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# Modification of the Physical Properties of Soy Protein Isolate by Acetylation

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The predictions of previously developed models for the hydration, gelation, and solubility of soy protein isolate regarding the effect of a chemical modification (acetylation) of the protein have been borne out by experiment. Acetylation decreased water binding, increased solubility in the pH 4.5–7 range, and decreased gel strength of soy protein isolate. A shift in molecular weight distribution of the protein to lower molecular weight species also occurred. This work shows that the functional properties (in a food use context) of soy proteins can be altered by chemical modification and that the effect of a particular chemical modification of these properties can be predicted.

In the fabrication of high protein foods, increasing use is made of soybean-derived material. It is possible to extract from soybeans the bulk of the intracellular protein in a form that contains no less than 90% protein. These protein isolates are of interest not only for their nutritional value, but because they possess physical properties that lend themselves to use in the fabrication of a variety of foods. Of particular interest is their ability, when dispersed in highly concentrated aqueous solutions (e.g., 20% protein), to form thermally irreversible, elastic gels upon heating (Wu and Inglett, 1974; Circle et al., 1964).

In addition to the ability of the protein to form gels, the water-binding ability of soy proteins is also of interest. It is well known that microbial growth in a food is dependent upon water activity ($a_w$) (Scott, 1957; Labuza et al., 1970; Troller, 1973). Binding of water to the protein and carbohydrate constituents of a food results in decreased $a_w$. Therefore, changes in water binding by food proteins could result in changes in microbial growth, depending upon the total water content of the food. These changes would be most apparent in the so-called intermediate moisture content regions ($0.6 \lesssim a_w \lesssim 0.9$).

Also of importance for the use of soy protein in foods is the solubility behavior as a function of pH (Wolf and Cowan, 1971). The solubility of typical soy protein isolates goes through a minimum at pH ~4.5 (Wolf and Cowan, 1971). This fact is useful in the preparation of most soy isolates, but also may restrict usefulness in many food applications. In applications where solubility is important it would be advantageous to be able to control solubility as a function of pH (e.g., increase solubility in the pH ~4.5–7 range without resorting to protein hydrolysis).

The purpose of the work presented here was to carry out a simple chemical modification of soy protein isolate and measure several properties of the modified protein relevant to its food use (e.g., gel behavior, solubility, water binding). Chemical modification of proteins has been used extensively for the purpose of determining functional groups of enzymes and the mode of action of drugs (Shaw, 1970). However, studies of the effect of chemical modification of proteins on their functional properties in a food use context have been more sparse. Acetylation was chosen as the chemical modification because of its specificity for free amino groups, principally lysine (Riordan and Vallee, 1971). Esterification of free amino groups with the neutral acetyl group will result in a reduction in the positive charge

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Table I. Amino Acid Composition (Rackis et al., 1961) and Moles Bound (Unfrozen at −35 °C) of Water per Gram of Protein (Kuntz, 1971) for Soy Protein Isolate

| | Residues/100000 g of protein | $10^4$ mol of bound $H_2O$/ g of protein |
|---|---|---|
| Ala | 50 | 7.5 |
| Arg | 52 | 15.6 |
| Asp | 97 | 58.2 |
| Cys | 4 | 0.4 |
| Glu | 159 | 119.3 |
| Gly | 61 | 6.1 |
| His | 18 | 7.2 |
| Hyp | | 0 |
| Ile | 38 | 3.8 |
| Leu | 60 | 6.0 |
| Lys | 39 | 17.6 |
| Met | 9 | 0.9 |
| Phe | 36 | 0 |
| Pro | 57 | 17.1 |
| Ser | 55 | 11.0 |
| Thr | 32 | 6.4 |
| Trp | 5 | 1.0 |
| Tyr | 40 | 12.0 |
| Val | 44 | 4.4 |
| Total | 856 | 294.5 |

of the isolate under acidic conditions. This loss of positively charged groups should result in a shift in the isoelectric point to lower pH values, thus increasing solubility in the pH ~4.5–7 range. Moreover, the loss of these charged groups should result in a decreased number of water molecules bound per protein molecule (Kuntz, 1971) and also reduce ionic attraction between neighboring molecules that are responsible in part for stabilization of protein gels (Catsimpoolas et al., 1970; Ferry, 1948). Therefore, we predict this modification to result in increased pH 4.5–7 solubility, decreased water binding, and inhibition of thermal gelation.

In order to simplify and organize our thinking about the gelation, water binding, and solubility properties of the heterogeneous material referred to as soy protein isolate, it is instructive to visualize it as a "model system" consisting of a randomly folded globular protein of molecular weight ~$10^5$ and amino acid composition given in Table I (average molecular weight and amino acid composition of the various soy proteins).

Models (discussed later) which have been developed for the hydration (Kuntz, 1971; Hansen, 1976) and for the gelation of protein (Ferry, 1948) can be used to predict the effect of a particular chemical modification of the protein on these properties. The agreement of these predictions with experimentally measured parameters related to hydration and gelation is a test of the correctness and utility of the models and should provide insight into means of tailoring the properties of the protein for specific food uses.

**Protein Preparation and Modification Procedure.** Soy protein concentrate (69% protein (% N × 6.25); 6.1% ash; 0.4% lipid) was suspended in 10 parts of water. This suspension was stirred continually with the pH adjusted to pH 11.0. The pH was held constant at 11.0 by the continuous addition of 1.0 N NaOH. After 1 h the slurry was spun at ~10000*g* to remove insolubles, leaving a clear amber supernatant. The supernatant was then adjusted to pH 7.0 with 1.0 N HCl. This solution was then doubled in volume by the addition of 100% ethanol. Protein precipitated and was collected by centrifugation. The pellet was resuspended in 50:50 water/ethanol to wash and then respun. This was repeated twice. The protein was then lyophilized. This protein isolate was 95% protein (% N × 6.25) and 3.4% ash.

The acetylation procedure used is similar to that of Riordan and Vallee (1971). Acetic anhydride was added slowly to a 2.5% dispersion of soy isolate in water with concurrent addition of sodium hydroxide to maintain the pH at 7.5. A sixfold molar excess of acetic anhydride was added based on an assumed average protein molecular weight of $10^5$ and 39 lysines per protein molecule. After reaction was complete (pH remained constant with time), the protein was precipitated by the addition of absolute ethanol to 50% by volume, collected by centrifugation at $10^4g$ for 20 min, washed with a 50% aqueous ethanol solution, recentrifuged, and freeze-dried (recovery of 80% of starting protein).

**Protein Assay Procedure.** The number of free amino groups present in the protein before and after modification was monitored by reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Fields, 1971). The protein was dissolved in 0.1 M $Na_2B_4O_7$ buffer at pH 9.5; 1.1 M TNBS was added with rapid mixing. The extent of reaction was followed by absorbance measurements at 420 nm (TNBS sulfite complex) on a Cary Model 15 spectrophotometer.

**Physical Measurements.** The hydration states of the original and modified protein were compared by: (1) NMR determination of unfrozen (bound) water at temperatures below 0 °C (Kuntz, 1971; Hansen, 1976); (2) measurements of water activity in protein samples hydrated to various total water contents using a Sina electric hygrometer. Gel strengths were determined qualitatively by preparing 20% dispersions of protein in water in 12-mm diameter test tubes, heating at 100 °C for 15 min and cooling to ambient temperature, followed by visual examination of the gels produced. Sedimentation velocity distribution was measured for 1.0% solutions of protein in phosphate buffer (pH 7.6, $\mu = 0.5$) using a Beckman Model E ultracentrifuge. The pH dependence of protein solubility was determined by absorbance measurements (Cary 15) at 263 nm on solutions originally containing 0.1% protein, adjusted to various pH values with 1 N HCl, and clarified by ultracentrifugation at 40000*g* when necessary.

## RESULTS AND DISCUSSION

**Extent of Modification and Effect on Quaternary Structure.** Assays of the unmodified and modified protein gave 41 and 0.6 mol of free amino groups per $10^5$ g of protein, respectively. The specificity for amino groups of the acetylation reaction was not determined. However, acetic anhydride has been shown by other workers to be relatively specific for amino groups of proteins under the conditions used here (Fraenkel-Conrat, 1959; Riordan and Vallee, 1971). Amino acid analysis revealed that the composition of the isolate was unaltered by acetylation, which suggests that acetylation is reversible upon acid hydrolysis. Acetylation may thus afford protection of the labile lysine residues in a food product, giving rise to a reduction in nonenzymatic browning and therefore to improved nutritional stability.

Bjarnason and Carpenter (1969) showed that acetylated bovine plasma albumin has, after heating, a higher nutritional equivalency than the unmodified protein, when fed to young rats on lysine-deficient diets. Moreover, Paik and Benoiton (1963) demonstrated that mammalian kidneys contain an $\epsilon$-lysine acylase that is capable of hydrolyzing $N^{\epsilon}$-formyl- and acetyllysines.

The sedimentation velocity distribution was measured to determine if extensive breakup of protein into lower molecular weight subunits occurred during reaction (see Figure 1). The 11S fraction of protein disappeared with a subsequent increase in lower molecular weight 2S and 7S components. This dissociation of the quaternary

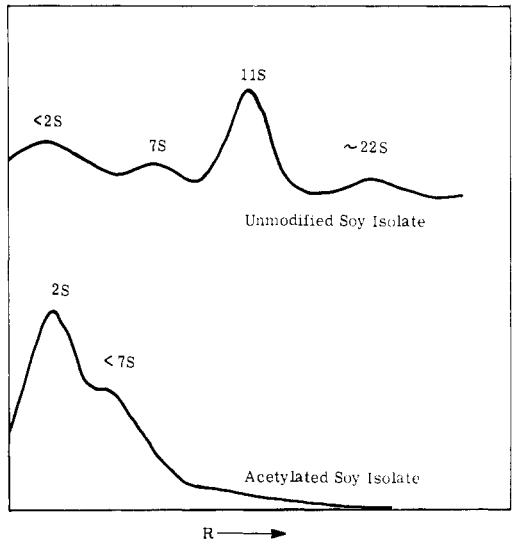


**Figure 1.** Sedimentation velocity distribution for unmodified and acetylated soy protein isolates.

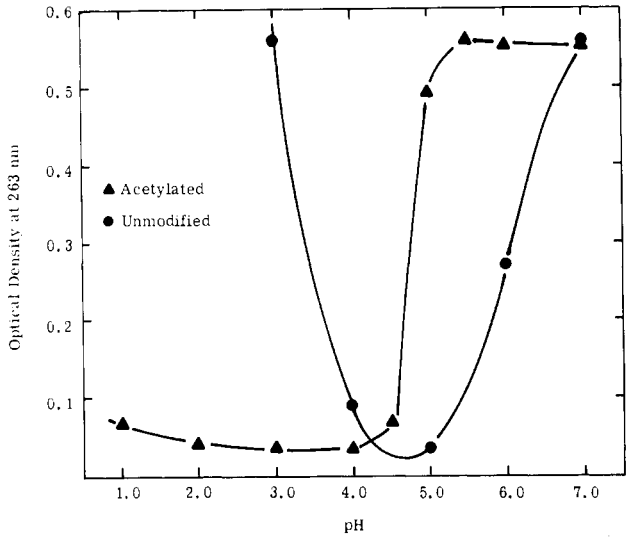


**Figure 2.** Solubility of unmodified and acetylated soy protein isolates as a function of pH.

**Table II. Water Binding and Activity Data for Soy Protein Isolate before and after Acetylation**

| $H_2O$ binding (at 2 g of $H_2O$/g of solids total hydration | Unmodified soy isolate: 0.49 g of bound $H_2O$/g of solids | Acetylated soy isolate: 0.39 g of bound $H_2O$/g of solids |
|---|---|---|
| Water act. ($a_W$) at | | |
| 2 g of $H_2O$/g of solids | ~1.0 | 0.98 |
| 0.5 g of $H_2O$/g of solids | 0.96 | 0.94 |

structure of the 11S globulins accompanying the acetylation suggests a role for lysine in stabilizing the multisubunit structures, perhaps through intrasubunit hydrogen bonds which were lost in acetylation.

The assay and sedimentation velocity distribution data show that the modification procedure was at least 98% complete in acetylating the free amino groups of the protein, with some breakdown of the 11S fraction of protein into lower molecular weight (2S and 7S) subunits.

**Protein Hydration.** The water binding (measured by NMR) and water activity data for soy isolate before and after acetylation are shown in Table II. The modification is seen to decrease the water binding capacity of the protein by about 20% (0.39 vs. 0.49 g/g) and to result in a somewhat lower water activity.

The hydration model treats initial water sorption as a site-binding phenomenon, in which discrete numbers of water molecules are associated with the various amino acid residues (Kuntz, 1971; Hansen, 1976). Knowledge of the overall amino acid composition of a protein then allows the maximum water binding capacity of the protein to be calculated assuming all sites are exposed (Table I). This calculated maximum water binding capacity of the unmodified soy isolate is 0.50 g of water/g of solids. The corresponding value calculated for the acetylated soy isolate (based on loss of hydration of lysine plus addition of amide linkages (Bull and Breese, 1968)) is 0.42 g of water/g of solids. The values measured for "bound" water (grams of unfrozen water at −50 °C/gram of solids) by NMR for unmodified and acetylated proteins are thus in good agreement with the prediction of the hydration model. The slightly lower water activities found for the acetylated isolate are unexpected in light of the decreased water binding, but may simply be the result of having more lower molecular weight species per unit mass of protein (i.e., an osmotic effect), or of residual sodium acetate (although ash values for the modified and original proteins were the same within ±10% of the ash values and much too low to account for the observed $a_w$ reduction).

**Solubility and Gelation.** Figure 2 shows the pH dependence of solubility (at the 0.1% level, expressed as optical density of the protein solution at $\lambda_{max}$ 263 nm. The solubility of the acetylated material is greatly increased between pH 5 and 7, dropping significantly below pH 4.5, whereas the solubility of the unmodified isolate begins to drop below pH 7.

Gel strengths of the acetylated and unmodified soy isolate at the 20% level, after heating to 100 °C, were compared qualitatively. The unmodified isolate gave a firm, rubbery, thermally irreversible gel, while the acetylated material did not change much upon heating and cooling, i.e., it remained highly viscous but did not gel. The apparent viscosities of 20% solutions of the acetylated soy isolate were considerably higher than those of the corresponding unmodified isolate at 20 and 75 °C, although a quantitative study as a function of temperature and protein concentration was not done. These results are consistent with findings of other workers on the effect of acylation of other food proteins on their heat stability. Succinylation of fish myofibrillar protein resulted in increased stability against coagulation or precipitation in water at pH 6–8.5 (Groninger, 1973). Acetylation of skim milk protein and serum albumin resulted in modified proteins which could be pasteurized (80 °C, 30 min) without coagulation (Van Roon, 1970). Reaction of egg white with 3,3-dimethylglutaric anhydride resulted primarily in substitution at lysine groups, giving a material less susceptible to heat coagulation whose foam formation properties were not seriously altered (Gandhi et al., 1968).

In conclusion, we have found that reducing the total positive charge of soy protein by acetylation of free amino groups did indeed increase solubility in the pH 4.5–7 range (lower isoelectric point). In addition, reduction of total charge should reduce the tendency to form ionic bonds (which are thought to be responsible in part for stabilization of protein gels) and thus inhibit thermal gel formation, which was observed to be the case. Water binding was decreased by the modification, as predicted. These results suggest that some control of the functional properties of soy proteins can be accomplished, in a

predictable way, by chemical modification.

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# Hexose–Amino Acid Degradation Studies Involving Formation of Pyrroles, Furans, and Other Low Molecular Weight Products

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Degradation reactions involving fructose and alanine, fructose and γ-aminobutyric acid, and rhamnose and alanine were carried out at pH 3.5; and the volatile, ether-soluble products, which included pyrrole and furan derivatives, were identified. The only derivatives formed in appreciable quantities by the rhamnose–alanine reaction were 5-methylfurfural and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone. The fructose–amino acid reactions produced several furans plus 2-acetylpyrrole and 5-methylpyrrole-2-carboxaldehyde, both of which have been reported as browning products in stored instant orange juice. A critical step in formation of the pyrroles by the fructose–alanine reaction is believed to be a Strecker degradation of alanine with a 1,2-hexosulose intermediate. By this reaction the hexosulose is reduced and incorporates the nitrogen atom.

Pyrroles and furans have been isolated from many foods whose flavors had been improved or impaired by nonenzymic browning (Shigematsu et al., 1972, and references therein). They are believed to be formed during nonenzymic browning, which results from interaction between sugars and amino acids present (Hodge, 1953). Since many pyrroles and furans have powerful flavors and aromas, more knowledge of their origin might enable food scientists to control their formation to either enhance desirable flavors or minimize off-flavors. Pyrroles and furans were shown to form in dehydrated instant orange juice (IOJ) during storage at ambient temperature (Tatum et al., 1967) and to contribute to its off-flavor which developed during storage (Shaw et al., 1970). In the latter study, *N*-ethylpyrrole-2-carboxaldehyde had the lowest flavor threshold of any browning product found in stored IOJ.

Model studies have shown that pyrroles can be produced by a Maillard-type reaction between hexoses and either amines or amino acids. Thus, Langner and Tobias (1967) identified 2-acetylpyrrole as a degradation product from heated model systems containing dextrose and lysine or lactose and lysine. Ferretti and Flanagan (1971, 1973) identified two 2-acetylpyrroles and three pyrrole-2-carboxyaldehydes from lactose–amino acid model systems. Kato and Fujimaki (1970) heated aqueous, glucose–alkylamine solutions and obtained several pyrrole carboxyaldehydes as degradation products. Shigematsu et al. (1972) roasted a glucose–alanine mixture in the dry state and obtained pyrazine and pyrrole derivatives. Kato (1967) treated pentoses and hexoses with alkylamines and proposed a mechanism for formation of the pyrrole-2-carboxyaldehydes that he found as degradation products of these reactions. The formation of furans in sugar–amino acid model systems has been reviewed by Anet (1964) and by Reynolds (1965). Acid-catalyzed degradations of sugars to furan derivatives have also been studied and related to nonenzymic browning in foods (Reynolds, 1965; Shaw et al., 1967). Many furans are formed from sugars in acid or amino acid catalyzed reactions that involve enolization and dehydration but not reduction, oxidation, or fragmentation (Anet, 1964; Hodge, 1953).

We report the degradation reactions of aqueous fructose–amino acid and rhamnose–amino acid solutions and propose a mechanism for formation of the pyrrole derivatives from hexose–amino acid degradation.

## EXPERIMENTAL SECTION

**General Conditions for Hexose–Amino Acid Degradation Reactions.** A solution of 0.81 mol of hexose (D-fructose or α-L-rhamnose) and 0.34 mol of amino acid (DL-α-alanine or γ-aminobutyric acid) in 325 mL of water

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