Effects of Progressive Succinylation on Some Molecular Properties of Soy Glycinin

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ABSTRACT

This study was conducted to determine the effectiveness of succinylation for improving the molecular flexibility of soy glycinin. Eighty and 90 percent succinylation of the free amino groups of glycinin was obtained with a 0.2:1 and 1:1 ratio of succinic anhydride to protein, respectively. Progressive succinylation up to 75% of free amino groups caused a gradual increase in specific viscosity of glycinin, but above 75% succinylation there was a sharp increase in viscosity. Succinylation of up to 50% of available amino groups gradually increased the hydrophobicity of glycinin. This was accompanied by gradual exposure of tyrosine and tryptophan residues to the polar medium as indicated by ultraviolet and fluorescence spectrophotometric analyses. The data indicated a gradual dissociation and/or unfolding of the component polypeptides of glycinin, with up to 50% succinylation followed by refolding or reassociation of polypeptides after further modification.

Soybean proteins are important functional ingredients for food formulation and fabrication. Both the physical properties and functional attributes of these proteins have been extensively studied (Kinsella 1979, Kinsella et al 1985, Nielsen 1985). The functional properties of the major globulin protein component, namely, glycinin or 11S protein (350–360,000 daltons), may be limited by their relatively stable oligomeric structure. The thermal stability and limited molecular flexibility of glycinin restricts its application in foods. For applications requiring surface activity, e.g., foaming, or emulsification, molecular flexibility is an important molecular prerequisite to enable the functional protein to orient and spread at an interface to form interfacial films (MacRitchie 1978). Glycinin has limited flexibility in its native state. However, appropriate modification may improve its surface-active properties. Thus, reduction of component disulfide bonds increases molecular flexibility (Kella et al 1986, Kim and Kinsella 1986). Modification of the net charge on glycinin may be a feasible approach for improving its functional behavior. For example, complete succinylation of soy protein isolate markedly improves its solubility and surface activity (Franzen and Kinsella 1976, Shetty and Kinsella 1979, Feeney and Whitaker 1985). Because of the effectiveness of succinylation in dissociating the structure of soy protein isolate, we studied the effects of the extent of succinylation of glycinin on its conformation and molecular properties.

MATERIALS AND METHODS

Chemical reagents of analytical grade were obtained from Eastman Kodak, Rochester, NY; Sigma Chemical Co., St. Louis, MO; or Fisher Scientific, Rochester, NY. Doubly distilled water was used for preparation of all solutions.

Preparation of Soy Glycinin

Glycinin was prepared from defatted, low-heat-treated soy flour (Central Soya, Ft. Wayne, IN) by the procedure of Thanh and Shibasaki (1978). The defatted flour (50 g) was suspended in 1 L of 0.03*M* Tris-HCl buffer, pH 8.0, containing 2 m*M* 2-mercaptoethanol. The solution was stirred for 1 hr at 24° C and then centrifuged at 15,000 × *g* for 20 min at 20° C. The supernatant was adjusted to pH 6.4 with 2*N* HCl and centrifuged at 15,000 × *g* for 20 min at 2° C. The glycinin precipitate was washed with Tris-HCl buffer, pH 6.4, and then dispersed in Tris-HCl buffer, pH 8.0. Aliquots of 2*N* NaOH were added while stirring until the protein was fully dissolved (pH 8.0). The protein solution was dialyzed against water adjusted to pH 8.0 at 4° C and lyophilized.

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Succinylation of Glycinin

Succinylation of the glycinin was performed as detailed in the method of Franzen and Kinsella (1976). To the glycinin (5 g in 70 ml) solution in 0.1*M* phosphate buffer (pH 8.2), known amounts (0.025, 0.05, 0.125, 0.25, and 0.5 g) of succinic anhydride were added in small (10 mg) increments. The solution was stirred and the pH maintained above 8.1 by adding 3*N* NaOH at 25° C. After the pH stabilized at 8.0 following addition of all the succinic anhydride, the solution was dialyzed for 24 hr against three changes of distilled water, adjusted to pH 8 at 5° C to remove excess reagent, and then lyophilized.

The extent of succinylation was quantified by determining the free amino groups with trinitrobenzenesulfate (TNBS) (Fields 1972). The TNBS (0.02 ml of a 1.1*M* solution) was added to 1 ml of the protein (0.1% w/v) in sodium borate buffer, mixed rapidly, held 5 min at room temperature, and the reaction was stopped by adding 2.0 ml of a freshly prepared 1.5 m*M* sulfite solution. The absorbance of the sample was read at 420 nm against a blank (i.e., borate buffer without protein, treated identically). The percent succinylation was calculated:

$$\% \text{ Succinylation} = \frac{A_{420}\,(\text{native}) - A_{420}\,(\text{succinylated})}{A_{420}\,(\text{native})} \times 100.$$

Viscosity

The specific viscosity of protein solution (0.1% in 20 m*M* phosphate buffer, pH 8.0) was determined using an Oswald-type viscometer. The time for the sample to flow through the capillary tube under gravity was used to calculate specific viscosity of glycinin according to the equation:

$$\text{Specific viscosity} = t_s/t_b - 1;$$

where t_s and t_b are the flow times for the sample and the buffer solution, respectively.

Measurements of Hydrophobicity

The hydrophobicity of proteins was determined using the fluorescence probe, *cis*-parinaric acid (Sklar et al 1977, Kato and Nakai 1980). Fluorescence intensity (FI) was monitored with a model 650-40 Perkin-Elmer fluorescence spectrophotometer. Freshly prepared *cis*-parinaric acid (10 ml of 0.0036*M* solution) was added to 2 ml buffer solutions (containing 0, 0.01, 0.02, 0.04, 0.06, 0.08, and 0.1% by weight of protein); these were excited at 330 nm, and the emission was measured at 420 nm (slit width of 5 nm). Peak emission values were plotted against protein concentration, and the hydrophobicity of the protein was calculated from the initial slope of the binding curve of FI versus protein concentration (Kato and Nakai 1980).

Turbidity

The solubility of proteins was determined by turbidometry (Damodaran and Kinsella 1982). The absorbance of protein solutions (0.05%) was measured at 540 nm after 1 hr of equilibration in buffer (i.e., sodium citrate 20 mM for pH 3–5 and sodium phosphate for pH 6–8). Water was used as the blank.

Ultraviolet Absorbance

The ultraviolet (UV) absorbance spectra of proteins (0.1% in 20 mM phosphate buffer, pH 8.0) were recorded in a Cary 219 spectrophotometer in the 230–330 nm range, using 1-cm quartz cuvettes of matched path length at 1-nm band width.

The effect on the absorbance at 290 nm of varying pH from 8 to 12 was monitored to determine the dissociation of tyrosine. The protein concentration was 0.1% in 50 mM borate buffer (pH 8.0–12.5). The percentage of tyrosine titrated at a particular pH x was calculated according to the equation:

$$\% \text{ Tyrosines titrated} = \frac{A_{295}\,(\text{pH } x) - A_{295}\,(\text{pH } 8)}{A_{295}\,(\text{pH } 12.5) - A_{295}\,(\text{pH } 8)} \times 100.$$

Fluorescence Spectra

The intrinsic emission fluorescence spectra of glycinin were determined in a Perkin-Elmer 650-40 recording fluorescence spectrophotometer. Protein concentration was 0.02% in 20 mM phosphate buffer (pH 8.0). The protein solutions were excited at 285 nm and the emission intensities were measured from 300 to 400 nm (slit width, 5 nm).

RESULTS AND DISCUSSION

The extent of succinylation of the lysine residues in glycinin increased with concentration of succinic anhydride (Fig. 1), and was quite high (80%) at relatively low ratios of reagent to glycinin, with 97% succinylation being obtained at a ratio of 1:1. This was comparable to the results of Rao and Rao (1979) for glycinin and to the succinylation of other proteins where 100% succinylation was easily achieved at relatively low ratios of succinic anhydride to protein (Means and Feeney 1971, Franzen and Kinsella 1976, Kinsella and Shetty 1979). These results indicated that most of the reactive groups, i.e., the amino group of lysine and N-terminal amino acids, were accessible for succinic anhydride at pH 8.0, or that the initial succinylation of exposed amino residues may have altered protein conformation and enhanced availability of otherwise inaccessible lysine groups.

The increase in molecular weight of glycinin upon succinylation was small. In the case of 100% succinylation, the increase in molecular weight was approximately 5.0% (based on number of succinic residues multiplied by the number of free amino groups in glycinin—160 residues—and a molecular weight of 320,000) (Iyengar and Ravestein 1981). The net charge on glycinin at pH 7.0 was altered following succinylation. Cationic amino groups were changed to anionic carboxyl groups after succinylation, and upon 100% succinylation the negative charges were increased by approximately 160 units per mole of glycinin at pH 8.0. From the electrometric titration data of Catsimpoolas et al (1971), glycinin carries a net negative charge of about 260 electron units per mole at pH 7.8; therefore, this change represents a significant increase in net negative charge of glycinin at pH 7.0.

The solubility of glycinin was significantly improved after succinylation as observed previously (Frazen and Kinsella 1976). Glycinin succinylated above 25% was completely soluble at all pH values in the pH range 4–8 at concentrations of 0.05%; whereas unmodified glycinin was insoluble in the range pH 4–5.

The hydrophobicity of succinylated glycinin progressively increased up to 50% succinylation but decreased upon further succinylation (Fig. 2). Apparently, with limited succinylation, i.e., up to 50%, hydrophobic sites previously inaccessible in native glycinin became available for binding by *cis*-parinaric acid. The decreased hydrophobicity above 50% succinylation may reflect conformational changes in glycinin induced by electrostatic repulsion between succinylated glycinin polypeptides.

Conformational Changes

Conformational changes of glycinin accompanying succinylation were determined by changes in specific viscosity, UV, and fluorescence spectra. The specific viscosity of glycinin slowly increased with increasing levels of succinylation with a sharp increase in viscosity at >75% succinylation (Fig. 2). This reflects an

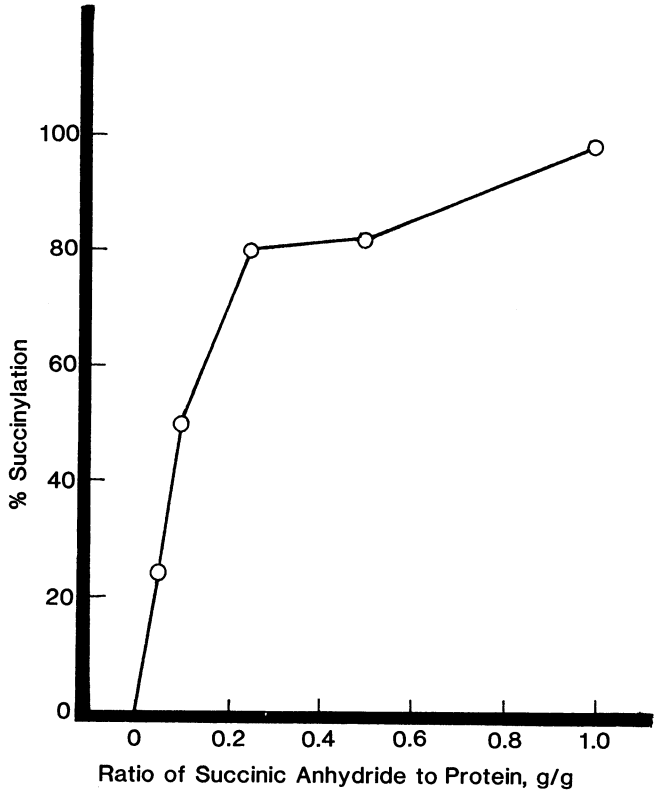

Fig. 1. The extent of succinylation of lysines in glycinin at pH 8.0 by increasing concentrations of succinic anhydride.

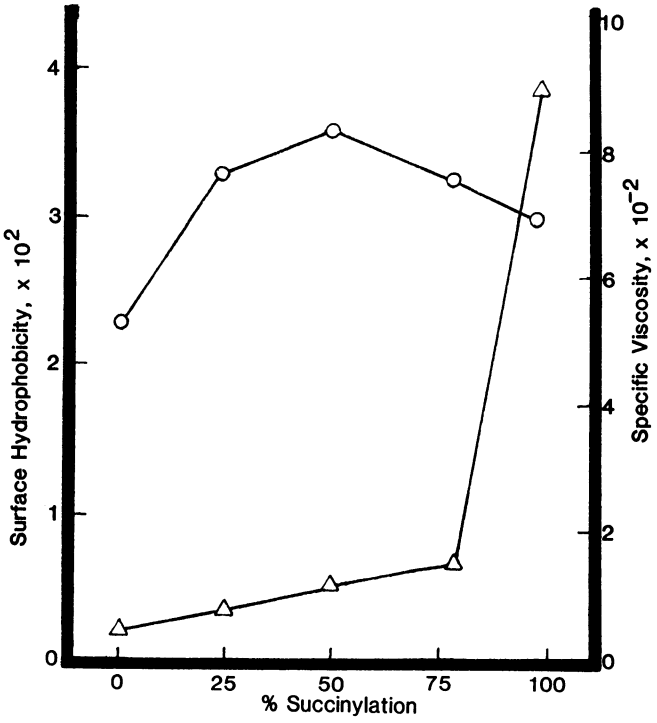

Fig. 2. The surface hydrophobicity (O) and specific viscosity (△) of native and succinylated glycinin. The protein concentration was 0.1% (w/v) in 20 mM phosphate buffer (pH 8) for specific viscosity and hydrophobicity.

increase in the hydrodynamic volume probably caused by some unfolding of the glycinin protein, as observed for arachin (Shetty and Rao 1978) and soy protein (Rao and Rao 1979). The alteration of the net negative charge by 160 after succinylation ostensibly destabilized the oligomeric structure of the native glycinin causing dissociation into half molecules, i.e., hexameric subunits (Rao and Rao 1979). Accompanying dissociation, the net repulsive charge may have caused some unfolding of acidic and basic subunits, which increased the hydrodynamic volume and viscosity.

The UV spectra of native and succinylated glycinin indicated that the λ_{max} progressively shifted to shorter wavelengths (blue shift) with increasing extent of succinylation, while the absorbance continuously decreased (Fig. 3). Glycinin contains approximately 60 tyrosine and 18 tryptophan residues per mole (360,000 Da), which are the principal chromophores absorbing at 290 nm. In accordance with Freifelder (1982) the decrease and shift in these spectra indicated that the tyrosine and tryptophan were shifted to a more polar environment with succinylation. The results are consistent with the dissociation and unfolding of the glycinin with progressive succinylation to expose the previously internal aromatic apolar groups to the aqueous phase. These data corroborate observations of hydrophobicity changes (Fig. 2), and the blue shift observed with the progressive succinylation of arachin (Shetty and Rao 1978).

Spectrophotometric titration of native and fully succinylated glycinin between pH 8 and 12 showed that the percent ionization of tyrosines increased following succinylation, especially between pH 10 and 12 (Fig. 4). There were negligible differences between the various succinylated glycinin polypeptides (data not shown). With ionization of tyrosines the λ_{max} changed from 274 to 295 nm with an increase in absorbance (Donovan 1973). Because the tyrosines on the protein surface have a pK of 9.6 (similar to free tyrosines), they should become fully titrated around pH 10, giving rise to increased absorbance. However, as indicated by the low ionization at pH 10, less than 10% of tyrosines were on the surface of the glycinin molecules in the samples; most tyrosine residues were in the interior of the glycinin and were not detectable spectrophotometrically below pH 10.5.

The marked increase in absorbance, i.e., ionization of tyrosines in unmodified glycinin between pH 11 and 12, indicated that the internal tyrosines were being exposed to the polar environment (Freifelder 1982). In modified glycinin the percent ionization, between pH 10 and 11.5, indicated that the number of tyrosines exposed to the polar environment was increased with succinylation. Negligible differences between the spectra of the various succinylated samples (25–98% succinylation) indicated that dissociation of the oligomeric structure of glycinin by succinylation, even at 25%, caused exposure of the tyrosines; further succinylation did not influence the environment of the tyrosine residues, or the changes that may have occurred were mutually cancelled in terms of net absorbance (Donovan 1973).

The fluorescence spectra of native and succinylated glycinin showed that the λ_{max} moved to the longer wavelengths with the extent of succinylation, whereas the relative fluorescence intensity increased, particularly up to 50% succinylation, but at 98% succinylation was less than native glycinin (Fig. 5). The maximum shift in λ_{max} was 5 nm. The red shift of λ_{max} following succinylation indicated that tryptophan residues in glycinin became exposed to a more polar environment because of dissociation of the native oligomeric structure and perhaps some unfolding following succinylation. Damodaran and Kinsella (1981) observed that the λ_{max} of fluorescence emission shifted from 335 to 350 nm after complete succinylation of total soy globulins. The shift observed in that study exceeded that observed in this study, probably because of the dissociating effects of the 2-mercaptoethanol (10 m*M*) that was used in the buffer. Rao and Rao (1979) observed similar shifts

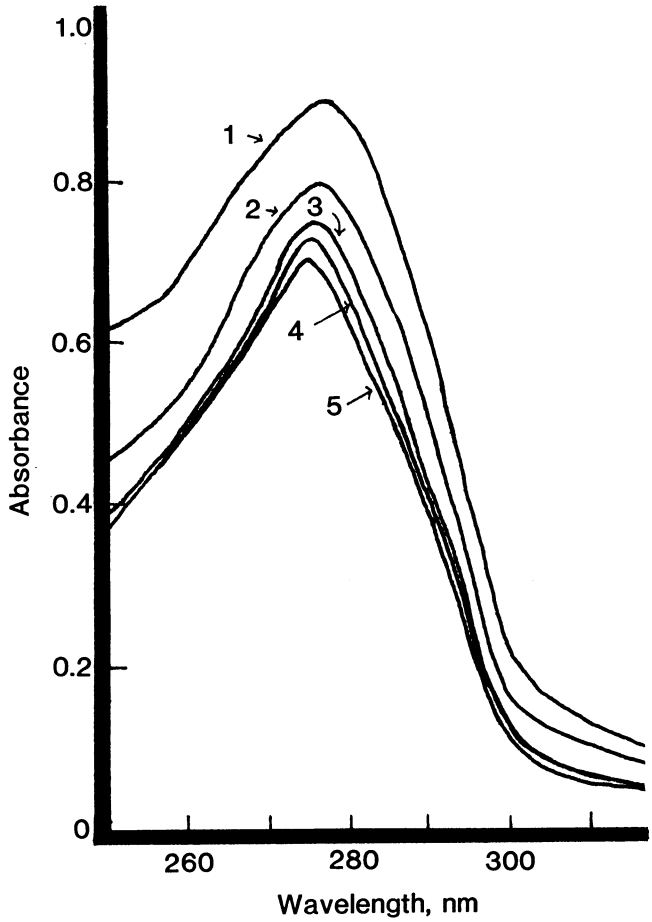

Fig. 3. Ultraviolet absorption spectra of native and succinylated glycinin: **1,** native; **2,** 25%; **3,** 50%; **4,** 80%; and **5,** 98% succinylated samples. Protein concentration was 0.1% (w/v) in 20 m*M* phosphate buffer (pH 8).

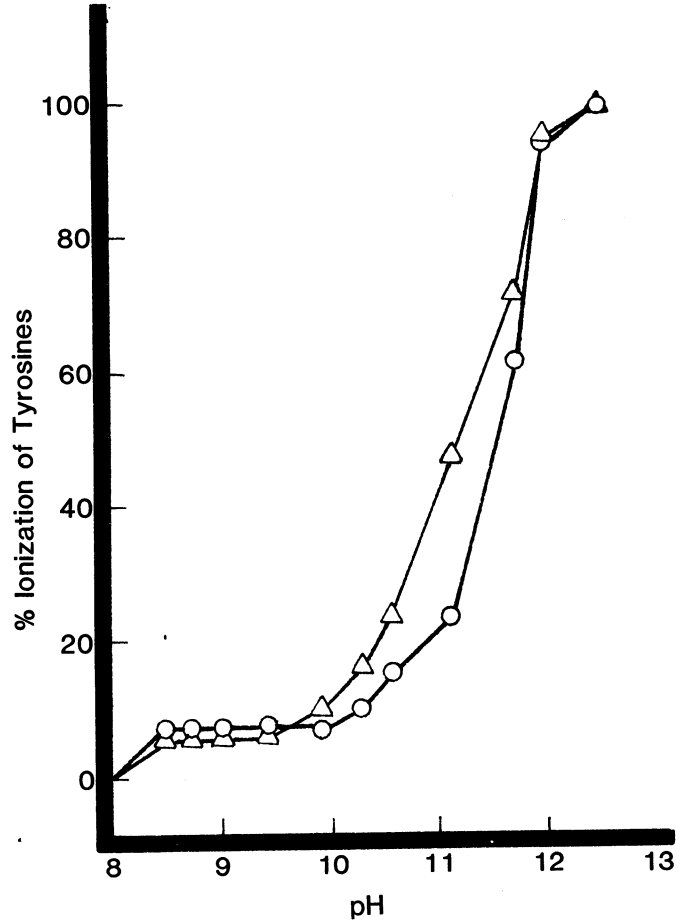

Fig. 4. Spectrophotometric titration of native (O) and succinylated (△) glycinin. Protein concentration was 0.1% (w/v) in 20 m*M* borate buffer.

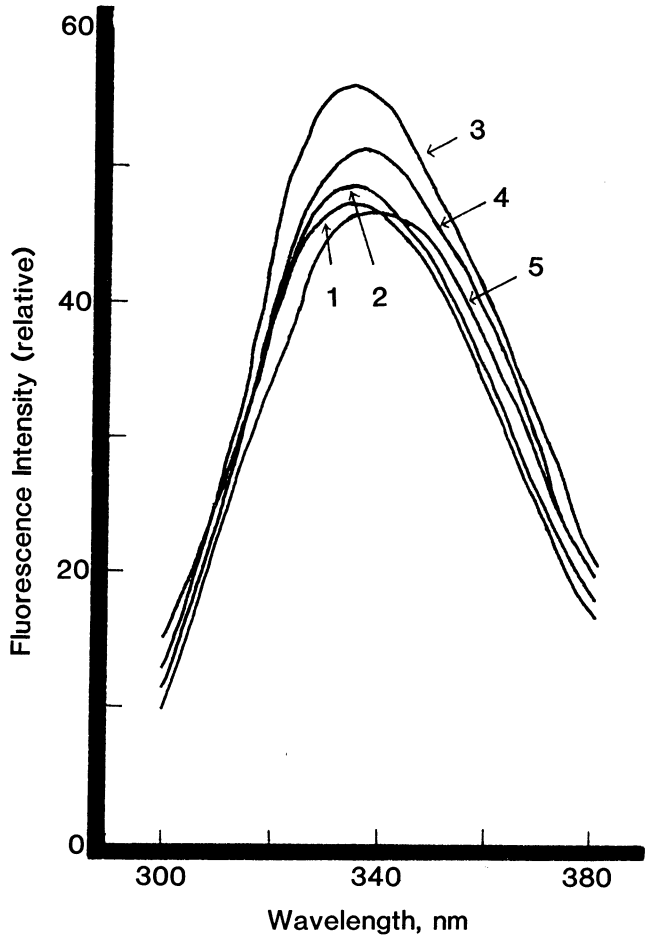

Fig. 5. The intrinsic fluorescence spectra of native and succinylated soy glycinin. **1,** native; **2,** 25%; **3,** 50%; **4,** 80%; and **5,** 98% succinylated samples. Protein concentration was 0.02% (w/v) in 20 m*M* phosphate buffer (pH 8).

in fluorescence emission after succinylation of glycinin.

The decrease in fluorescence intensity of glycinin above 50% succinylation was consistent with reassociation of the unfolded polypeptides to occlude some of the tryptophan residues. However, interpretation of intrinsic fluorescence based on the quantum yield is complex and defies simple conclusions, because not all of the tryptophan contributes equally to fluorescence; hence, it is difficult to identify the particular residues involved (Freifelder 1982).

These studies indicated that the oligomeric structure of glycinin is partly dissociated with some unfolding following succinylation. The significant increase in net charge and electrostatic repulsion causes the molecules to expand and undergo conformational changes as reflected by the increased viscosity changes in the hydrophobicity and spectrophotometric data. The spectroscopic studies indicated that apolar residues were exposed partly because of the dissociation of the oligomeric structure and unfolding, reflecting a more open structure after succinylation. Therefore, as succinylation was increased soy glycinin adopted a more open and expanded structure. The effects of these conformational changes on the surface properties will be reported in a subsequent paper.

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