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Registry No. Vitamin A, 68-26-8; α-carotene, 7488-99-5; β-carotene, 7235-40-7; 15-*cis*-β-carolene, 19361-58-1; γ-carotene, 472-93-5; lutein, 127-40-2.

Hydrogen Ion Equilibria of α-Globulin from *Sesamum indicum* L.

V. Prakash* and M. S. Narasinga Rao

Central Food Technological Research Institute, Mysore 570 013, India

The hydrogen ion equilibrium of the high molecular weight protein α-globulin of sesame seed has been investigated in the pH range 2.0–12.0. Analysis of the data gave pK_{int} values of 4.37, 7.47, and 9.60 for carboxyl, imidazole, and ε-amino groups, respectively. The number of residues containing these groups agreed well with the values obtained from the amino acid analysis. Tyrosyl phenolic groups had a pK_{int} of 9.41 as determined from spectrophotometric titration. The value of the electrostatic interaction parameter, w, varied with pH, and this is discussed in relation to the hydrodynamic properties of the protein.

The major protein of *Sesamum indicum* L. seed is α-globulin, which constitutes nearly 65% of the total protein (Nath et al., 1957; Prakash and Nandi, 1978). The protein has been isolated to homogeneity and characterized for its physicochemical properties. Its behavior in solutions of electrolytes, detergents, urea, guanidine hydrochloride, polyhydric alcohols, acid, or alkali has also been studied in detail (Prakash and Narasinga Rao, 1986; Prakash, 1985; Lakshmi et al., 1985a,b). The protein undergoes association–dissociation and denaturation in the presence of acid or alkali. Thus the binding of hydrogen ion by the protein has an effect on its stability and quaternary structure.

In this study we have investigated the hydrogen ion equilibrium of the protein by electrometric titration and have attempted to interpret the data in terms of the changes that the protein undergoes at acid/alkaline pH.

MATERIALS AND METHODS

(a) Isolation of α-Globulin. Sesame seeds were obtained from the National Seeds Corp. Ltd., Bangalore, India, and α-globulin was isolated by the method of Prakash and Nandi (1978). The protein was found to be homogeneous by analytical ultracentrifugation, polyacrylamide gel electrophoresis, and ion-exchange chromatography (Prakash and Nandi, 1978). It was dialyzed repeatedly against 0.1 M KCl solution.

(b) Protein Concentration. The concentration of α-globulin was determined by macro-Kjeldahl nitrogen estimation and using a factor of 6.25 to convert nitrogen to protein value (Prakash and Nandi, 1978). A calibration curve relating the milligrams of protein present in the solution with the ultraviolet absorbance of the protein at 280 nm was obtained; proper correction for scattering between 380 and 310 nm was applied. The absorption coefficient of $E_{280}^{1\%,1cm} = 10.0$ obtained thus was used for the routine determination of protein concentration. This was also confirmed by a gravimetric method.

(c) Amino Acid Analysis. The amino acid analysis of the protein was performed in an LKB amino acid analyzer by following the standard procedure of hydrolyzing the protein in 6 N HCl (Spackman et al., 1958).

(d) Hydrogen Ion Titration. The pH meter was standardized with standard buffers of pH 4.01, 6.50, and 9.05. All solutions were prepared with distilled and deionized water. The hydrogen ion titration of the protein was conducted at 28 ± 0.1 °C in 0.1 M KCl solution using extensively dialyzed protein solution. To 25 mL of 1% protein solution in 0.1 M KCl was added either standard HCl (0.1 M) or standard NaOH (0.1 M) in increments of 10 μL via an Agla micrometer syringe. After each addition, sufficient time was allowed for stabilization of pH and the pH value was noted. Nitrogen gas was flushed over the solution for measurements above pH 6.0. A blank titration was carried out with 25 mL of 0.1 M KCl in a fashion similar to that described above for the protein solution. From the difference in pH values, the number of hydrogen ions bound/dissociated per 250 000 g of protein was calculated (Prakash and Nandi, 1978; Prakash, 1985; Tanford, 1962).

(e) Spectrophotometric Titration. This was carried out by adjusting the pH of the protein solution in 0.1 M KCl with 1 M NaOH to different pH values and measuring the absorbance of the solution at 295 nm in a Perkin-Elmer 124 double-beam spectrophotometer (Tanford, 1962; Wetlaufer, 1962). Nitrogen was used to flush the solutions. The pH of the solution was measured both before and after absorbance measure-

* Address for correspondence: Biophysical Chemistry Unit, Food Chemistry Department, CFTRI, Mysore 570 013, India.

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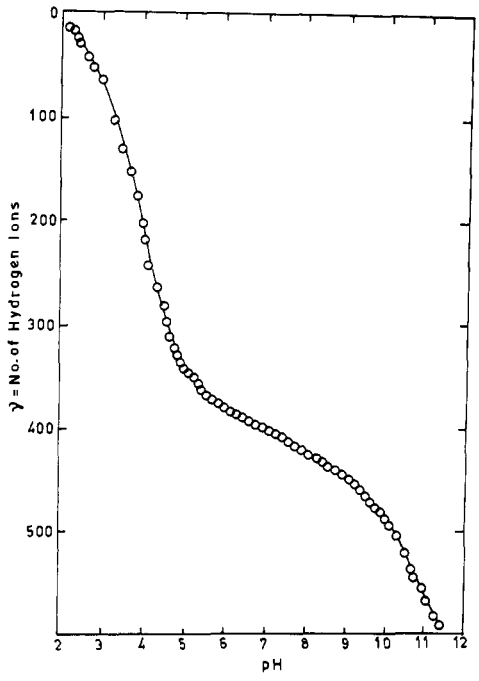

Figure 1. Hydrogen ion titration curve of α-globulin at an ionic strength of 0.1 M at 28 ± 0.1 °C: (O) experimental data points; (—) calculated curve.

ments. The measurements were made in an air-conditioned room at an ambient temperature of 28 °C.

(f) Partial Specific Volume. The partial specific volume $\bar{V}$ of the protein at different pH values was measured by following the procedure of Prakash (1982). Protein solutions were prepared by adding buffers of different pH to preweighed samples. The concentration of the protein was confirmed by gravimetric analysis and absorption at 280 nm. The density of the solutions was determined at 20 ± 0.1 °C with an Anton Paar DMA 55 density meter. The apparent partial specific volume $\bar{V}$ of the protein was calculated from the density of the protein.

RESULTS AND DISCUSSION

Potentiometric Titrations. The hydrogen ion titration curve of α-globulin determined in 0.1 M KCl solution is shown in Figure 1. Titration commenced at pH 7.0 and was carried out till pH 2.0 or 12.0 on either side of this pH. The protein was soluble over the total pH range except between pH 4.5 and 2.8. Precipitation of the protein occurred at pH 4.5, and this redissolved when the pH was below 2.8. Even in the pH range where precipitation occurred, the pH measurements were reproducible.

At the acid end point (pH 2) a maximum of 387 hydrogen ions were bound. With this as the reference point, the number of hydrogen ions dissociated (r) was estimated as a function of pH. Figure 1 is such a plot of r vs pH. The curve can be divided into three regions, acidic (pH 2–6), neutral (pH 6–8), and alkaline (above pH 9).

The analysis of the titration curve was performed by the method developed by Linderstorm and Lang as described by Tanford (1962) using the relation

$$\mathrm{pH} - \log \frac{r_i}{n - r_i} = \mathrm{p}K_{\mathrm{int}}^{(i)} - 0.868wZ \qquad (1)$$

where r_i is the number of titratable groups i dissociated whose intrinsic dissociation constant is $\mathrm{p}K_{\mathrm{int}}^{(i)}$, n is the total number of groups of type i, z is the net charge on the protein, and w is the electrostatic interaction factor. A plot of pH − log $[r_i/(n - r_i)]$ vs Z yields $\mathrm{p}K_{\mathrm{int}}$ as a intercept at $Z = 0$ and $-0.868w$ as the slope.

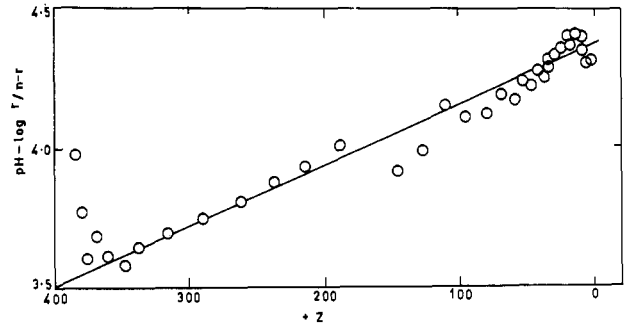

Figure 2. Plot of pH − log $[r/(n - r)]$ vs Z for titration of side-chain carboxyl groups.

Table I. Titratable Groups[a] and Their Characteristics in α-Globulin at 28 ± 0.1 °C and 0.1 M Ionic Strength

group	n from titration	n from amino acid analysis	$\mathrm{p}K_{\mathrm{int}}$	w
carboxyl	387 ± 10	370 ± 20	4.37	0.0025
imidazole	45 ± 2	45 ± 2	7.47	0.0096
ε-amino	50 ± 3	50 ± 3	9.60	0.0040
phenolic	55 ± 2	55 ± 3	9.41	0.0103

[a] The number of groups is calculated for 250 000 g of protein.

On the basis of eq 1, we have estimated a total of 387 groups titrated in the acidic region, 45 groups in the neutral region, and 105 groups in the alkaline region up to pH 11.9.

(a) pH 2–6. In the region pH 2–6, carboxyl groups dissociate. From the amino acid composition of α-globulin the total number of aspartic and glutamic acid residues, after correction for the number of amide groups, is 370 ± 20. This error is principally due to the uncertainty in the estimation of amide ammonia in an automatic amino acid analyzer. Calculations were made by using the value of n from 370 to 390. The net charge on the protein Z was taken as equal to the number of hydrogen ions bound. Thus the binding of other ions such as K^+ has not been taken into consideration. Preliminary experiments using a K^+-sensitive electrode did not indicate any significant binding of the ion compared to H^+. A plot of pH − log $[r/(n - r)]$ vs Z was made (Figure 2). The experimental data points were fitted with a linear regression line, giving an intercept at $Z = 0$ of $\mathrm{p}K_{\mathrm{int}} = 4.37$ and a slope of 0.0022. With these values of $\mathrm{p}K_{\mathrm{int}}$, $0.868w$, and n, r, the number of hydrogen ions dissociated, was calculated from eq 1. The calculated line is shown as a solid line in Figure 2. Excellent agreement was observed between the experimental data and calculated data with $n = 387$. This agreed well with the amino acid composition data. The $\mathrm{p}K_{\mathrm{int}}$ value of 4.37 for the side-chain carboxyl groups was well within the range of values of 4.0–4.8 for many proteins as also for a normal carboxyl group as reported by Tanford (1962). In the case of groundnut and soybean proteins, values of 4.96 and 4.30 have been reported for conarachin and glycinin, respectively (Shetty and Narasinga Rao, 1977; Catsimpoolas et al., 1971). Thus the carboxyl dissociation of α-globulin did not exhibit any abnormal features (Table I). The titratable carboxyl groups also include α-carboxyl groups. Since α-carboxyl groups form a very small portion of the total groups titrated, their $\mathrm{p}K_{\mathrm{int}}$ values cannot be usually derived from experimental data (Tanford, 1962).

(b) pH 6–8. The dissociation of carboxyl groups extends to the neutral region. In this region imidazole groups also dissociate hydrogen ions. Hence from the experimental r values, r due to carboxyl dissociation was

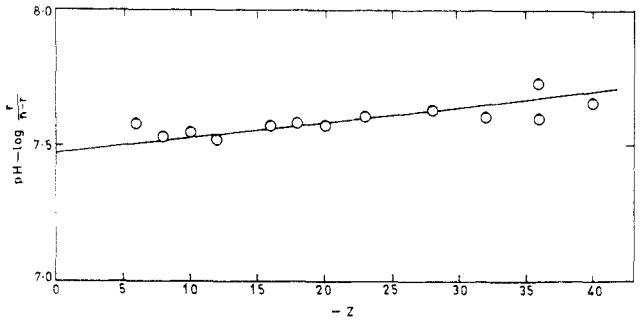

Figure 3. Plot of pH − log $[r/(n - r)]$ vs Z for titration of imidazole groups.

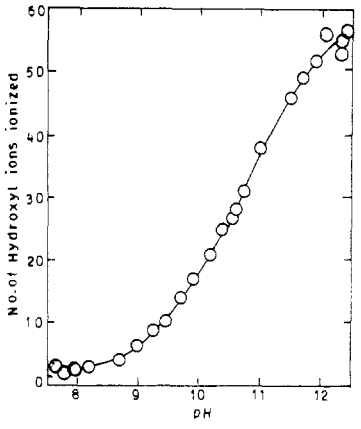

Figure 4. Plot of hydroxyl ions ionized as a function of pH as determined spectrophotometrically.

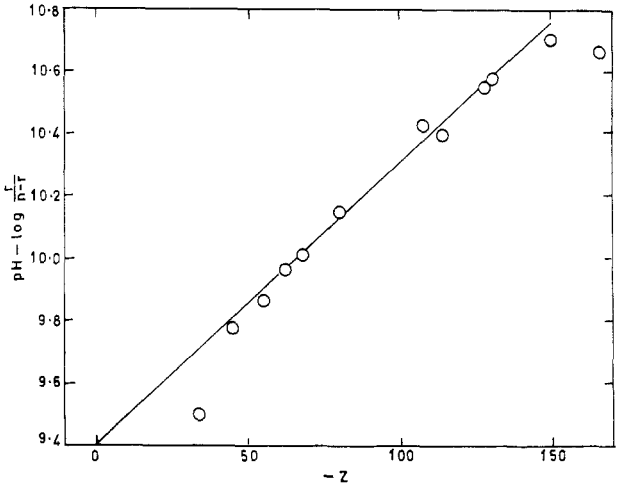

Figure 5. Plot of pH − log $[r/(n - r)]$ vs Z for titration of tyrosyl groups.

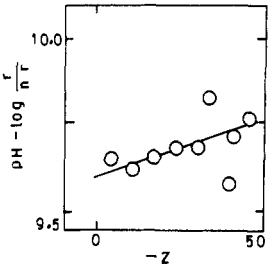

Figure 6. Plot of pH − log $[r/(n - r)]$ vs Z for titration of amino groups.

deducted, and the values due to imidazole dissociation only were obtained. With those values and $n = 45$ for imidazole, a plot of pH − log $[r/(n - r)]$ vs Z was made (Figure 3). Values of $pK_{int} = 7.47$ and $0.868w = 0.00855$ were obtained (Table I). For a normal imidazole group $pK_{int} = 7.0$ (Tanford, 1962). The value obtained by us is slightly higher, possibly indicating that the imidazole groups are in a more hydrophobic environment. The protein has a hydrophobicity index of 872 cal/residue (Prakash, 1985).

(c) Above pH 8.0. Spectrophotometric Titration. In the region above pH 8.0 both ϵ-amino groups of lysine residues and phenolic residues of tyrosine dissociate (Figure 4). The tyrosyl dissociation was followed independently by spectrophotometric titration by measuring the absorbance at 295 nm as a function of pH. From the change in molar extinction value upon increasing the pH from neutral to alkaline conditions and with a value of 2300 for the difference in molar extinction coefficient of ionized tyrosine, a value of 55 tyrosine residues per mol of protein was obtained. Analysis of the titration data with the Linderstrom–Lang equation with $n = 55$ gave a value of $pK_{int} = 9.41$ and $0.868w = 0.009$ for the tyrosyl group (Figure 5, Table I). This value appears to be well within the range of pK_{int} for normal phenolic groups (Tanford, 1962). This value is significantly different from the values of 10.5 and 11.3 obtained for other seed proteins like conarachin (from groundnut) and glycinin (from soybean), respectively (Shetty and Narasinga Rao, 1977; Catsimpoolas et al., 1971).

From the electrometric titration curve in the region above pH 8.0 the number of lysine groups titrated was obtained by subtracting the dissociated tyrosyl groups obtained from the spectrophotometric titration curve and the data analyzed as before with eq 1. $pK_{int} = 9.60$ and $0.868w = 0.0035$ were obtained (Figure 6, Table I). When $n = 50$ was used, the pK_{int} was within the range reported for a normal lysyl group (Tanford, 1962), and the n value agreed with the amino acid data (Table I). However, the value of w was higher than that obtained in the acid and neutral range of titration of α-globulin.

With the values of n, pK_{int}, and w for carboxyl, imidazole, and amino groups given in Table I, the titration curve was calculated from pH 2.0 to 11.9. The calculated titration curve agreed fairly well with the experimental data (Figure 1).

From Table I, it is apparent that the value of w steadily increases from acid pH to neutral pH, from a value of 0.003 to 0.006, and in the alkaline pH range (pH 9–10.5), there appear to be two values, 0.004 and 0.009. This may be related to the sensitivity of α-globulin in the pH range 9–10 to dissociation and denaturation, which would alter the w value. Now, if one were to calculate w from the equation (Tanford, 1962)

$$w = \frac{\epsilon^2}{DRKT}\left(1 - \frac{kR}{1 + ka}\right) \qquad (2)$$

where w is the electrostatic interaction parameter, ϵ is the unit of electron charge, D is the dielectric constant of the solvent, K is Boltzmann's constant, T is the absolute temperature, k is the Debye–Hückel parameter, and a is the radial distance of closest approach to the center of the sphere to the center of the average ion of the salt that is being used to create the ionic strength, one gets a value of 0.07 for α-globulin, which is considerably higher value than the experimental value (Table I). However, according to Tanford (1962), in view of the uncertainty in the calculation of R (eq 2) and Z (eq 1) and the approximate nature of the theory, the experimentally determined w values are generally one-third the value predicted by eq 2. The experimental values obtained are much less than the calculated value (Table I). Since α-

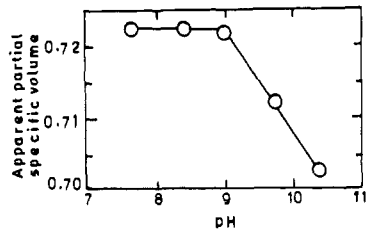

Figure 7. Effect of increasing pH on the apparent partial specific volume of α-globulin.

globulin has been shown to undergo association–dissociation as well as denaturation at acidic and alkaline pH, the large variation in w might be attributed to this (Prakash, 1976; Prakash and Nandi, 1977a,b; Prakash et al., 1980). Also α-globulin has been shown to reassociate at extreme acid pH (Prakash, 1976; Prakash and Nandi, 1977b). In the alkaline region of pH 9.0–11.0, the protein has a transition in its denaturation profile (Prakash and Nandi, 1977b). Even small changes in the pH can result in a large change in the size of the molecule and hence a sharp change in w value. This also has a bearing on the swelling of the α-globulin molecule both at acid and alkaline pH (Prakash, 1985; Prakash and Nandi, 1977b).

In order to obtain these data the partial specific volume of α-globulin was determined at different pH values between pH 7.5 and 10.5. The results are shown in Figure 7. From the figure it is apparent that the partial specific volume of the protein is 0.725 ± 0.002 in 0.02 M phosphate buffer pH 7.5 containing 0.1 M KCl. This is in accordance with the value obtained earlier (Prakash, 1982). Above pH 9.0, the value sharply decreases to nearly 0.700 ± 0.002 at pH 10.5. There appears to be change in the $\bar{V}$ of the protein between pH 7–9 and pH 9–10.5 corresponding to nearly the imidazole region and the tyrosyl region, respectively. Further, if one calculates the ΔV at pH 7 and pH 10.5, one gets a difference in value of −4000 ± 1000 mL/mol of α-globulin. This along with conformational change (Prakash et al., 1980) is sufficiently large to accommodate the observed change in w in the imidazole group and tyrosyl group titration range and may possibly explain these large changes of w values.

There is a special difficulty in the interpretation of titration data of multimeric seed proteins. These are of two kinds: (1) high amide content and (2) dissociation of the protein at acid and alkaline pH. Table I shows that there is considerable uncertainty in the estimation of aspartic and glutamic acids by amino acid analysis. This is due to uncertainty in the estimation of amide ammonia in an automatic amino acid analyzer. It is reported that some ammonia is liberated due to the destruction of serine and threonine also (Spahr and Edsall, 1964). This is likely to contribute to higher amide ammonia and a decrease in the estimated number of aspartic and glutamic acid residues. Also seed proteins have a high content of amide nitrogen (Pernollett and Mosse, 1983), which also leads to uncertainty in the estimation of aspartic acid and glutamic acid residues. The sharp changes in the w values as indicated in Table I show that the molecule does change in its electrostatic interaction parameter in a narrow range of pH. Such changes could be thought of as due to the varying properties of the subunits, each of which could behave differently in a narrow range of pH.

It is rather difficult to have α-globulin in either dissociated or aggregated or denatured form *only*. When it is dissolved in a solvent such as 6 M guanidinium hydrochloride solution, dissociation and denaturation occur simultaneously (Prakash and Nandi, 1977b). Although it would be nice to have the titration curve of the only dissociated or denatured α-globulin and compare it with that of the protein in the native form, experimentally it would be difficult to do so. Further, changes in pH of the protein solution even in the absence of a dissociating/denaturing reagent cause dissociation/denaturation of the molecule.

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