.9^{ab}$ | 0.53 |
| Yuuzuru | $34.4^{b}$ | 2.34 | $14.0^{d}$ | 0.95 |
| Tokachi-nagaha | $36.2^{ab}$ | 1.20 | $16.3^{b}$ | 0.53 |
| Wase-kogane | $3.14^{c}$ | 0.65 | $15.7^{bc}$ | 0.33 |
| Hodgson | $34.8^{ab}$ | 2.84 | $14.3^{cd}$ | 1.17 |
| Corsoy | $33.5^{bc}$ | 1.58 | $13.9^{d}$ | 0.67 |
| Coles | $32.7^{bc}$ | 2.52 | $13.8^{d}$ | 1.06 |
| Vinton | $38.3^{a}$ | 3.14 | $17.8^{a}$ | 1.45 |
| Weber | $33.0^{bc}$ | 1.21 | $13.5^{d}$ | 0.50 |

[a] Values sharing common superscripts were not significantly different by Fisher's least significant difference test, $a = \alpha/2 = 0.025$.

The amount of glycinin per whole dry soybean also was calculated, and the results are presented in Table II. The percentage soy as glycinin measures the influence of total protein on the glycinin available in the seed. When analysis of variance was performed on glycinin/dry soy values, significant differences between varieties were found to exist at the 99% confidence limit. Fisher's least significant difference analysis results are presented in Table II. The differences observed in percent soy as glycinin were observed over a range from 13.5 to 17.8%. There were significant differences between groups at the low percentage end vs. the high percentage end. It is interesting to note that Wase-Kogane, which was lowest in total protein as glycinin, still had significantly higher total glycinin content per gram of seed than the five lower percentage glycinin per gram of seed due to the high total protein content of Wase-Kogane. These percentage soy as glycinin values would be of particular benefit to the soybean user more interested in the bulk of glycinin than the ratio of this protein to the others in the seed.

## CONCLUSIONS

The results of this investigation indicate that lineage does have some impact on glycinin content in soybeans. Ancestry does not seem to be the sole influence on glycinin content. Wolf et al. (1961), utilizing ultracentrifugal analysis, observed greater differences in glycinin content of soybeans with different ancestry and environmental influences. We observed a maximum difference of 7% in glycinin per unit of protein (gram/gram). Wolf et al. (1961) reported a 10% maximum difference in a glycinin per unit of protein.

Saio et al. (1969) have reported that glycinin has an important impact on gel formation in tofu. It has not been determined if the differences in glycinin content observed in this investigation would have a significant influence on the production of tofu. Future work with glycinin quantitation will involve correlation of these two factors. It is hoped that the information from this investigation, combined with future work, will ultimately make possible a more informed selection of soybean varieties for soy food production.

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# Phosphorylation of Casein and Lysozyme by Phosphorus Oxychloride

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Casein and lysozyme were phosphorylated by phosphorus oxychloride at pH 6–8 and 3–20 °C. Up to 7.4 and 6.2 mol of phosphate/mol of protein were covalently attached to casein and lysozyme, respectively. $^{31}P$ NMR spectral data and pH stability studies of the phosphate residues indicated that in phosphorylated casein, the phosphate was exclusively bound to hydroxyl oxygen as monophosphate and diphosphate. The phosphate linkages were stable at pH 2.0–8.5. In contrast, the majority of the phosphate in phosphorylated lysozyme appeared to be bound to nitrogen. In addition to mono- and diphosphate, triphosphate bonds were present. Gel electrophoresis in the presence of sodium dodecyl sulfate and urea indicated that protein cross-linking occurred during phosphorylation. Although there was a considerable decrease in the initial rates of both trypsin- and $\alpha$-chymotrypsin-catalyzed hydrolysis of phosphorylated casein, the extent of hydrolysis after 24 h was the same for control and phosphorylated casein. In the bioassay, *Tetrahymena thermophili* grew as well on the phosphorylated casein as on Hammarsten casein. Dispersions of phosphorylated casein had significantly higher viscosities than control casein. In contrast, the viscosity of lysozyme was not affected by phosphorylation. Both phosphorylated proteins adsorbed more moisture than the corresponding control proteins. The emulsifying capacity of phosphorylated casein was lower than that of control casein.

The feasibility of using alternative sources of proteins (e.g., trash fish, grain, microbes, and leaf) as food proteins is often limited due to their low biological value, undesirable organoleptic properties, toxic constituents, and poor functional properties. These problems may be overcome by physical or mechanical treatment or by microbial, enzymatic, or chemical modification. Modification of proteins by phosphorylation is examined in this paper as a means to improve functional properties.

Phosphorylation of proteins has been achieved by using a variety of chemicals: phosphorus oxychloride (Bechhold, 1901; Neuberg and Pollak, 1910; Neuberg and Oertel, 1914; Rimington, 1927; Heidelberger et al., 1941; Mayer and Heidelberger, 1946; Boursnell et al., 1948; Salák et al., 1965; Willmitzer and Wagner, 1975; Woo et al., 1982), phosphorus pentoxide dissolved in phosphoric acid (Ferrel et al., 1948; Dickson and Perkins, 1971; Rao et al., 1975), phosphoric acid with trichloroacetonitrile as a coupling agent (Ullman and Perlman, 1975; Yoshikawa et al., 1981), monophenyl phosphodichloride (Bourland et al., 1949), phosphoramidate (Müller et al., 1956; Rathlev and Rosenberg, 1956), diphosphoimidazole (Taborsky, 1958), and trisodium trimetaphosphate (Sung, 1982). Changes in functional properties (Heidelberger et al., 1941; Mayer and Heidelberger, 1946; Ferrel et al., 1948; Salák et al., 1965; Sung, 1982) and changes in in vitro digestibility (Neuberg and Oertel, 1914; Rimington, 1927; Taborsky, 1958; Sung, 1982) due to phosphorylation have been studied only occasionally.

According to previous studies, the phosphorus bound to proteins by chemical derivatization could be attached to the hydroxyl oxygen (Rimington, 1927; Ferrel et al.,

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