Physical and Hydrodynamic Studies on Salt-Soluble Proteins of Mungbean

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ABSTRACT

Proteins were isolated from salt-soluble mungbean (*Phaseolus aureus*), variety 54. Gel filtration showed the proteins to be homogeneous, having a molecular weight of 62,500, a Stokes' radius of 33 Å, and pI of 5.85. The presence of two fractions was suspected from the starch gel electrophoresis. Partial specific volume, intrinsic viscosity, kinematic viscosity, voluminosity, conformation, and polarity ratios were determined. These proteins were spherical, with a radius of 25 Å, and had a water shell 8 Å thick, corresponding to about three layers of water molecules. Their conformation was stable in the temperature range 35–85° C. About 36% of the polar residues were on the surface of the protein.

Soluble fractions of mungbean proteins were investigated by Mitchell (1948) and by Pant and Tulsani (1969). By ultracentrifugation, Kumar et al (1975) found only one fraction of protein, designated 5.8 S. By a similar technique, Vaintaub et al (1976) detected two fractions, designated 7 S and 11 S. Literature on physicochemical and hydrodynamic properties of salt-soluble proteins of mungbean is rather scanty. In the present study, the salt-soluble fraction of mungbean proteins was investigated.

MATERIALS AND METHODS

Materials

A 5% salt (NaCl) solution was used to extract the proteins from powdered mungbean variety 54, obtained from the Department of Plant Breeding, Punjab Agricultural University. The proteins were isolated essentially according to the procedure of Mitchell (1948). A powdered sample was defatted with petroleum ether (40–60° C). The albumins from the defatted sample were removed by repeated extractions with distilled water and the residue was successively extracted with 5% NaCl solution. The pooled extracts were centrifuged at 3,000 rpm in a refrigerated centrifuge and the supernatants filtered through Whatman No. 1 filter paper. Globulins were precipitated with acetone and repeatedly dispersed in water and reprecipitated with acetone until the sample was free of chloride ions. The isolate was then washed with ethyl ether, dried at room temperature, and kept in a refrigerator for further use.

Protein Solutions

The protein fraction (1 g, db) was dissolved in 5% NaCl solution and the volume made to 100 ml. The contents were filtered through Whatman No. 1 filter paper. This gave a clear solution which was diluted suitably and degassed before use in the subsequent experiments.

Protein Content

The micro-Kjeldahl method of McKenzie and Wallace (1954) was followed to determine the nitrogen content, which was converted into protein content with the factor 6.25.

Sugar Moiety

Sugars were determined using paper partition chromatography (Partridge 1948), with *n*-butanol/acetic acid/water (4:1:5) as the solvent system. To identify the sugars, the chromatograms were sprayed with benzidine/trichloroacetic acid solution (Bacon and Edelman 1951).

Isoelectric pH (pI)

This was determined according to the method of Pusztai and Watt (1970).

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Molecular Weight and Stokes' Radius

Sephadex G-100 was suspended in 5% NaCl solution, shaken for 6 hr, packed in the column, and washed for 48 hr with 0.025*M* phosphate buffer (pH 7) containing 5% NaCl. A test solution of 10 mg of protein in 0.05*M* phosphate buffer containing 5% NaCl was transferred to the top of the column and eluted with the same solvent system. Five milliliters of the eluent was used for determining the protein content by the method of Lowry et al (1951).

Sephadex column dimensions were 43 × 2.6 cm. Separations were conducted at 20° C.

Constants K_D and K_{av} were determined according to the method of Siegel and Monty (1966). The void volume (V_o) was determined using Dextran Blue 2,000 (Pharmacia). The elution volume (V_e) was experimentally determined (Table I). Standard curves were prepared using literature values for reference proteins. One curve plotted $(K_D)^{1/3}$ vs (mol wt)$^{1/2}$ and the other V_e/V_o vs log mol wt.

The Stokes' radii ('a'), taken from literature values, were plotted against $(-\log K_{av})^{1/2}$ and against $(K_D)^{1/3}$. Stokes radii for the mungbean proteins were obtained from these plots using the values determined for K_{av} and K_D.

Starch Gel Electrophoresis

Gels containing 15% hydrolyzed starch and 6*M* urea in formate buffer (pH 3.1) were prepared as described by Poulik (1966). A solution of 2.0 mg of protein per 0.3 ml of formate buffer (pH 3.1) containing 3*M* urea and a drop of mercaptoethanol was subjected to electrophoresis for 12 hr, using 8–10 mA current. Amido black dye solution was used to stain the protein bands; the excess dye was removed by repeated washings with 2% acetic acid solution.

Partial Specific Volume ($\bar{v}$)

This was determined in 5% NaCl solution pycnometrically by the method of Charlewood (1957). Tanford's equation (1955) was used for calculating the $\bar{v}$ of the proteins from the viscosity data. The values thus obtained were compared with the experimentally determined values.

Density

A pycnometer was used to determine the density (d) in duplicate.

TABLE I
Experimentally Determined Elution Volumes (V_E), K_D[a], and K_{av}[a] of Various Proteins on Sephadex G-100 in 0.025*M* Phosphate Buffer Containing 5% NaCl

Sample	Molecular Weight	V_e (ml)	K_D	K_{av}
Bovine serum albumin	69,000	96	0.1399	0.1316
Egg albumin	45,000	126	0.3499	0.3289
Pepsin	36,000	131	0.3849	0.3618
Trypsin	23,400	156	0.5598	0.5263
Cytochrome C	13,300	191	0.8047	0.7566
Dextran blue	···	76	···	···

[a] Constants determined by the method of Siegel and Monty (1966).

Protein and Salt Contents

Moisture content (A_1) of protein samples was determined by the AACC method (1972) and the salt content (A_2) by Mohr's method (Vogel 1962). From the density of the solution, the concentration of the protein in the solution was calculated as follows:

$$\text{Protein content g/ml} = (100 - [A_1 + A_2]) \frac{d}{100} \qquad (1)$$

Intrinsic and Kinematic Viscosities

A multi-gradient Ubbelohde viscometer was used to determine the values of intrinsic viscosity (η) and kinematic viscosity (η/d) in the temperature range of 35–85° C.

Voluminosity

This was determined in the temperature range of 35–85° C using Simha's equation (1940) and by Eiler's equation as modified by Dewan et al (1973). The latter equation was further modified by the authors to:

$$V_E = \lim_{C \to 0} \frac{(\eta_{rel})^{1/2} - 1}{C[1.35(\eta_{rel}) - 0.1]} \qquad (2)$$

where

V_E = voluminosity (Eiler's equation)

η_{rel} = relative viscosity, ie, t/t_o (obtained from viscometric data)

C = concentration

The bound water content (δ) of 5% NaCl-soluble frations was calculated, using the equation:

$$V_E \text{ or } V\eta = (\bar{v} + \delta \bar{v}_1) \qquad (3)$$

where

δ = grams of H_2O per gram of protein

$\bar{v}_1$ = partial specific volume of water (1 ml/g).

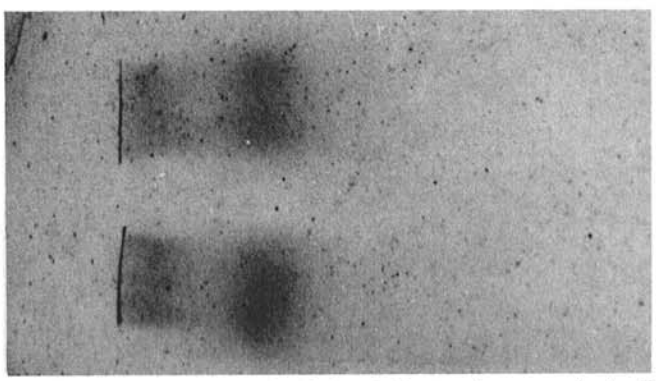

Fig. 1. Starch gel electrophoresis of salt-soluble mungbean proteins in pH 3.1 formate buffer.

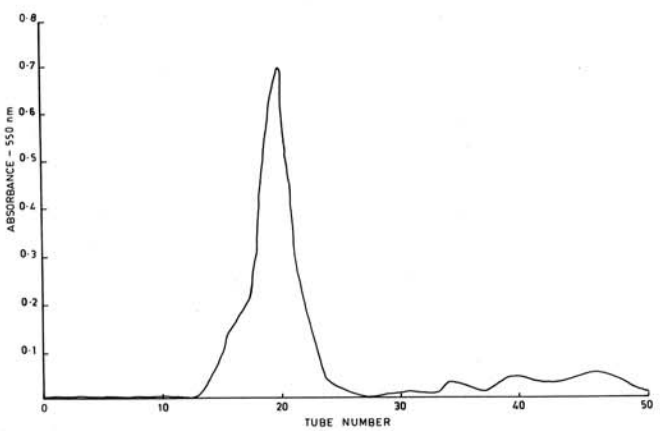

Fig. 2. Elution profile of salt-soluble mungbean proteins on Sephadex G-100 column.

Polarity Ratio

This was calculated by the method of Fisher (1964), using 33 Å as Stokes' radius of the 5% NaCl-soluble proteins. All data presented here are average values.

RESULTS AND DISCUSSION

The dried protein preparation was cream colored and remained free-flowing when kept for about a year at room temperature. The solubility of freshly separated protein in 5% NaCl solution was low and in water almost negligible. Its pI was 5.85. The sample had 82.5% protein content (db). Because sugar from the protein fraction could be released by mild acid hydrolysis, the sugars are probably linked via C-N bond to the protein (Marks et al 1963). Mannose was identified as the principal carbohydrate moiety in the protein preparation. Electrophoresis revealed the presence of two components with close mobilities. (Fig. 1).

The results of gel filtration (recovery 98.3%) showed that only a single fraction (Fig. 2) of protein, with a molecular weight of 62,500 and a Stokes' radius of 33 Å, was present.

The plots of $(\text{mol wt})^{1/2}$ vs $(K_D)^{1/3}$ and log mol wt vs V_e/V_o (Fig. 3) were linear. The respective molecular weights deduced from these plots were 62,500 and 62,370.

Values for Stokes' radii were 33.0 Å from the plot of 'a' vs $(-\log K_{av})^{1/2}$ and 33.2Å from the plot of 'a' vs $(K_D)^{1/3}$ (Fig. 3). The shapes of the plots, which exhibited a slight scattering of points (Fig. 4), were in fair agreement with those predicted by Porath (1962), Laurent and Killander (1964), and Squire (1964). The values of Stokes' radii used in the standard curves (Fig. 4) are those reported in literature, and these are not considered ideal for obtaining the standard plots (Brissac et al 1976, Burnett et al 1976).

From the data obtained by gel filtration, the relation between K_{av} and K_D was found to be:

$$K_{av} = 0.94\ K_D$$

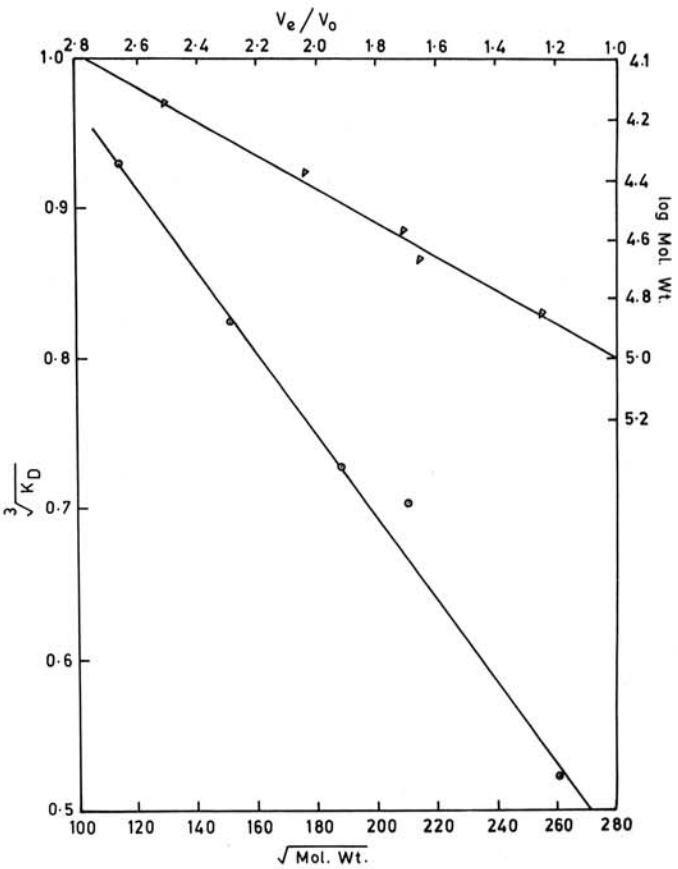

Fig. 3. Relationship between molecular weight and V_e/V_0 and K_D. ⊙ = $(K_D)^{1/3}$ vs $(\text{mol wt})^{1/2}$, △ = V_e/V_o vs log mol wt.

This compared with the value of $K_{av} = 0.96\ K_D$ previously reported by Siegel and Monty (1966).

Partial Specific Volumes

These values were in the range of 0.695–0.665 ml/g, with an average value of 0.680 ml/g, as the temperature increased from 35–85° C (Fig. 5). Using Tanford's equation (1955), a range of 0.623–0.657 ml/g, with an average of 0.643 ml/g, was obtained. The calculated values so obtained are only slightly lower than the values determined experimentally.

Below a protein concentration of 0.8% in 5% NaCl solution, the protein molecules appeared to have a high degree of conformational stability in the temperature range 35–85° C.

Bradbury et al (1965), who have used the plot of partial specific volumes vs temperature to locate the helix coil transitions in poly-γ-benzyl-L-glutamate and have reported that changes in partial specific volumes at different temperatures reflect associations and other experimental changes. Hvidt (1975) used volume changes in model system of proteins as evidence of hydrophobic interactions. Contrary to Portzehl (1950), who had reported that the isoionic polyelectrolytes the partial specific volumes were independent of concentration, we found that the partial specific volumes decreased with the increase in the concentration of the protein in the solution (Figs. 6 and 7). The thermodynamic equation indicates that in very dilute solutions Δ G is independent of the number of particles and remains zero at constant temperature.

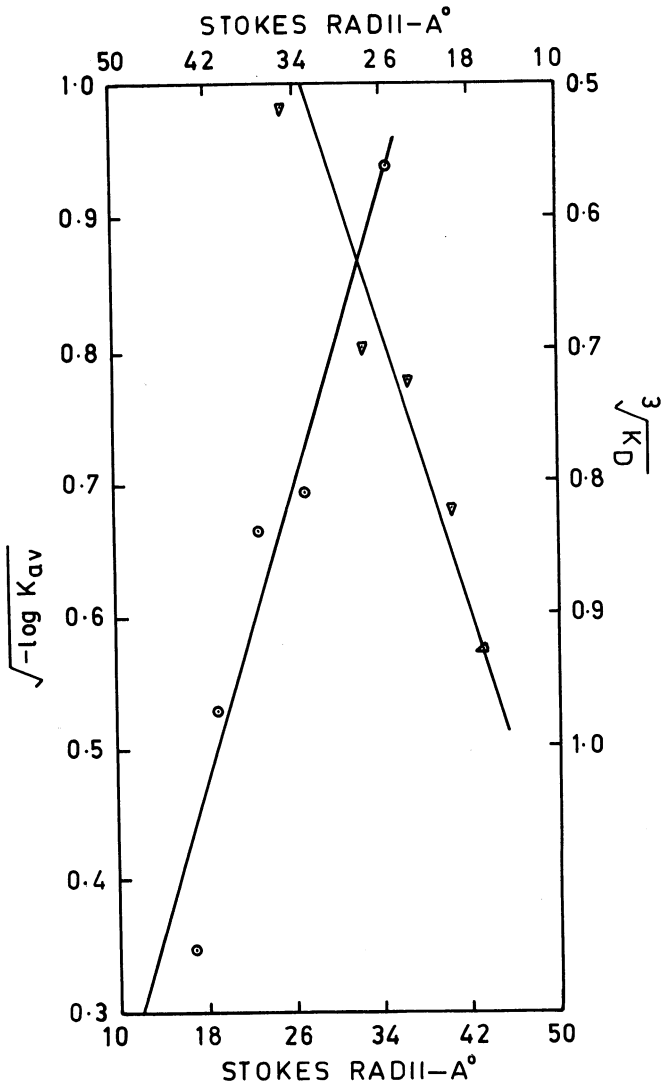

Fig. 4. Relationship between Stokes' radii 'a' and K_{av} and K_D of various standard proteins. ⊙ = $(-\log K_{av})^{1/2}$ vs 'a', Δ = $(K_D)^{1/3}$ vs 'a'.

$$dG = \sum_{i=1}^{N} \left(\frac{dG}{dni}\right)_{T,P,nj} \cdot dn_i \; i \neq j \qquad (4)$$

and

$$\Delta G = \Delta E + P\,\Delta V - T\,\Delta S \qquad (5)$$

where

G = free energy
T = temperature
P = pressure
V = volume
S = entropy
E = energy

Because Δ G is a function of Δ V and Δ S, a decrease in Δ V should cause a decrease in Δ S, indicating that the system is tending towards a more orderly state. This is found to be the case, as shown by the shape of the plots in Figs. 6 and 7. Stronger protein-protein

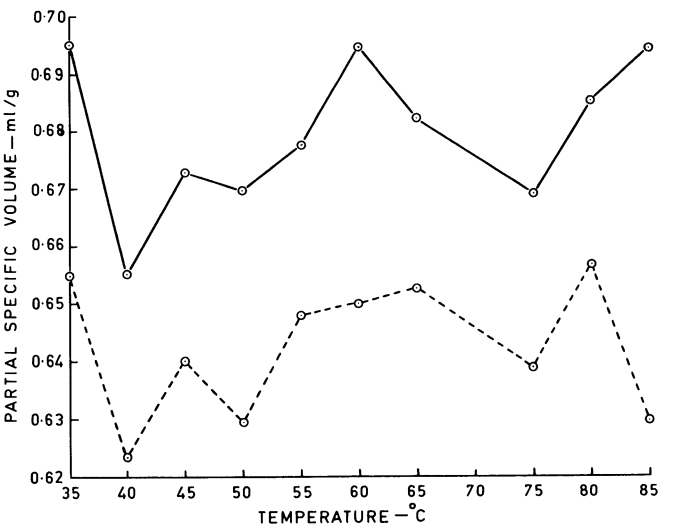

Fig. 5. Effect of temperature on partial specific volumes of salt-soluble mungbean proteins. —⊙— = experimental partial specific volumes, ---⊙--- = calculated partial specific volumes.

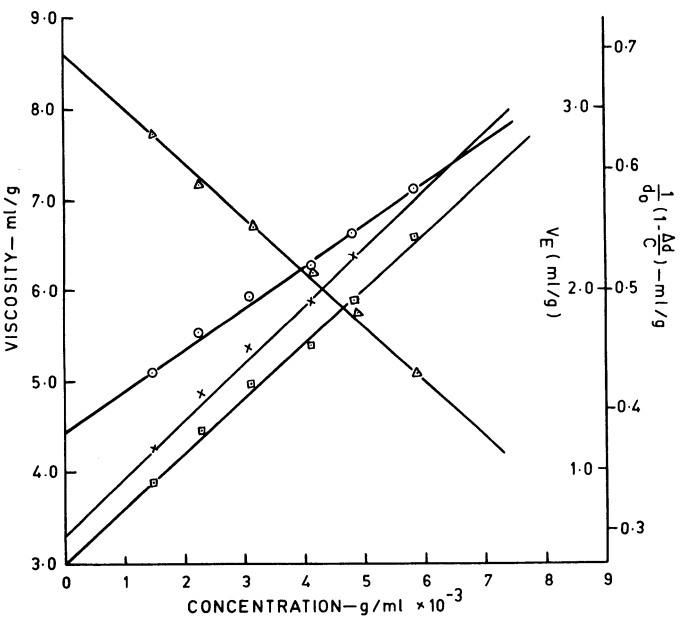

Fig. 6. Plots for determining partial specific volume (Δ), intrinsic (×) and kinematic (⊡) viscosities, and voluminosity (V_E, ⊙) at 35° C. Density = 1.02907.

interactions in the salt-soluble mungbean proteins are indicated. This is in keeping with the observed negligible solubility of these proteins in water and low solubility in 5% NaCl solution. At a protein concentration of 1% and above, a thick gel is formed; stronger protein-protein electrostatic interactions are therefore involved. Microheterogeneity probably also exists in such proteins.

Intrinsic and Kinematic Viscosities

The values for intrinsic and kinematic viscosities between 35 and 85° C were in the range of 3.17–4.62 ml/g (Table II), the average

TABLE II
Variation in Intrinsic and Kinematic Viscosities with Temperature

Temperature (° C)	Viscosity (ml/g)	
	Intrinsic	Kinematic
35	3.32	3.00
40	4.00	3.65
45	3.17	2.83
50	3.47	3.12
55	3.63	3.30
60	3.17	2.83
65	4.00	3.67
75	3.40	3.05
80	4.62	4.28
85	3.73	3.37

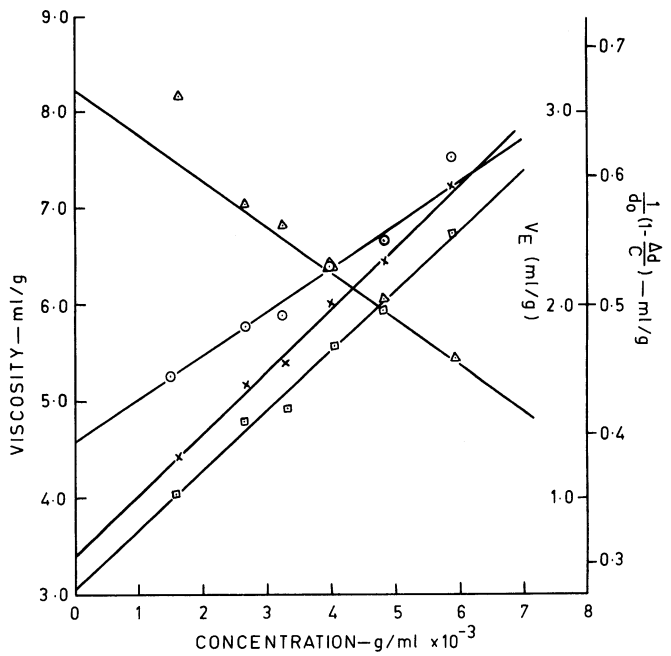

Fig. 7. Plots for determining partial specific volume (△), intrinsic (×) and kinematic (⊡) viscosities, and voluminosity (V_E, ⊙) at 75° C. Density = 1.010705.

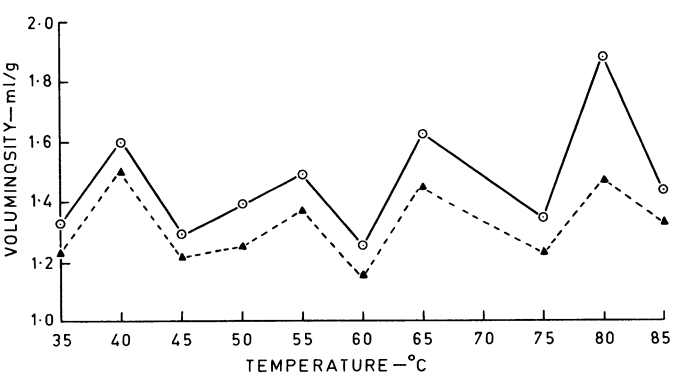

Fig. 8. Effect of temperature on the voluminosity (ml/g) of salt-soluble mungbean proteins. ⊙ = viscosity voluminosity, ▲ = Eiler's voluminosity.

being 3.65 ml/g. These values are slightly higher than those theoretically predicted by Einstein (1906 and 1911) for rigid spheres but low enough to be explained on the basis of random coiling of the protein molecules as suggested by Pusztai and Watt (1970).

The average V_η (Fig. 8) of the protein was 1.45 ml/g with a range of 1.27–1.85 ml/g. A value of 33 Å was obtained for the Stokes' radius, which agreed closely with the value obtained by the Sephadex gel filtration method. The plot of voluminosities (Fig. 8) indicated a number of sharp peaks oscillating about the mean value. These results support the conformational stability of the 5% NaCl soluble proteins of mungbean.

The validity of Eiler's equation as modified by Dewan et al (1973) could not be established from the voluminosity results.

The modified voluminosity equation eliminated the problem of solute-solute interactions. The value of V_E so obtained showed a good correlation with the V_η values obtained from viscometric studies (Figs. 6 and 7). The average value of V_E in the temperature range 35–85° C was 1.35 ml/g, with a range of 1.20–1.81 compared to the V_η value of 1.46 ml/g and range of 1.27–1.85 ml/g. Equation 2 dispensed with the need for the determination of densities in order to determine the voluminosities of the proteins. The values of δ obtained were 0.78 and 0.82 g/g of protein, based respectively on the experimentally determined and the calculated values of partial specific volumes. The difference between the two sets of values was only about 5%. The average value of the bound water content was, therefore, taken as 0.80 g/g of protein, which was somewhat different from the similarly calculated average value of 0.69 g/g of protein based on the V_E values. Kuntz and Kauzmann (1974) emphasized that the bound water values of protein varied with the method of determination.

On the basis of average partial specific volumes (Fig. 5), calculated by using Tanford's equation (1955), the average value of the radius of dry protein was accepted as 25 Å. Using the value of 33 Å, a polarity ratio (p) of 0.64 was obtained by Fisher's method (1964). The p values of a number of proteins reported by Fisher (1965) varied from 0.90–1.4. From the p values obtained in this study, we inferred that polar residues on the exterior of protein constituted about 36% of the volume and the remaining 64% was accounted for by the nonpolar core.

Some inconsistencies appeared in the conclusions, based respectively on viscosity, partial specific volumes, and pI data of protein in the 5% NaCl solutions. According to these values, the protein molecules must have a high proportion of amino acid residues carrying charge at the exterior of the protein to produce a viscoelastic system at low concentrations. On the other hand, the inference drawn above from the value of p indicated that only about one third of the residues were charged residues on the molecular surface. However, under certain conditions of environment or due to conformational changes, the value of p probably does not truly reflect the extent of the polar residues on the surface of the molecule when dissolved in salt solution. The salt soluble protein fractions of mungbean thus showed a high degree of stability over a wide temperature range (35–85° C). A reasonable assumption is that under such conditions the ion-pair interactions in the interior of the molecule, on the one hand, lead to the observed conformational stability and on the other hand, to a lowered value of p.

The difference between the Stokes' radius of the hydrated protein and of the dry protein was calculated to be 8 Å, which approximately represented the thickness of the water shell on the protein molecule. Taking the thickness of water molecule as 2.76 Å, as reported by Bernal and Fowler (1933), with approximately 1 Å as the length of the hydrogen bond, and on the basis of 25% of broken hydrogen bonds in water (Hindman 1966), the average thickness of water molecule was taken as 2.5 Å which gave an approximate value of three molecules of water in the shell.

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