The fourth band and the greater mobility observed after deesterification of the water- and acid-soluble pectins could be explained via the hypothesis that the basic structure of pectins might involve linkages of different types and of different degrees of vulnerability and molecular configuration (Kertesz, 1951). Treatment with alkali may liberate sterically hindered esterified carboxyl groups which can only be deesterified after they are exposed thus increasing the net molecular charge and giving rise to changes in molecular size and shape.

## CONCLUSIONS

The study showed that tomato pectic substances extracted from alcohol-insoluble solids according to their solubilities in water, 0.2% ammonium oxalate, 0.05 *N* hydrochloric acid, and 0.05 *N* sodium hydroxide can be further fractionated by using the techniques of gel filtration and disc gel electrophoresis.

Gel filtration, based on the properties of Sephadex G-200, demonstrated that the water, 0.2% ammonium oxalate, 0.05 *N* hydrochloric acid, and 0.05 *N* sodium hydroxide soluble pectins are heterogenous in molecular size with a majority of the pectic substances having molecular weight of $2 \times 10^5$ or more. Small differences in the pectic substances of the two cultivars were detected by using gel filtration. These small differences were observed in each of the solubility fractions.

Disc gel electrophoresis indicated at least three distinct groups of pectic compounds possessing slightly different molecular charges, further confirming the heterogeneity of each pectic fraction. Electrophoretic patterns of the pectic fractions from the two varieties showed quantitative differences when the intensities of their colored bands were compared. Deesterification of the same pectic fractions showed differences between the number of component bands of the water-soluble pectins from both cultivars and of the hydrochloric acid soluble pectin from the Chico III variety.

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# Fractionation and Characterization of Major Reserve Proteins from Seeds of *Phaseolus vulgaris*

Keizo Ishino*[1] and María Luisa Ortega D.

The globulin fraction (75% of the total protein) of "negro mecentral" bean (*Phaseolus vulgaris*) consisted of four major components, $\alpha$, $\beta$, $\gamma$, and $\delta$, designated in order of decreasing electrophoretic mobility, which accounted for 50, 19, 10, and 12% of the globulin, respectively. The $\alpha$ component (14.55% of nitrogen) had a $s_{20,w}{}^{0.53\,\%} = 7.42$ S and an approximate molecular weight of 170,000. Also this component was a glycoprotein which contained 4.95% of carbohydrate (as mannose) and 1.19% of hexosamine (as glucosamine), and was deficient in the sulfur-containing amino acids. Although neither 8 *M* urea nor 0.2 *M* 2-mercaptoethanol treatments induced dissociation of the $\alpha$ component, alkali treatment (pH 12.5) caused dissociation into the subunits, although not completely even after 24 hr. The $\beta$ and $\gamma$ components dissociated immediately with alkali. At pH 4.1, 62% of the globulin remained soluble in acetate buffer with $\mu = 0.5$, and 57% with $\mu = 0.1$. The resolubility of acid precipitated protein was 65% in the phosphate buffer (pH 7.5), $\mu = 0.5$. The $\alpha$ and $\beta$ components seemed to form insoluble complexes during acidification.

In Latin American countries, many kinds of bean have been used for food from prehistoric times and, together with corn, still hold an important position in the diet of the people. At present, processing of beans is limited to boiling, but it is possible that new, more highly processed foodstuffs could be developed based on the reserve proteins in the seeds. Moreover, studies of bean protein comparable to those of soybean have not been done. Therefore, we think that it is important to investigate the nature of the reserve proteins of bean.

As constituents of seed protein (*Phaseolus vulgaris*), phaseolin, phaselin, and conphaseolin have been known for a long time (Osborne, 1894; Waterman *et al.*, 1923; Jones *et al.*, 1937–1938). It has been reported by Osborne (1894) that phaseolin accounts for about 20% of the seed dry weight, that is, 85% of crude total protein. The two other fractions, phaselin and conphaseolin, make up 2%

Laboratorio de Bioquímica, Rama de Botánica, Colegio de Postgraduados, Escuela Nacional de Agricultura, Chapingo, México.
[1] Present address: Asahimatsu-Koritofu Co., Ltd., Matsuo Iida-City, Nagano-Prefecture, Japan.

(Osborne, 1894) and 0.35–0.40% (Waterman *et al.*, 1923) of the dry seed. However, it has not been proved whether or not these protein fractions were homogeneous by modern experimental methods. Recently, glycoproteins from the bean seeds (*P. vulgaris*) have been investigated by Pusztai (1965a–c, 1966), Pusztai and Watt (1970), and Racusen and Foote (1971). Racusen and Foote isolated the major glycoprotein which accounted for 35% of the total seed protein and mentioned that it was the most abundant protein reserve. Also, McLeester *et al.* (1973) fractionated two globulin fractions which were called legumin and vicilin (Danielson, 1949) with an ascorbic acid–NaCl medium from beans (*P. vulgaris* and *Vicia faba*). But as mentioned in the report, use of these terms commonly for classification of globulins obtained from seeds of different species is not recommended because the individual globulins have not been characterized enough.

In the present report, in order to identify major components of the seed protein, the proteins were extracted under the least altered condition and then fractionated by means of gel filtration and polyacrylamide gel electrophoresis. Some characteristics of these proteins were studied.

## MATERIALS AND METHODS

**Materials.** The black bean, "negro mecentral" (*P. vulgaris*), harvested in the experimental field at Chapingo was used in this study. This bean is eaten in the central region of Mexico.

**Extraction of Seed Protein.** The seed coats were removed before milling, because they have a deep violet pigment that combines with proteins during salt extraction. The finely milled flour (less than 70 mesh) was defatted by *n*-hexane at 2°. The proteins were extracted by stirring the defatted flour in 0.5 *M* NaCl (solvent-to-meal ratio, 10:1) at pH 7.5 for 1 hr at 2°. Insoluble materials were removed by centrifugation at 10,000*g* for 30 min. The globulin and acid precipitated protein were prepared by dialysis or acidification at pH 4.1 by HCl from the salt extract.

**Determination of Solubility.** Solubilities of the globulin fraction and total salt-extractable proteins at different pH values were determined by the following procedure. The pH of a globulin solution suspended in a standard buffer (phosphate buffer pH 7.5, $\mu = 0.5$) or the pH of an aliquot of the crude salt extract was adjusted to the desired value by addition of HCl containing 0.5 *M* NaCl. After leaving overnight at 2°, the solution was centrifuged at 10,000*g* for 30 min. The protein concentration in the supernatant was determined spectrophotometrically at 280 nm on a Beckman D.U. spectrophotometer. Solubility was indicated by N.S.I. (nitrogen solubility index = soluble nitrogen/total nitrogen × 100).

**Polyacrylamide Gel Electrophoresis.** Polyacrylamide gel electrophoresis was carried out using 7.5% acrylamide and 0.25% *N,N'*-methylenebisacrylamide with a current of 4 mA per tube for 2 hr at 2° with Tris-glycine buffer (pH 8.3). The gel was stained with 1% Amido Black 10B and destained with 7% acetic acid. A densitometer scan was made with a Photovolt Densicord 542.

**Gel Filtration.** Gel filtration chromatography on a Sephadex G-200 column (2.5 × 96 cm) equilibrated with the standard buffer was performed to determine the composition of prepared protein fractions. The sample was eluted by the same buffer. Each 5-ml fraction was collected and analyzed at 260 and 280 nm.

**Ultracentrifugal Analysis.** Sedimentation velocity measurement was carried out using a Spinco Model E ultracentrifuge at 56,000 rpm at 20° in phosphate buffer (pH 4.9), $\mu = 0.5$.

**Amino Acid Analysis.** After dialysis against distilled water and lyophilization, the protein was hydrolyzed with 6 *N* HCl for 24 hr at 110° in a hydrolysis tube under high vacuum. The hydrolysate was analyzed by a ligand method on a Hitachi KLA-3B amino acid analyzer.

**Preparation of Phaseolin and Conphaseolin.** Phaseolin and conphaseolin were prepared according to the method of Waterman *et al.* (1923).

**Quantitative Determination of Carbohydrate, Hexosamine, and Phosphorus.** Carbohydrate content was estimated by the phenol–$H_2SO_4$ method (Hodge and Hofreiter, 1962) using D-mannose as a standard. Hexosamine content was determined by the method of Randle and Morgan (1955) following hydrolysis of the lyophilized protein according to the conditions of Racusen and Foote (1971). For the determination of phosphorus, the lyophilized globulin and acid-precipitated protein (0.5–1.0 g) were dry-ashed at 600° for 4 hr following dialysis against deionized water to eliminate noncombined phosphorus. The residue was dissolved in 0.2 *N* HCl, filtered though a paper, and made up to 25 ml. Phosphorus was determined by the Fiske and Subbarow method (1925). Nitrogen was determined by the micro-Kjeldahl method.

## RESULTS AND DISCUSSION

The dried, defatted flour contained 27.0% crude protein. This value was calculated on the basis of a conversion of 6.87 g of protein/g of nitrogen, since the globulin fraction, which accounted for most of the total protein (described later), contained 14.6% of nitrogen.

**Extraction of Proteins from Seeds.** Six extractions of the 0.5 *M* sodium chloride solution dissolved 88.4% of the total nitrogen from finely ground bean flour at pH 7.5. The extractability of protein from seeds by saline solution has been reported by others with varying results. For example, in studies on the navy bean (*P. vulgaris*), Powrie (1961) extracted 74.1–74.5% of the total meal nitrogen with 0.5 *M* sodium chloride and Evans and Kerr (1963) reported that 74–76% of the total nitrogen was extracted with 0.19–1.52 *M* sodium chloride solution. Also in the case of kidney bean (*P. vulgaris*), Smith *et al.* (1959) mentioned that 76.2% of the total nitrogen was extracted with 0.5 *M* sodium chloride. However, as pointed out by Djang *et al.* (1953) and Cacampang *et al.* (1966), particle size of the powdered sample, meal–solvent ratio, extraction times, and temperature of extraction are critical factors in determining yields by extraction, so these values would be changeable. The salt extract was fractionated into albumin and globulin fractions by dialysis against distilled water at 2° for 3 days. The obtained precipitate (globulin fraction) was 85% of the salt extractable proteins. Therefore, at least 75% of the total protein of this bean is in the globulin fraction.

A cold precipitated protein as found in peanut (Inoue *et al.*, 1970) and the 11S component of soybean protein (Eldrige and Wolf, 1967) could not be obtained from the salt extract even with low ionic strength ($\mu = 0.1$).

**Changes in Solubility.** In order to get acid precipitated protein from the salt extract, the solubility of the salt extractable proteins at different pH values was investigated. As shown in Figure 1, precipitation began near pH 5.5, reached its maximum at pH 4.1, and then decreased with further decreases in pH. The tendency for the globulin was virtually identical, suggesting that the globulin and acid precipitated protein have similar constituents. Amino acid compositions of both fractions were almost similar except for the sulfur-containing amino acids (Table I). However, at pH 4.1, 65% of the protein extracted by 0.5 *M* NaCl was still soluble. Furthermore, in acetate buffer, 62% of the globulin remained soluble with $\mu = 0.5$ and 57% with $\mu = 0.1$. Evans and Kerr (1963) found that only 30% of the protein extracted by 1.5% sodium chloride solution precipitated at pH 3.5–4.5, and 73% of the protein extracted by water was precipitated at pH 3.8. Recently Anderson *et al.* (1973) reported that the extractability of soybean protein increased by adding salt even near the isoelectric point (pH 4.5).

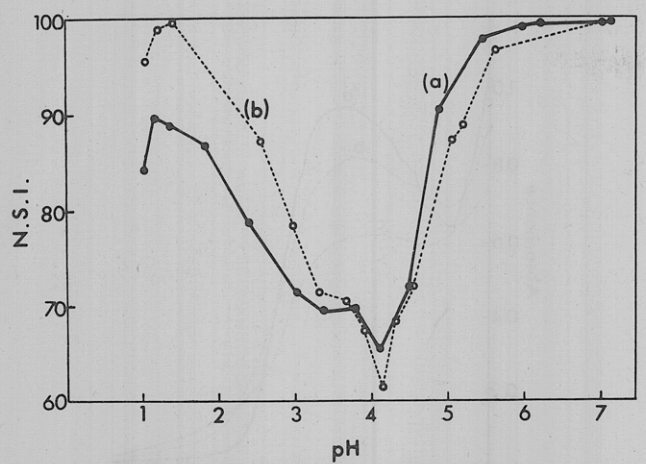


**Figure 1.** Changes of N.S.I. of the total salt-extractable proteins (a) and globulin fraction (b) depending on pH. Acidification was made by adding hydrochloric acid and allowing to stand 1 hr.

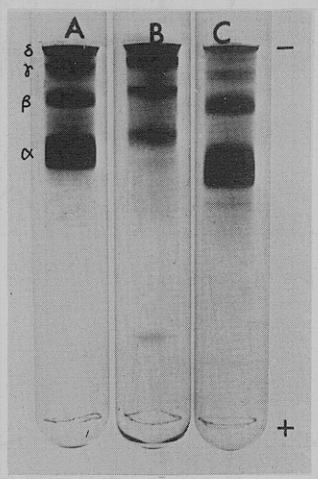


**Figure 2.** Polyacrylamide gel electrophoretograms of: (A) globulin fraction, (B) acid precipitated protein prepared at pH 4.1, and (C) globulin treated with 8 *M* urea for 1 hr. Migration went down from the top.

**Table I. Amino Acid Composition of the Total Seed, Globulin, Acid Precipitated Protein, and α Component[a]**

| Amino acids | Total seed | Globulin | Acid precipitated protein | α component | Glycoprotein II[b] |
|---|---|---|---|---|---|
| Lys | 4.33 | 5.58 | 5.79 | 5.31 | 4.70 |
| His | 2.06 | 2.33 | 2.17 | 2.31 | 2.09 |
| $NH_3$ | 8.90 | 12.00 | 10.21 | 11.58 | 11.85 |
| Arg | 4.07 | 3.55 | 3.98 | 3.81 | 3.51 |
| Asp | 8.87 | 9.40 | 8.54 | 10.09 | 11.86 |
| Thr | 4.80 | 3.41 | 4.58 | 3.95 | 3.64 |
| Ser | 6.37 | 6.36 | 5.54 | 6.78 | 8.26 |
| Glu | 14.88 | 13.30 | 12.99 | 12.88 | 13.76 |
| Pro | 6.80 | 4.32 | 5.39 | 4.08 | 3.20 |
| Gly | 6.66 | 5.41 | 6.38 | 5.30 | 4.99 |
| Ala | 5.49 | 5.60 | 6.14 | 5.36 | 4.50 |
| Cys | 0.42 | 0.40 | 0.87 | 0.12 | 0.28 |
| Val | 6.74 | 6.01 | 6.22 | 5.99 | 5.61 |
| Met | 1.11 | 0.98 | 1.94 | 0.71 | 0.57 |
| Ile | 5.15 | 5.20 | 5.05 | 5.11 | 5.36 |
| Leu | 7.70 | 8.58 | 7.28 | 8.28 | 8.70 |
| Tyr | 2.00 | 3.47 | 3.20 | 3.68 | 2.23 |
| Phe | 3.73 | 4.10 | 4.03 | 4.18 | 4.90 |

[a] Values are mole per cent of the protein. [b] Calculated from the data of Pusztai and Watt (1970).

**Polyacrylamide Gel Electrophoresis.** With polyacrylamide gel electrophoresis as shown in Figure 2, the globulin fraction separated into four major components, α, β, γ, and δ, designated in order of decreasing electrophoretic mobility. These components constituted about 50, 19, 10, and 12% of the globulin, respectively, as a result of densitometric analysis of the electrophoretic pattern of gel. As the α component accounted for 38% of the total seed protein and, furthermore, this amount coincided almost with that of the glycoprotein isolated by Racusen and Foote (1971), this component should be the major reserve protein. The banding pattern of globulin was quite similar to those of the total seed proteins of seven cultivars of *P. vulgaris* (Hall, 1970) and that of a red kidney bean (Juo and Stotzky, 1970). Hall mentioned too that the major band which corresponded to the electrophoretic band of the α component in our experiment was the major reserve protein in the mature seed.

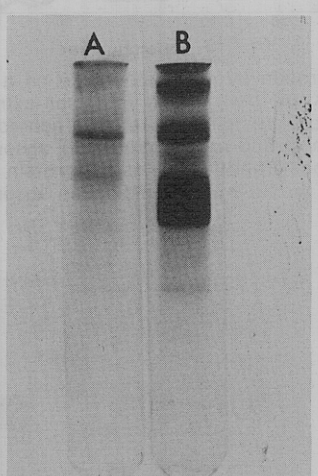


**Figure 3.** Polyacrylamide gel electrophoretograms of (A) conphaseolin and (B) phaseolin.

The acid precipitated protein was resolubilized in the standard buffer and then the insoluble fraction was removed by paper filtration. The electrophoretic pattern of the filtrate, that is, the soluble fraction of acid precipitated protein in the above buffer, was almost identical with that of the globulin fraction (Figure 2B). However, the amount of α and β components was considerably less than that found in the globulin.

Phaseolin and conphaseolin were demonstrated by electrophoresis not to be homogeneous fractions (Figure 3). Phaseolin contained all major components. Conphaseolin left for 24 hr at 2° after precipitation with ammonium sulfate was very insoluble in the standard buffer.

**Gel Filtration Chromatography with Sephadex G-200.** The total salt extract, globulin, and acid precipitated protein were examined by gel filtration chromatography on a Sephadex G-200 column. The elution patterns are shown in Figure 4. Gel filtration of the globulin yielded two large peaks and five small peaks. Disc electrophoresis demonstrated that peaks 1, 2, 3, and 4 corresponded to the δ, γ, β, and α components, respectively (Figure 5). Almost the same elution pattern was obtained from the total salt extract except for the low molecular weight fractions. The determination of protein content by the method of Lowry *et al.* (1951) revealed that peak 1 contained a smaller amount of protein than peak 4. Ultraviolet spec-

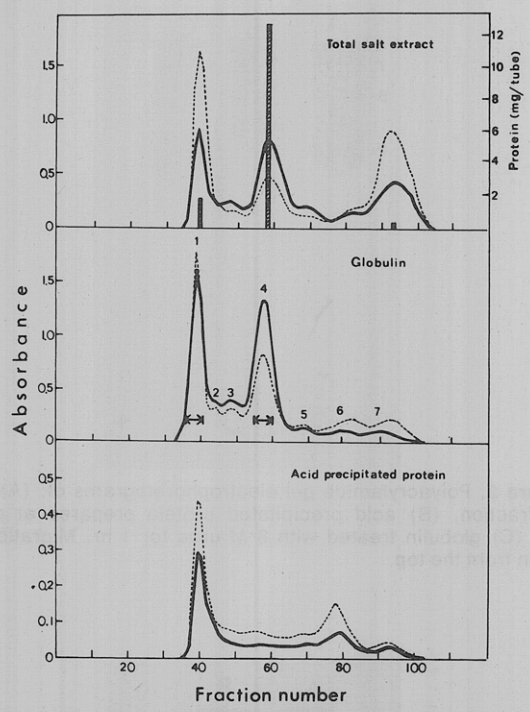


**Figure 4.** Separation by chromatography on a Sephadex G-200 column (2.5 × 96 cm) of the total salt-extractable proteins, globulin, and acid precipitated protein prepared at pH 4.1: (solid line) absorption at 280 nm; (dotted line) absorption at 260 nm. Histogram (upper graph) indicates the protein content (by Lowry's method) in the tube having maximum absorption of peaks 1, 4, and 7.

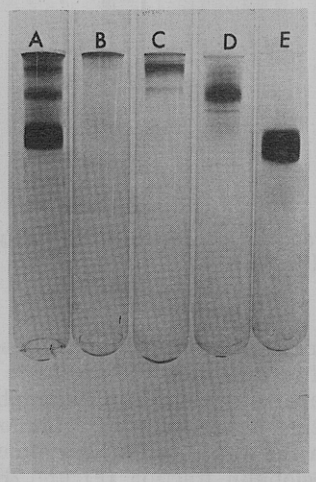


**Figure 5.** Polyacrylamide gel electrophoretograms of major components fractionated from the globulin fraction (Figure 4) by Sephadex G-200 gel filtration: (A) globulin fraction; (B) peak 1; (C) peak 2; (D) peak 3; (E) peak 4.

trophotometric analysis demonstrated contamination of peak 1 with nucleic acid (Morita and Yoshida, 1968; Obara and Kimura, 1967; Mohammad *et al.*, 1973). The maximum absorption of peak 1 was between 275 and 267 nm (Figure 6), and the extinction ratio, $E_{280nm}/E_{260nm}$, was less than 0.95 within the range indicated by a horizontal arrow in Figure 4. Also, as found with rice globulin (Morita and Yoshida, 1968), soybean (Koshiyama, 1969), and coconut globulin (Hagenmaier *et al.*, 1972), the eluent constituting this first peak was turbid. Morita and Yoshida (1968) suggested that the protein in this peak might be a denatured product of some other components. The maximum absorption of peak 4 shifted to 278 nm and the extinction ratio, $E_{280nm}/E_{260nm}$, was more than 1.55 within the range indicated by a horizontal arrow in Figure

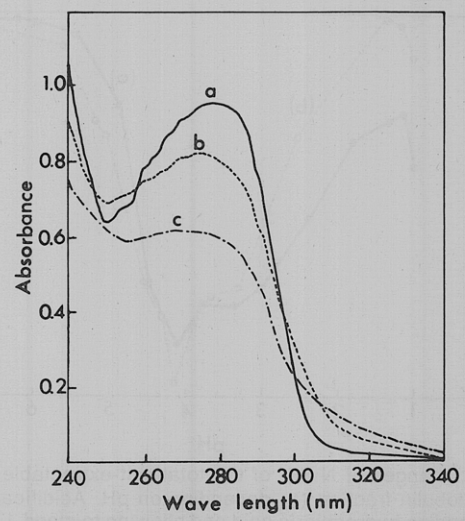


**Figure 6.** Ultraviolet absorption spectra of the globulin fraction, peaks 1 and 4. All spectra were measured in the standard buffer: (a) peak 4; (b) globulin fraction; (c) peak 1.

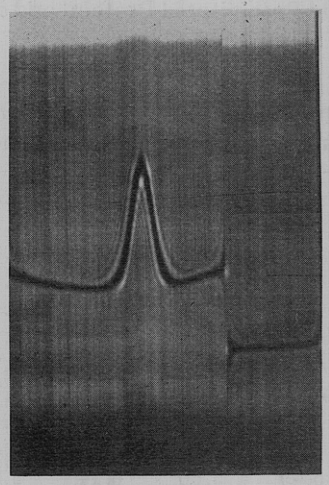

**Figure 7.** Ultracentrifugal pattern of a 0.53% solution of the $\alpha$ component. Photograph was taken at 36 min after reaching 56,000 rpm at a 70 bar angle.

4. This shows that of the total protein and nucleic acid contained in the fractions, 0.53% was nucleic acid (Dawson *et al.*, 1969). The nearly nucleic acid free condition of the fraction contrasts with peak 1 which contaminated more than 3.7% of nucleic acid (Dawson *et al.*, 1969). The $\alpha$ component contained 14.55% of nitrogen and also 4.95% of carbohydrate (as D-mannose) and 1.19% of hexosamine (as glucosamine), which shows that this component is a glycoprotein. The approximate molecular weight was estimated at 170,000 according to the method of Determann and Mitchel (1966). The small shoulders between 270 and 250 nm in peak 4 (Figure 6a) should be due to the absorption of phenylalanine (Wetlaufer, 1962; Koshiyama, 1968). The absorption curve of the globulin was intermediate between those of peaks 1 and 4.

The elution pattern of the soluble fraction of the acid precipitated protein described previously was quite different from those of the total extract and globulin. Peaks 3 and 4, which corresponded to the $\beta$ and $\alpha$ components, disappeared almost completely. The acid precipitated protein contained much more phosphorus ($3.4 \times 10^{-4}$ g/g of protein) than the globulin fraction ($0.5 \times 10^{-4}$ g/g of protein), and the resolubility of the former was 65% as compared with 100% of the latter. Considering these findings and the results of electrophoresis and gel filtration, the $\alpha$ and $\beta$ components might form insoluble protein–phytin

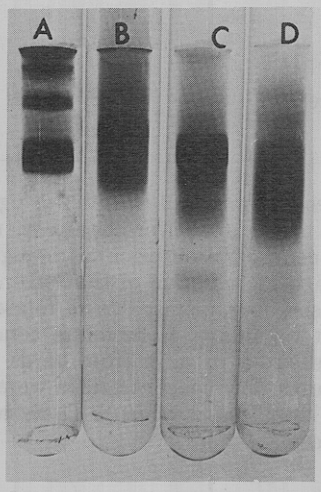


**Figure 8.** Polyacrylamide gel electrophoretograms of alkali treated (pH 12.5) globulin fraction and the $\alpha$ component. Alkali treated protein was dialyzed against the standard buffer before running: (A) undenatured globulin fraction; (B) globulin treated at pH 12.5 for 1 hr; (C) $\alpha$ component treated at pH 12.5 for 1 hr; (D) $\alpha$ component treated at pH 12.5 for 24 hr.

complexes (Smith and Rackis, 1957). Phytic acid has been found in the seed of *P. vulgaris* (Bourdillon, 1951). Also, as mentioned by Nash *et al.* (1971) and Anderson *et al.* (1973), protein modification caused during acidification may contribute partly to insolubilization to the acid precipitated protein.

**Ultracentrifugal Analysis.** The analysis was performed at pH 4.90 which seemed to be near the isoelectric point of the $\alpha$ component (Pusztai and Watt, 1970) to avoid an interaction between solvent and protein molecules by net charge. The $\alpha$ component showed a symmetrical peak and had a $s_{20,w}{}^{0.53\,\%} = 7.42$ S (Figure 7). This value is similar to that of the glycoprotein II isolated by Pusztai and Watt (1970). But McLeester *et al.* (1973) reported that the G-1 globulin (legumin fraction), which had an electrophoretic mobility (about the major band of their pattern) corresponding to that of the $\alpha$ component, had a sedimentation coefficient of 12.2 S. Under our conditions we could not detect oligomers of the $\alpha$ component as demonstrated by Pusztai and Watt (1970).

**Amino Acid Analysis.** The amino acid composition of the $\alpha$ component was similar to that of the glycoprotein II and was deficient in the sulfur-containing amino acids. The acid precipitated protein contained more cystine and methionine compared to the others (Table I).

From these results of physicochemical properties and chemical composition above, it is considered that the $\alpha$ component corresponds to the glycoprotein isolated by Pusztai and Watt (1970) and Racusen and Foote (1971).

**Quaternary Structure of Major Components.** To obtain some information about the quaternary structure of these components, the total globulin fraction was treated with 8 *M* urea. The treatment resulted in some decreases in the $\gamma$ and $\beta$ (the original electrophoretogram showed a noticeable relative decrease) components, and some minor components dissociated. However, the $\alpha$ component was not influenced at all electrophoretically. Addition of 2-mercaptoethanol (0.2 *M*) to the globulin dissolved in the standard buffer did not cause any changes in the major components. Although the $\gamma$ and $\beta$ components dissociated within 1 hr with alkali treatment at pH 12.5, the $\alpha$ component did not dissociate completely even after 24 hr (Figure 8B–D). Considering the cleavage reaction of the disulfide bond by alkali (Donovan, 1967) and the results of the addition of 2-mercaptoethanol, the subunits of these components, especially the $\alpha$ component, might be bound by disulfide bonds located in the interior of the molecule, which cannot contact with the solvent without unfolding of polypeptide chains. If this were the case, alkali could cause unfolding by, for instance, increasing an electrostatic repulsive force between polypeptide chains. Our results suggest that the $\alpha$ component has a very compact structure. This consideration is supported by the report of Jaffé and Hanning (1965) that the resistance of water- and saline-soluble protein fractions of a kidney bean against enzymatic hydrolysis would not be altered significantly after a 6 *M* urea treatment.

As described above, the $\alpha$ component reported here and the major protein fraction reported by other authors have similar electrophoretic mobilities (Juo and Stotzky, 1970; Hall, 1970; McLeester *et al.*, 1973) or physicochemical properties and chemical compositions (Pusztai and Watt, 1970; Racusen and Foote, 1971). Therefore, the $\alpha$ component seems to be the major constituent that is contained commonly in the seed of *P. vulgaris* as fixed genetic information like the 7S and 11S components of soybean. In the future, a specific name will be required instead of the traditional ones.

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