Acetylation of Amino Groups and Its Effect on the Structure of Soybean Glycinin

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Purified soybean glycinin (11S globulin) was acetylated at three degrees; low (21~51 %), middle (60~81 %) and high (90~92 %) acetylation in lysine residues. With increasing the acetyl content, the β -structure gradually decreased and the random structure increased resulting in the exposure of tyrosine residue. These were determined from the results of optical rotatory dispersion, intrinsic viscosity, ultraviolet and fluorescence measurement. Gel filtration, ultracentrifugation and gel electrophoresis studies showed drastic conformational changes of highly acetylated 11S (over 90 %), in which most of the modified protein (75 %) polymerized, and the other dissociated into 3S protein. The close relation between the conformation of acetylated 11S and its emulsifying properties was discussed.

Recently, studies on the functional properties of soybean protein have been growing, because the protein is a useful ingredient in the manufacture of processed food. However, there is little information concerning the relationship between the basic protein structure and the functional behavior.

In a previous work¹⁾ the emulsifying properties of acetylated soybean proteins have been measured. Acid precipitated protein, 7S and 11S globulin were modified by low (30 %), middle (60 %) and high (90 %) degrees of acetylation. As the acetyl contents of modified 7S increased, the emulsifying capacity and the emulsion stability became higher. These properties of modified 11S increased with increasing modification until the extent of acetylation became middle degree. However, the high acetylation of this protein gave the remarkable decrease-effects on the emulsifying properties. Interest in this phenomenon led us to study on the molecular structure of the modified 11S with varying acetyl content.

Soybean proteins have been modified by various chemical reactions including carboxymethylation,²⁾ deamination,³⁾ succinylation⁴⁾ and acetylation.^{4,5)} These modified proteins were also tested for some functional properties

in their reports. However, these reactions have been applied to a heterogeneous protein mixture. Further, some chemical reactions may provide too drastic changes for the structure of the protein to understand the relation between functional properties and the chemical structure, because in these cases many factors affect the structure. Among the chemical reactions, the acetylation can selectively modify the ϵ -amino group in lysine residues in a mild condition without any side reactions.

The purpose of this research is to investigate the effect of acetylation on the molecular structure of purified 11S protein, and the relation between the change of molecular structure and emulsifying properties is discussed.

MATERIALS AND METHODS

Defatted soybean meal. Soybean (var. Raiden) harvested in 1974 grown at Motoyoshi, Miyagi was dehulled, flaked, defatted with hexane, and ground further in a coffee mill to pass through 40-mesh sieves.

Preparation of 11S globulin. Defatted soybean meal was agitated with 0.03 M Tris-HCl buffer (pH 8.0) containing 0.01 M 2-mercaptoethanol (ME) (meal: the buffer, 1: 20) for 1 hr and centrifuged (10,000 rpm, for 20 min at 20°C) to obtain buffer extract. The extract was adjusted to pH 6.4 with 2 N HCl and stirred

for 1 hr at 5°C.⁹⁾ The suspension was centrifuged (10,000 rpm, 20 min at 5°C) to precipitate the 11S protein and washed with 0.01 M Tris-HCl buffer (pH 6.4) three times.

The crude 11S was purified on a column (5.5 × 50 cm) of DEAE-Sephadex A-50 equilibrated with 0.035 M phosphate buffer (pH 7.8) containing 0.1 M NaCl and 0.01 M ME. Elution with NaCl in gradient concentration of 0.1 to 0.7 M was carried out using a mixer chamber (containing 2 liter of the equilibrated buffer) and a reservoir chamber (containing 2 liter of the phosphate buffer made 0.7 M in NaCl, pH 7.8). The protein in the main peak from the column was dissolved in the phosphate buffer (standard buffer, 2.6 mM KH₂PO₄, 32.5 mM K₂HPO₄, 0.4 M NaCl, 0.01 M ME, pH 7.6, $\mu=0.5$) and to this solution ammonium sulfate was added until 51% saturation. The supernatant was adjusted to 66% saturation with ammonium sulfate and the precipitate was dissolved in the standard buffer (pH 7.6, $\mu=0.5$). This purified 11S showed a single peak in ultracentrifugal analysis as shown in Fig. 6. The protein concentration was measured by the dry weight method after dialysis against water and lyophilization, or ultraviolet spectrum measurement at 280 nm.

Acetylation of 11S protein. The purified 11S protein was acetylated according to the methods of Ansari *et al.*⁷⁾ Proteins were dissolved (1%) in the standard buffer (pH 7.6, $\mu=0.5$) at 0~4°C with constant stirring. Acetic anhydride was added in small aliquots and the pH of the reaction mixture was maintained between 7.4~7.8 by the addition of 1~2.5 N NaOH using a pH stat. The reaction was carried out for 30 min, and then the solution was dialyzed against half-saturated sodium acetate solution and distilled water. In the case of 11S, the prolonged dialysis against water sometimes gave some protein precipitation. Therefore, after short time dialysis (1 hr) against water, the solution was dialyzed against the standard buffer ($\mu=0.3$) containing 0.2 M NaCl instead of 0.4 M described above. This solution was used throughout the following experiments.

Determination of extent of chemical modification. TNBS (2, 4, 6-trinitrobenzene 1-sulfonic acid) method⁹⁾ was used to quantify the extent of chemical modification. Sodium borate solutions (0.15 M, 2 ml) were added to a protein solution (0.5 ml). After addition of a mixture (1 ml) of 0.01 M Na₂SO₃ and 0.10% TNBS (1:1, by volume), the solution was kept at 37°C for 2 hr and the absorbance was determined at 420 nm. The slopes, m and m' , for native and modified protein preparations may be related to percent modification by the expression:

$$\text{Percent modification} = 100 (1 - m'/m)$$

Ultraviolet (UV) spectrum measurements. UV

spectrum measurements were made in 10 mm-silica cells by a Hitach Model-124 automatic recording spectrophotometer in the wave length range of 250~310 nm. UV difference spectrum measurements were carried out by the same spectrophotometer with reference to the native protein.

Fluorescence measurements. The excitation and emission fluorescence spectra of the proteins were recorded with a Hitach Model-204 spectrofluorometer.

Optical rotatory dispersion (ORD) analysis. ORD of the 300~600 nm region was measured. The sample solutions in the standard buffer ($\mu=0.3$) and a cell of 10-mm path length were used, and the resulting data were treated by means of Moffitt-Yang equation.¹⁰⁾

$$[m']_{\lambda} = \frac{3}{n^2 + 2} \cdot \frac{M}{100} \cdot \alpha = \frac{a_0 \lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0 \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

in which $[m']_{\lambda}$ is the mean residue rotation at the wave length of λ corrected for the refractive index (n) of solvents, λ_0 is the absorption wave length associated with the rotation assumed to be 212 nm, and M_0 is the mean molecular weight. The parameter a_0 and b_0 were calculated from the intercepts and slopes of $[m']_{\lambda} \cdot (\lambda^2 - \lambda_0^2)^{-1}$ vs. $(\lambda^2 - \lambda_0^2)^{-1}$ plots. Contents of β -structure (x^{β}) and α -helix (x^H) were calculated from the following equation.¹⁰⁾

$$x^{\beta} = (a_0 + 560 + 1.1 \cdot b_0) / 800$$

$$x^H = b_0 / -630$$

Gel filtration. Protein solutions (1 ml, 2%) were applied on a column of Litex 10 (Litex Co., the molecular range, 10,000~300,000) and eluted with the standard buffer ($\mu=0.3$) in 3 ml fractions, which were monitored at 280 nm.

Reduced viscosity. Viscosity was measured by using an Uberode viscometer at 30 ± 0.01°C in the phosphate standard buffer ($\mu=0.3$).

Sedimentation analysis. Ultracentrifugal analysis was performed with a Hitachi UCA-1A centrifuge at 55,430 rpm. Proteins were dissolved in the standard buffer at ionic strength $\mu=0.3$.

Polyacrylamide gel electrophoresis. Gel electrophoresis was carried out in 7.5% polyacrylamide gels using a slab gel apparatus (15 × 12 cm) with the discontinuous buffer system according to Ornstein and Davis.^{11,12)}

RESULTS

Extent of chemical modification

Figure 1 shows the degree of N-acetylation

FIG. 1. Determination of Acetyl Content in Soybean 11S Protein.

Lysine residues in 11S protein were determined by the TNBS method. Native 11S (—), 21% Ac-11S (---), 60% Ac-11S (— · —) and 90% Ac-11S (····). The inset shows the amount of acetic anhydride.

TABLE I. RELATION BETWEEN THE AMOUNT OF ACETIC ANHYDRIDE AND EXTENT OF MODIFICATION AND EXTINCTION COEFFICIENT.

Anhydride (ml/g protein)	Modifi- tion (%)	$E_{280\text{nm}}^{1\%}$	Abbreviations ^a
0	0	8.04	N-11S
0	0	8.02	0% Ac-11S ^b
0.05	21	8.02	21% Ac-11S
0.17	60	7.90	60% Ac-11S
1.91	90	6.62	90% Ac-11S

^a The abbreviations used are as follows: N-11S, native 11S protein; *n*% Ac-11S, *n*% acetylated 11S protein.

^b Prepared by the same acetylation procedure without acetic anhydride in the reaction.

caused by treatment of the protein with increasing amounts of acetic anhydride. The extent of modification is given in Table I. Three kinds of acetylated protein were prepared; low (21%), middle (60%) and high (90%) acetyl content. Under the acetylation condition of Ansari *et al.*,⁷ only lysine residues would be modified, since concentrated acetate ions in dialysis solution hydrolyze *O*-acetyl residues. The abbreviations of these modified proteins are shown in Table I. The modified protein, 0% Ac-11S means the product prepared by the same acetylation procedure and dialysis without acetic anhydride in the reac-

tion. The other preparations of 25, 51, 81 and 92% Ac-11S were used for the studies of intrinsic viscosity and gel electrophoresis.

UV Spectra

The ultraviolet absorption changes caused by acetylation of 11S protein are shown in Fig. 2. Acetylation produces a slight change in the absorbance at low and middle acetyl contents, and a significant change is observed at high acetyl content of 11S protein. The decrease at 280 nm is 17.7% for 90% Ac-11S (Table I). For 90% Ac-11S, the absorption maximum shows a blue shift. The difference spectra are shown in Fig. 2. The spectra give negative peaks near 278 and 286 nm at high acetyl content. In most proteins, negative peaks at 278 and 286 nm are ascribed to an exposure of tyrosine residues from a hydrophobic region. Therefore, the highly acetylated proteins expose the tyrosine residues from the buried region in the molecule. Negative peaks at 292 and 284 nm are ascribed to increased hydrophilic circumstances of tryptophan, whereas the acetylated proteins showed no peak at 292 nm, which is specific for tryptophan.

FIG. 2. Ultraviolet Absorption Spectra (A) and Difference Spectra (B) of Native and Acetylated 11S Proteins.

Sample (0.09%) were dissolved in the phosphate buffer (pH 7.6, $\mu=0.3$ and 0.01M 2-mercaptoethanol). Difference spectra were measured with reference to the native protein. Native 11s (—), 21% Ac-11s (---), 60% Ac-11S (— · —) and 90% Ac-11S (····).

Fluorescence spectra

The excitation and emission spectra of 11S protein and various acetylated 11S are shown in Fig. 3. The quantum yield of 11S increased with increasing acetyl content, appreciably for 90% Ac-11S. The emission maximum for 90% Ac-11S shows a small (about 2 nm) red shift. These results suggest a very little change of environment of tryptophan or some other residues in the modified proteins.

FIG. 3. Excitation and Emission Spectra of Native and Acetylated 11S Proteins.

Samples (0.09%) were dissolved in the phosphate buffer (pH 7.6, $\mu=0.3$ and 0.01 M 2-mercaptoethanol). Left: emission spectra (excitation wavelength is 280 nm). Right: excitation spectra (emission wavelength is 335 nm). Native 11S (—), 21% Ac-11S (---), 60% Ac-11S (— · —) and 90% Ac-11S (----).

Optical rotatory dispersion

ORD was measured to determine the secondary structure of the acetylated proteins. The results in Fig. 4 show a decrease of β -structure and an increase of random structure with increase of acetyl content. However, α -helix does not change appreciably. The contents of β -structure, α -helix and random structure changed from 40, 6, and 55% for native to 24, 8, and 68% for 90% Ac-11S, respectively.

Gel filtration

Figure 5 shows the elution patterns of the native and acetylated 11S proteins. For 21

FIG. 4. Effect of Acetylation on the Secondary Structure of Acetylated 11S Proteins.

Samples (0.3%) were dissolved in the phosphate buffer (pH 7.6, $\mu=0.3$ and 0.01 M 2-mercaptoethanol). The contents of secondary structure were calculated from the ORD parameters. A, β -structure; B, α -helix; C, random structure.

FIG. 5. Chromatogram of Native and Acetylated 11S Proteins by Gel Filtration.

A column (Litex 10, 1×110 cm) was eluted with the phosphate buffer (pH 7.6, $\mu=0.3$ and 0.01 M 2-mercaptoethanol) in 3 ml fractions. Native 11S (—), 21% Ac-11S (---), 60% Ac-11S (— · —) and 90% Ac-11S (----).

and 60% Ac-11S, the main peaks become more broad. Pronouncedly, different from the others, the main peak for 90% Ac-11S moves to the position of the void volume, accompanied with a little peak at the latter position. These indicate that the most of 90% Ac-11S (75.3% for fraction number 11~16 based on the UV measurement) polymerizes and the some other part (24.7% for fraction number 19~24) dissociates.

Ultracentrifugation

Ultracentrifugal analyses of the native and

FIG. 6. Ultracentrifugal Patterns of Native 11S (A), 60% Ac-11S (B) and 90% Ac-11S (C). Samples (1.0%) were dissolved in the phosphate buffer (pH 7.6, $\mu=0.3$ and 0.01 M 2-mercaptoethanol). Photographs were taken at 45 min after reaching 55,430 rpm.

acetylated proteins are shown in Fig. 6. Native 11S protein shows a single peak. The sedimentation pattern of 60% Ac-11S gives the two peaks of 11S and $\overline{7S}$. The latter is a half molecule of 11S, and this agrees with the fact that the main peaks become broad in gel filtration pattern. For 90% Ac-11S, $\overline{7S}$ disappears and a little 3S peak appears and the most of the modified protein moves to the bottom. These indicate the polymerization.

Reduced viscosity

Viscosity data of the native and the modified proteins are shown in Fig. 7. For 51% Ac-11S

FIG. 7. Reduced Viscosity of Native and Acetylated 11S Proteins.

Viscosity was measured with an Ubbelohde viscometer at $30 \pm 0.01^\circ\text{C}$ in the phosphate buffer (pH 7.6, $\mu=0.3$ and 0.01 M 2-mercaptoethanol). Native 11S (—), 51% Ac-11S (---), 81% Ac-11S (···) and 92% Ac-11S (— · — ·).

a little decrease is shown and 91% Ac-11S gives a definite increase than native 11S. The decrease of the viscosity for 51% Ac-11S may be ascribed to dissociation to $\overline{7S}$ and the increase for 91% Ac-11S may depend on the polymerization, which is suggested by the results of gel filtration, ultracentrifugation, and gel electrophoresis.

Gel electrophoresis

Figure 8 shows the slab gel electrophoresis

FIG. 8. Slab Gel Electrophoretic Patterns of Native and Acetylated 11S Proteins.

Slab gel (15 × 12 cm) was run in 7.5% polyacrylamide gel with the discontinuous buffer system of Davis. Native 11S (A), 25% Ac-11S (B), 60% Ac-11S (C) and 92% Ac-11S (D).

patterns by Davis's system. Although the native 11S protein showed one peak by the ultracentrifugal analysis, two bands of 11S and $\overline{7S}$ appeared by gel electrophoresis. This may depend on the gradual dissociation of 11S to $\overline{7S}$ with time, because the native protein shows less $\overline{7S}$ band just after preparation of 11S. The dissociation is also ascribed to electrophoretic conditions of Davis's system which has lower ionic strength and higher pH than those of the stable conditions of 11S.

The electrophoretical mobility of a protein in gel depends on the net charge and size of the protein molecule. Upon acetylation ammonium cations are replaced by neutral acetyl groups which provide an increase of the more net anions in the molecule. This agrees with the mobility of acetylated proteins from native to 60% Ac-11S, where the higher acetyl contents showed the higher mobility with respect to the 11S and $\overline{7S}$ band. The band of $\overline{7S}$ moved at higher rate than that of 11S. The $\overline{7S}$ seemed to be acetylated more easily than 11S. For 91% Ac-11S, the presence of polymerized protein at starting line and dissociated protein at the fast moving end agrees with the results of the gel filtration and the centrifugation analyses.

DISCUSSION

Native and 0% Ac-11S showed almost the same properties as far as the experimental results of ultraviolet spectra, fluorescence spectra, gel filtration and viscosity. Therefore these results were not shown. This means that acetylation and dialysis against half-saturated sodium acetate don't result in any conformational changes of the modified proteins. Therefore, the conformational change can be ascribed to the effect of acetylation of lysine residues in 11S protein.

For acetylated 11S preparations, UV and fluorescence measurements show the gradual conformational changes with the extent of acetyl content, significant at high acetylation, resulting in the exposure of tyrosine residues. ORD measurement shows a gradual decrease

of ordered structure and an increase of random structure. It is possible that the acetyl groups make steric misfits for the ordered structure, just as proline and hydroxyproline for the α -helical conformation of proteins. Among the acetylated preparations, the highly acetylated 11S shows marked changes in the UV absorption, the fluorescence spectra and the ultracentrifugal analyses. However, the extent of the conformational change of the highly acetylated 11S is far less than that of denatured 11S with 8 M urea. Fukushima¹³⁾ reported that the 11S changes the parameter, $-a_0$, from 290 to 540 in 8 M urea, whereas the highly acetylated protein changed the $-a_0$ from 205 for the native protein to 312. Therefore, the different value in the change is 250 for 8 M urea denaturation and 107 for 90% acetylation. However, drastic changes are observed in the molecular size of acetylated 11S by means of gel filtration, ultracentrifugation, intrinsic viscosity and gel electrophoresis. The half molecule of 11S ($\overline{7S}$) is shown at middle acetylation. The high acetylation causes the polymerization of the most of 11S accompanied by the dissociation to 3S.

Recently, we have reported the emulsifying properties of acetylated soybean proteins¹¹⁾. As the acetyl contents of modified 7S increased, the emulsifying capacity and the emulsion stability became higher. These properties of modified 11S increased with increasing modification until the extent of acetylation became middle degree, thereafter decreased. This characteristic decrease consists with the drastic change in the 11S molecular size by the polymerization and a little dissociation. The increased emulsifying activity, as much as middle acetyl content, is supposed to depend on the increase of hydrophobic properties of the acetyl group and on the neutralization of ammonium cation. However, the decreased emulsifying properties in high acetylation mean that the emulsifying properties are ascribed to not only the hydrophobic property but also the molecular structure. The polymerization of 11S protein causes the decrease of emulsifying properties. The polymerization may reduce the number

of attaching site of oil. It was reported that the moderate partial hydrolysis of soybean protein with mineral acid or enzyme increased its emulsifying properties.^{14~16)} Recent paper has also reported that the dissociation of vegetable proteins with denaturing agents provides greater emulsion stability.¹⁷⁾ This degradation or dissociation seems to be accompanied by an increase of hydrophobic residues.

The subunit structure of 11S may be stabilized by noncovalent interactions such as ionic and hydrogen bonds and hydrophobic interaction, together with the covalent bond of disulfide. The removal of positive charges by high acetylation over 90% would destroy the molecular structure of 11S protein. This is an evidence that the ionic bonds contribute to stabilize the 11S structure. Eleven S protein consists of acidic and basic subunits,¹⁸⁾ on the other hand, 7S protein consists of no definite acidic or basic subunits.¹⁹⁾ Therefore, the high acetylation may affect rather 11S than 7S. This agrees with the fact that the emulsifying properties of highly acetylated 11S showed the lowest value, whereas, highly acetylated 7S gave the highest value compared with the preparations of middle or low acetyl content.

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