Effect of Chemical Modification of Sunflower 11S Protein on the Binding of Chlorogenic Acid

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The binding of chlorogenic acid by sunflower 11S protein and succinylated and *N*-ethylmaleimide-treated protein was measured at pH 4.0 in 0.1 M acetate buffer. Succinylation reduced binding, whereas *N*-ethylmaleimide treatment did not. Analysis of the binding data showed that succinylation reduced the number of binding sites without affecting the binding affinity. *N*-Ethylmaleimide treatment reduced neither the number of binding sites nor the binding affinity. Succinylation dissociated the 11S protein, whereas *N*-ethylmaleimide treatment did not. The secondary structure of *N*-ethylmaleimide-treated protein was different from that of the unmodified protein.

INTRODUCTION

The 11S protein, the major fraction of sunflower (*Helianthus annuus*) seed proteins, is a high molecular weight protein; its molecular weight is 350 000. It consists of 10 nonidentical subunits (Rahma and Narasinga Rao, 1981). It is free from phosphorus impurities. A method has been developed to isolate polyphenol-free 11S protein from defatted sunflower flour (Shamanthaka Sastry and Narasinga Rao, 1990a), and the interaction of chlorogenic acid (CGA) with the protein has been reported (Shamanthaka Sastry and Narasinga Rao, 1990b). The results presented in the previous paper suggested that the binding of CGA by the 11S protein did not involve any ionic linkages and possibly only hydrogen bonding was involved. Studies with other proteins have suggested that the phenolic groups of CGA might possibly combine with the ϵ-amino groups of the lysine residues (Sosulski, 1979). Similarly SH groups of the protein are reported to be highly susceptible to oxidation by quinones (Vithayathil and Gupta, 1981). Therefore, the ϵ-amino groups and SH groups of the sunflower 11S protein were blocked by succinylation and *N*-ethylmaleimide (NEMI), respectively, and the interaction of CGA with the modified proteins was studied.

MATERIALS AND METHODS

Materials. Sunflower seed of EC-68414 variety was obtained from the Karnataka Agro Industries Corp., Mysore. Succinic anhydride, *N*-ethylmaleimide, and chlorogenic acid were purchased from Sigma Chemical Co.

Protein Isolation. Polyphenol-free 11S protein was prepared from defatted sunflower seeds by the method of Shamanthaka Sastry and Narasinga Rao (1990a). Homogeneity of this preparation was checked both by electrophoresis and by ultracentrifugation.

Succinylation. Succinylation of the 11S protein was carried out at room temperature (~26 °C) between pH 7.0 and 8.0 with succinic anhydride according to the method of Hass (1964). Two grams of succinic anhydride was added in small increments to 15 mL of 1–2% protein solution in phosphate buffer of pH 8.0. The solution was kept stirring. The pH of the solution was maintained at 8.0 by the addition of 2 N NaOH. After the complete addition of succinic anhydride, the reaction mixture was left for 2 h with constant stirring. The solution was then dialyzed against the buffer at room temperature to remove excess succinic anhydride.

Estimation of the Degree of Succinylation. The extent of succinylation was measured by determining the "available lysine" content of the protein by Carpenter's procedure (1960).

Sulfhydryl Group Modification. To 10 mL of 1% protein solution in phosphate buffer of pH 7.6, 7 mL of β-mercaptoethanol and 12.5 mg of NEMI were added in sequence and incubated at room temperature for 1 h (Gregory, 1955). At the end of this time, the solution was dialyzed against 1M NaCl solution or the desired buffer. The extent of interaction was determined by the method of Beveridge et al. (1974).

Equilibrium Dialysis. Two-milliliter aliquots of 1% protein solution in 0.1 M acetate buffer of pH 4.0 were dialyzed in Visking sausage dialysis tubing at 30 °C against 5 mL of buffer solution containing varying amounts of CGA. Corresponding "blanks" containing only the buffer solution were also run. At the end of the equilibrium period (40 h) the concentration of CGA in the solution outside the dialysis bag was estimated. From the difference in CGA concentration of the blank and the "experimental", the ν value, the number of CGA molecules bound by 350 000 g of protein, was calculated.

Protein Concentration. The absorption coefficient of 1% protein solution at 280 nm, $E^{1\%}_{1cm}$, was found to be 8.2. Protein concentration was routinely estimated by absorbance measurement at 280 nm.

Concentration of CGA. The concentration of CGA in water and buffer solutions was determined by measuring the absorbance at 324 nm and using a molar extinction coefficient of 1.85×10^4 (Barnes et al., 1950).

Ultracentrifugation. The experiments were done at room temperature in a Spinco Model E analytical ultracentrifuge fitted with phase plate Schlieren optics and rotor temperature indicator and control (RTTC) unit. One percent protein solution was centrifuged at 59 780 rpm. Photographs of the sedimentation velocity patterns were taken at different intervels of centrifugation. From the enlarged tracings of the patterns the proportions of the various components were estimated by area measurements.

Fluorescence Spectrum. Perkin-Elmer spectrofluorometer Model 203 was used. The emission spectrum was recorded in the range 300–400 nm after excitation at 280 nm. Protein concentration of 0.1 absorbance at 280 nm was used.

RESULTS AND DISCUSSION

Succinylation. In Figure 1 the degree of succinylation is given as a function of the ratio of succinic anhydride to protein (grams per gram). Succinylation increased with increase in the ratio of succinic anhydride. At the ratio of 1, nearly 80% of the lysine groups had been succinylated.

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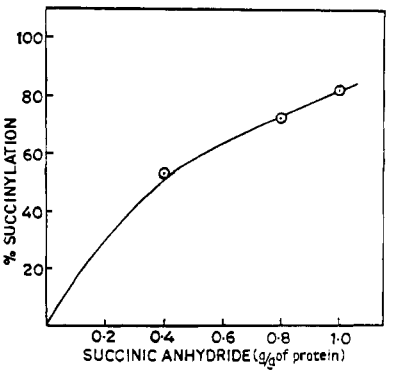

Figure 1. Degree of succinylation of sunflower 11S protein as a function of ratio of succinic anhydride to protein.

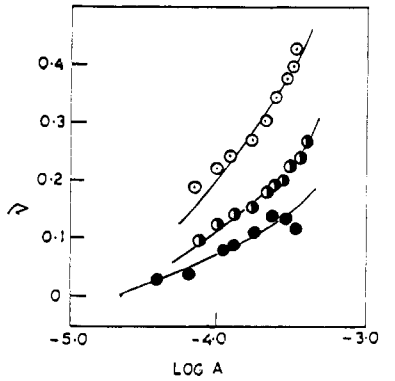

Figure 2. Binding of CGA by succinylated 11S protein (0.1 M acetate buffer, pH 4.0; 30 °C). (⊙) 53% succinylation; (◑) 71% succinylation; (●) 80% succinylation. (—) Calculated curve.

Effect of Succinylation on the Binding of CGA. The 11S protein has 59 lysine residues per 350 000 g. The available lysine content of the protein was 2.65 g/16 g of N. At 53% succinylation the available lysine content was 1.25 g/16 g of N, at 71%, 0.77 g/16 g of N, and at 80%, 0.53 g/16 g of N.

By use of the protein succinylated to 53%, 71%, and 80% binding studies were carried out at pH 4.0 (0.1 M acetate buffer). The binding data are presented in Figure 2 as a plot of ν as log A, where A is the equilibrium concentration of CGA. Succinylation of the protein decreased the binding of CGA by the protein. At 53% succinylation the maximum binding that could be observed experimentally was $\nu = 0.4$. Increase in the extent of succinylation reduced the binding even further. With the 80% succinylated protein, the maximum binding that could be observed was $\nu = 0.15$. Since the binding was low, the error in the estimation of ν values was high.

The Scatchard plot of the binding data indicated the absence of positive cooperativity of binding (Figure 3) which was observed with the unmodified protein. In fact, a negative cooperativity was observed. By the method of least-squares analysis, the binding constant K and the number of maximum binding sites N were determined. To check the accuracy of the determined values, these were used to calculate ν as the function of A by using the equation $\nu = KNA/(1 + KA)$. Such calculated curves are given as the solid line in Figure 2. The agreement between the experimental data and the calculated curves was satisfactory. The values of K and N are given in Table I.

In spite of the poor experimental accuracy, because of low binding, one observation emerged from the data of Table I. The low binding was due to a decrease in N values with increase in the degree of succinylation. However,

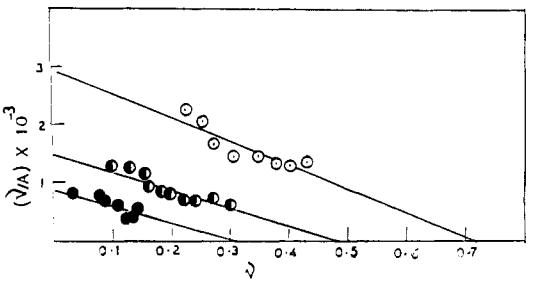

Figure 3. Scatchard plot of binding data of succinylated 11S protein. (⊙) 53% succinylation; (◐) 71% succinylation; (●) 80% succinylation. (—) Least-squares line.

Table I. Value of N and K for the Binding of CGA by Modified 11S Protein of Sunflower Seed[a]

protein	N	K
unmodified protein	15	5.5×10^3
53% succinylated 11S protein	0.71	4.2×10^3
71% succinylated 11S protein	0.48	3.2×10^3
80% succinylated 11S protein	0.31	3.7×10^3
NEMI-treated 11S protein	14	2.6×10^3

[a] 0.1 M acetate buffer of pH 4.0; 30 °C.

the K values were nearly constant and are comparable with the value for the unmodified protein.

In the binding of polyphenolic compounds by several proteins lysine groups have been implicated (Pierpoint, 1969). Superficially our results also indicate that perhaps ϵ-amino groups of lysine residues are involved in the interaction with CGA. However, this conclusion is not supported by other evidence. The total number of lysine residues per 350 000 g of protein is 59. The N value with the unmodified protein is 15. In the case of binding studies it is generally observed that the N value obtained by the Scatchard plot agrees with the total number of amino acid residues involved in the binding of the ligand. Even if the value of $N = 15$ is subject to considerable uncertainty, still the difference is too large to be explained satisfactorily. Also, it has been shown earlier (Shamanthaka Sastry and Narasinga Rao, 1990b) that salts like NaCl do not affect the *binding affinity*, which would indeed by the case if any ionic interaction was occurring with the lysine groups.

The decrease in binding is more likely to be a charge effect. When the protein is succinylated, the positive charge on the ϵ-amino groups is neutralized and a negative charge is introduced. This reduction in positive charge (or increase in negative charge) may affect the binding of the negatively charged CGA molecules. All other evidence suggests that the interaction between CGA and the 11S protein is by hydrogen bonding (Shamanthaka Sastry and Narasinga Rao, 1990b). It has been suggested (Oh et al., 1980) that hydrogen bonds may be pH dependent, in other words, charge dependent.

Effect of SH Group Blocking on Binding. Sulfhydryl groups are the most reactive side groups commonly found in proteins. Therefore, the interactions of CGA with 11S protein in which the SH groups had been blocked NEMI treatment was studied. The SH content of the 11S protein was 7 groups per 350 000 g of protein. After NEMI treatment, it was 0.3 group. Thus, nearly 96% of the SH groups had been blocked. The binding curve (Figure 4) shows that binding was not markedly affected by the modification.

The Scatchard plot of the binding data did not show any cooperative binding (Figure 5). The data could be fitted to a straight line with a negative slope. Values of $K = 2.6 \times 10^3$ and $N = 14$ were obtained from the slope and

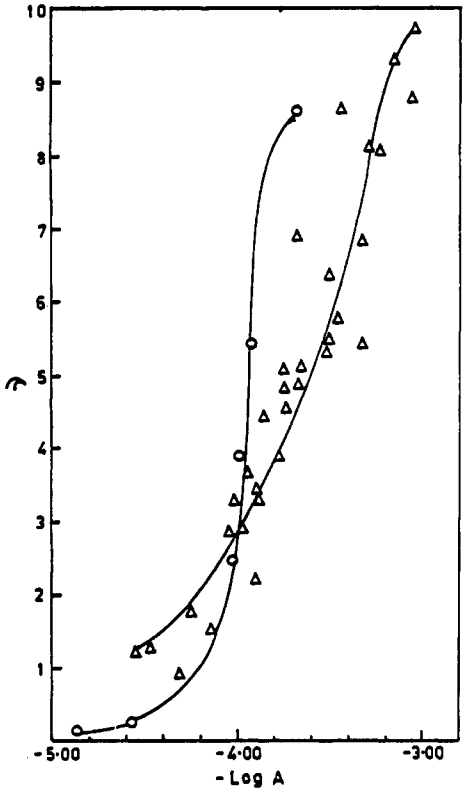

Figure 4. Binding of CGA by NEMI-treated 11S protein (0.1 M acetate buffer, pH 4.0; 30 °C). (○) 11S protein; (△) NEMI-treated 11S protein. (Curve calculated with $N = 14$ and $K = 2.6 \times 10^3$.)

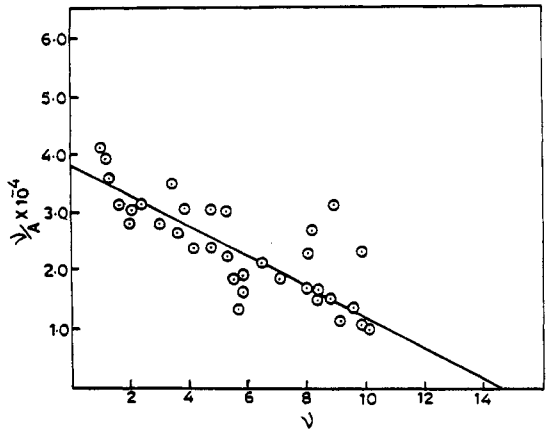

Figure 5. Scatchard plot of binding data of NEMI-treated 11S protein.

intercept. The binding isotherm calculated with these values fitted the experimental data reasonably well (Figure 4).

The values of K and N are nearly the same as those for the unmodified protein. Thus, SH group modification did not affect these values. Only cooperativity of binding was abolished. This was confirmed further by analysis of the binding data with the Hill equation (Hill, 1910). The Hill plot (Figure 6) gave a value of 1 for the Hill coefficient.

It has been reported that succinylation of arachin (Shetty and Narasinga Rao, 1978) and of glycinin (Appu Rao and Narasinga Rao, 1979) resulted in dissociation and denaturation of the protein. Therefore, the modified protein was analyzed by ultracentrifugation and fluorescence spectroscopy.

The sedimentation velocity pattern of the unmodified 11S protein in 0.1 M acetate buffer of pH 4.0 consisted

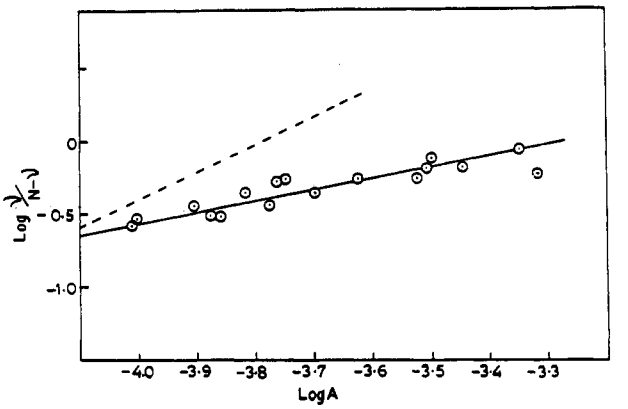

Figure 6. Hill plot of binding data of NEMI-treated 11S protein. (- - -) Data with unmodified protein.

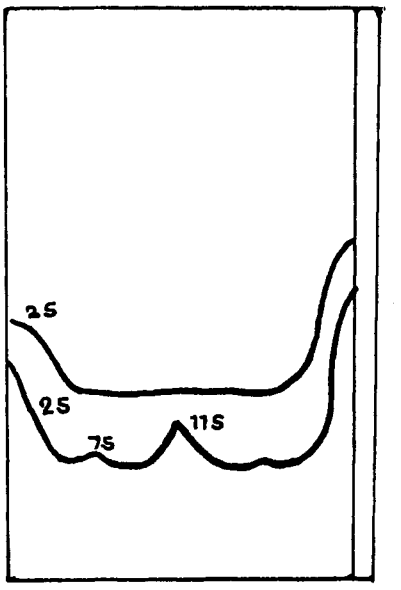

Figure 7. Sedimentation velocity pattern of 11S protein. (Lower) 11S protein at pH 4.0 (0.1 M acetate buffer); (upper) 80% succinylated 11S protein at pH 4.0 acetate buffer. (Sedimentation proceeds from left to right.)

of three peaks with S values of 2S, 7S, and 11S; their proportions were 60%, 5%, and 36%, respectively (Figure 7). The 80% succinylated protein gave a pattern that consisted of a single peak of 2S (Figure 7). Thus, the protein was completely dissociated. The NEMI-treated 11S protein gave a pattern that was the same as that of 11S protein (Figure 8). Thus, SH group blocking did not affect the oligomeric structure of the protein.

The fluorescence spectra of unmodified and succinylated 11S protein are given in Figure 9. The unmodified protein had emission maximum at 330 nm. The spectra of 53% and 80% succinylated 11S protein also had almost the same fluorescence intensity as the unmodified protein and also the maximum was at 330 nm. Thus, there was possibly no difference in the secondary structure of the succinylated and unmodified 11S protein. On the other hand, NEMI-treated 11S protein gave a spectrum with enhanced fluorescence intensity, although the emission maximum remained unchanged at 330 nm. Fluorescence emission is due to tryptophan and tyrosine residues embedded in the interior of the protein molecule (Teale and Weber, 1967). When the protein is denatured, these groups are exposed to the solvent medium, leading to quenching of fluorescence. Enhancement of fluorescence intensity would mean that the protein acquires a more

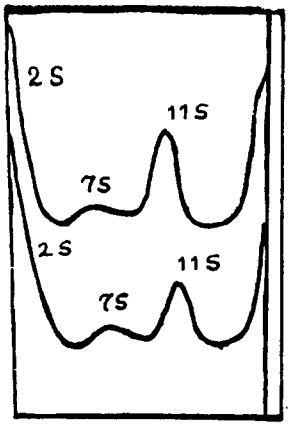

Figure 8. Sedimentation velocity pattern of 11S protein and NEMI-treated 11S protein at pH 4.0 (0.1 M acetate buffer). (Top) NEMI-treated 1uS; (bottom) 1uS protein. (Sedimentation proceeds from left to right.)

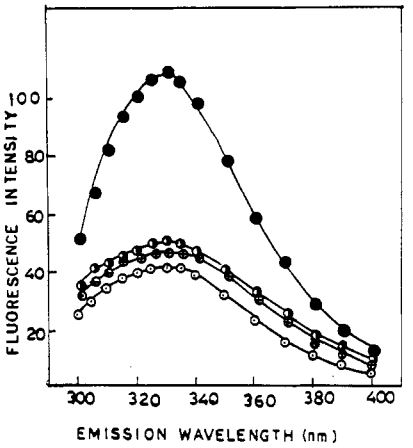

Figure 9. Fluorescence emission spectrum of 11S protein (0.1 M acetate buffer, pH 4.0). (◑) Unmodified 11S protein; (⊕) 53% succinylated 11S protein; (⊙) 80% succinylated 11S protein; (●) NEMI-treated 11S protein.

ordered structure. Possibly NEMI treatment caused a conformational change in the protein, and this change abolishes positive cooperativity of binding.

The blocking of SH groups of the 11S protein does not dissociate the protein. Also there is no change in the binding of CGA. On the other hand, succinylation of the lysine residue of the 11S protein dissociates it to low molecular weight proteins and also reduces the binding of CGA. Dissociation of the protein, if anything, should increase the binding since a greater number of binding sites becomes available on dissociation. If the amino groups of lysine residues were indeed the groups with which CGA binds by ionic interaction, the N value should approximate the lysine content of the 11S protein. Also, the binding *affinity* would not remain the same after modification. These observations argue against ionic interaction and thus indirectly suggest the importance of hydrogen bonds in the interaction. Urea, a hydrogen-bond breaker, completely abolishes binding of CGA although it does not interact with the amino groups of lysine residues.

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Received for review April 10, 1989. Revised manuscript received February 27, 1990. Accepted June 6, 1990.

Registry No. Chlorogenic acid, 327-97-9.