Reversible Modification of Lysine in Soybean Proteins, Using Citraconic Anhydride: Characterization of Physical and Chemical Changes in Soy Protein Isolate, the 7S Globulin, and Lipoxygenase

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The effects of reversible acylation of lysine in soybean proteins, using citraconic anhydride, were studied. Approximately 95% of the lysine residues in soy protein isolate, the 7S soy globulin, and lipoxygenase-1 could be acylated near pH 8.0. Optimal conditions for the complete decitraconylation of soy protein isolate were determined. Electrophoretic, fluorescent, and pH solubility studies showed a significant alteration in the charge and structure of the 7S globulin after citraconylation. Upon deacylation under relatively mild acidic conditions, the properties characteristic of the native proteins were mostly restored. The effects of decitraconylation on the activity of lipoxygenase-1 were somewhat obscured by partial inactivation at the pH of deacylation, but a substantial dependence of activity on ionic strength was found with the citraconylated enzyme. The results are discussed in terms of reversible acylation as a potential method of improving the processing, functional, and nutritional properties of food proteins.

The growing demand for food grade proteins as supplements to protein deficient diets and as nutritional and functional ingredients in formulated foods has led to the investigation of several novel protein sources or to the reevaluation of traditional ones. Nonanimal proteins, because of their potentially greater availability and lower cost, have generated particular interest, especially soybean proteins (Wolf and Cowan, 1975; Kinsella, 1979). However, limited functional properties and inferior organoleptic qualities have been major factors preventing the maximum utilization of these and other novel proteins (Kinsella, 1976; Rackis et al., 1979).

Methodology currently employed to improve the functional characteristics of proteins, such as partial hydrolysis by proteolysis or extremes of pH, has limitations because of the formation of undesirable products, e.g., enzymatically formed bitter-tasting peptides (Arai et al., 1975) or alkali-induced lysinoalanine (Provansal et al., 1975). Hence, innovative methods are needed to circumvent these problems and to facilitate the exploitation of less functional and underutilized proteins.

Chemical derivatization of ϵ-amino groups of lysine residues by acylation with succinic anhydride has been suggested as a method of improving the functionality of food proteins (Kinsella and Shetty, 1978). The enhancement of some functional properties by succinylation of the following proteins was observed: casein (Creamer et al., 1971), vegetable proteins (Melnychyn and Stapley, 1973), wheat flour proteins (Grant, 1973), fish myofibrillar protein (Groninger, 1973; Groninger and Miller, 1975; Chen et al., 1975), leaf and soy protein (Franzen and Kinsella, 1976a,b), and yeast protein (Shetty and Kinsella, 1979). Acylation of lysine residues was also shown to minimize heat damage in proteins, i.e., prevent Maillard reactions (Bjarnason and Carpenter, 1969), and was suggested as a possible method of reducing the formation of lysinoalanine during alkali treatment (Friedman, 1977).

Ideally, chemical modification of food proteins should enhance functional properties without impairing their nutritive value (Feeney, 1977). Therefore amino acylating reagents which are structurally similar to succinic anhydride but form an acid labile amide bond with the lysine ϵ-amino group of proteins, e.g., citraconic (Dixon and Perham, 1968) and maleic (Butler et al., 1969) anhydrides, have potential applications because of the relative ease of deacylating the lysine derivatives under mildly acidic conditions. In addition to the possible use of these reagents as blocking groups to protect lysine residues during heat or alkali processing, or as a means of improving protein functionality, the possible spontaneous deacylation of proteins derivatized with these reagents in the acidic environment of the stomach to an extent which would allow complete digestion and absorption to occur is significant. Hence, modification of food proteins with reversibly acylating anhydrides may provide a novel method of enhancing functional properties and minimizing lysine side reactions during processing without sacrificing nutritional quality. Of the various reversible acylating reagents available, citraconic anhydride appears to be the most effective and practical (Habeeb and Atassi, 1970; Gibbons and Perham, 1970; Singhal and Atassi, 1971; Nakagawa and Perlmann, 1972; Nakagawa et al., 1972).

This study was conducted to determine the effects of reversible citraconylation of lysine residues on some physical and chemical properties of various soybean proteins (soy protein isolate, the 7S globulin, and lipoxygenase) in order to assess the potential of reversible acylation as a method of modifying the physical properties of food proteins and/or protecting lysine against the Maillard reaction.

MATERIALS AND METHODS

Reagents. Citraconic anhydride was purchased from Eastman Chemicals (Rochester, NY). Trinitrobenzenesulfonic acid (TNBS) and linoleic acid (99% pure) were obtained from Sigma Chemical Co. (St. Louis, MO). Electrophoresis reagents were purchased from Bio-Rad Laboratories (Richmond, CA). All other chemicals were of analytical grade.

Proteins. Soy protein isolate (SPI) was prepared according to Puski and Melnychyn (1968) from minimally heat-treated, defatted soy flour (Central Soya, Chicago, IL). The major 7S soy globulin (β-conglycinin) was purified from soy flour by the method of Thanh and Shibasaki (1976a). Soybean lipoxygenase-1 (EC 1.13.1.13), highest purity form, was purchased from Sigma Chemical

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Co. (St. Louis, MO) and used without further purification.

Protein Determination. Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. Similar results were obtained by the micro-Kjeldahl method (AOAC, 1970), using a factor of 6.25.

Citraconylation. Citraconic anhydride was directly added from a microliter syringe to solutions of SPI and 7S globulin (8–10 mg/mL in 0.1 M sodium phosphate, 0.02% sodium azide, pH 8.0) and to solutions of lipoxygenase (1 mg/mL in 0.1 M sodium phosphate, 0.5 M NaCl, pH 8.0, plus a few drops of toluene) in amounts necessary to obtain desired molar ratios of anhydride to lysine. The pH was maintained at 7.6–8.0 with 1.0 N NaOH until the reaction was complete (5–10 min). The excess reagent was removed by dialysis for 12 h against buffer or water (adjusted to pH 8). The derivatized protein was used immediately or lyophilized.

Decitraconylation. After modification with an excess of citraconic anhydride and dialysis (12 h) against the same phosphate buffer, citraconylated SPI was treated under various conditions to determine the optimum pH, temperature, and time required for deacylation (i.e., decitraconylation) of lysine, e.g., 2 h at 35 °C in the pH range 3.0–6.8, 2 h at pH 3.8 at 5–50 °C, and pH 3.8 at 35 and 50 °C for 0–4 h (Figures 2–4). For example, the citraconylated 7S globulin was most effectively deacylated by incubation at pH 3.8 for 3 h at 35 °C Acidification was done with 1.0 N HCl. Native, i.e., unmodified, control samples were treated similarly. After deacylation treatment aliquots of the protein solutions were readjusted to pH 8.0 with 1.0 N NaOH. In a few cases, where if necessary, the samples were brought to pH 9–10 to enhance solubilization and then were readjusted to pH 8.0 with 1.0 N HCl. The deacylated 7S was used directly or dialyzed against water and lyophilized for subsequent study.

After treatment with a 10 molar excess of citraconic anhydride to lysine and dialysis, the citraconylated lipoxygenase samples were deacylated at 35 °C for 0–56 h at pH 6.0 and pH 8.0, respectively. Subsequently aliquots of the deacylated enzyme were adjusted to pH 8–9 with 1.0 N NaOH and assayed for free lysine and specific activity.

Lysine Assay. The extent of lysine modification or deacylation was determined by a modification of the trinitrobenzenesulfonic acid (TNBS) method of Hall et al. (1973). Protein solutions (pH 8) containing 40–200 μg of lysine in 10-mL graduated tubes with sintered glass stoppers were diluted to 0.8 mL with distilled water, followed by 1.0 mL of 4% $NaHCO_3$ and 0.2 mL of 1.25% TNBS. The samples were incubated for 2 h at 40 °C, and the reaction was stopped by the addition of 3.5 mL of concentrated HCl. After hydrolysis at 103–105 °C for 4 h the samples were diluted to 10 mL, cooled, and extracted twice with 5 mL of anhydrous diethyl ether. Blanks were prepared by switching the order of TNBS and HCl additions. The absorbance of duplicate samples and blanks was read at 415 nm against water. The percentage of free lysine was calculated from the ratio of absorbance of the sample to that of the unmodified native protein after standardization of the protein concentrations.

Electrophoresis. The native, modified and deacylated proteins were examined by electrophoresis. The buffer system of Davis (1964) was used for polyacrylamide gel electrophoresis (PAGE) of 7S globulin derivatives on a 1.0 mm thick slab containing separating and stacking gels of 7.0% and 2.5% acrylamide, respectively. EDTA (1 mM) was included in the gels and running buffer. Lyophilized protein samples were dissolved (1–2 mg/mL) in 50 mM Tris–HCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 20% sucrose, 0.0125% bromphenol blue, pH 6.7, and were applied to the stacking gel in 10–30-mL aliquots. The gels were run at 8–10 mA for 3.5–4.0 h, fixed, and stained in 0.05% Coomassie Brilliant Blue R-250, 10% acetic acid, 50% methanol, then destained in 10% acetic acid, 10% methanol.

A modification of the method of Laemmli (1970) was used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis ($NaDodSO_4$–PAGE). Linear gradient separating gels were prepared by using equal volumes of 5% and 20% acrylamide solutions, each containing 0.38 M Tris-HCl, 0.1% $NaDodSO_4$, 1 mM EDTA, 0.025% (w/v) *N,N,N′,N′*-tetramethylethylenediamine (TEMED), and 0.046% ammonium persulfate (added immediately before gradient formation). The 5% and 20% solutions contained 0.067% and 0.267% bisacrylamide, respectively, and the latter solution was made 9.7% (v/v) in glycerol. Stacking gells were composed of 0.125 M Tris-HCl, 0.1% $NaDodSO_4$, 1 mM EDTA, 0.025% (w/v) TEMED, 0.046% ammonium persulfate, and 4.0% acrylamide with 0.106% bisacrylamide. The purified 7S globulin (1–2 mg/mL) was made up in 10 mM Tris-HCl, 2.0% $NaDodSO_4$, 1.0% 2-mercaptoethanol, 8 M urea, 1 mM EDTA, 0.025% bromphenol blue, pH 6.8 dissociating buffer and incubated at 40 °C for 20 min prior to loading.

The citraconylated 7S protein bands in the polyacrylamide gel slices were subsequently separated on $NaDodSO_4$–PAGE by a modified method of Cleveland et al. (1977). After fixing and staining the excised gel slices, those containing the 7S protein were rinsed with distilled water, swirled for 30 min in 10 mL of 0.125 M Tris-HCl, 0.1% $NaDodSO_4$, 1 mM EDTA, pH 6.8 buffer, and then crushed with a glass rod in 25 μL of dissociating buffer. After 20 min at 40 °C the crushed gel slices were placed into the wells of the previously described 5–20% $NaDodSO_4$–PAGE gel and overlaid with 20 μL of dissociating buffer.

All samples were electrophoresed at 15 mA for 3.5–4.0 h with 25 mM Tris-HCl, 0.192 M glycine, 0.5% $NaDodSO_4$, 1 mM EDTA, pH 8.3 running buffer. Gels were fixed in 10% acetic acid and 50% methanol, stained in 0.05% Coomassie Brilliant Blue R-250 and 10% acetic acid, and 50% methanol, and destained in 10% acetic acid, 10% methanol.

pH Solubility. Solutions of 7S derivatives were adjusted to pH values ranging from 3 to 7 (with 0.1 N HCl and 0.1 N NaOH) and brought to final concentrations of 0.5 mg/mL protein in 2.0 mL of 17 mM sodium phosphate. The sample turbidity was then measured at 600 nm against water.

Fluorescence Spectrophotometry. The fluorescence spectra of 7S derivatives were recorded with a Model 650-40 fluorescence spectrophotometer with a Model 56 recorder and a Model 150 xenon power supply (Perkin-Elmer, Norwalk, CT), using an excitation wavelength of 285 nm. Samples having 280 nm (1 cm) absorbance values of 0.100 were prepared in 0.1 M sodium phosphate, pH 8.0, and their spectra were recorded in quartz cuvettes at 25 °C.

Lipoxygenase Assay. The activity of lipoxygenase-1 derivatives was determined by the method of Chan (1973), using 0.1 mM linoleic acid in 0.2 M borate, pH 9.0 at 25 °C. Between 5 and 20 μL of approximately 0.1% enzyme solutions were added to the substrate solution to give a 3.00-mL final volume. The samples were immediately mixed, and the initial linear rate of increase in absorbance

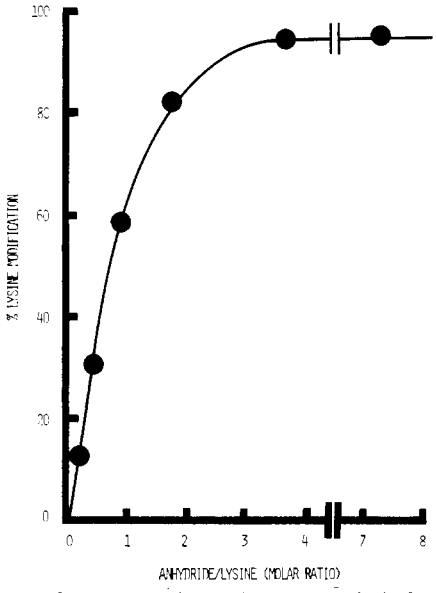

Figure 1. Modification of lysine in soy protein isolate at pH 8.0 with various molar ratios of citraconic anhydride. Citraconylation and lysine determination were performed as described under Materials and Methods.

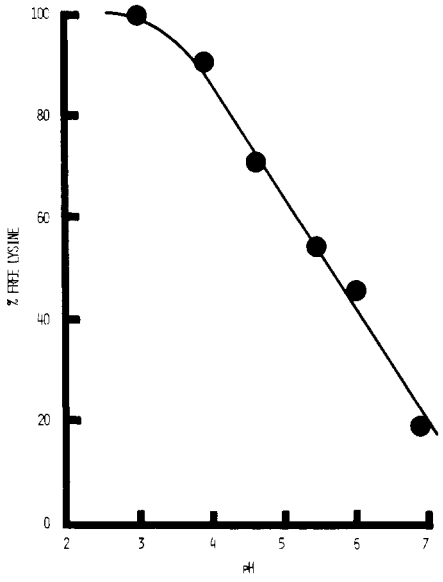

Figure 2. Extent of lysine decitraconylation in citraconylated soy protein isolate after 2 h at 35 °C as a function of pH. Decitraconylation was performed as described under Materials and Methods.

at 234 nm was determined. One unit of activity was defined as a 0.001 increase in absorbance per minute, which is equivalent to the oxidation of 0.12 μmol of linoleic acid per minute. Specific activity was defined as units of activity per milligram of protein.

RESULTS AND DISCUSSION

Citraconylation. The acylation of lysine residues in SPI with varying levels of citraconic anhydride at pH 8.0 was 95% complete at molar ratios of anhydride to lysine near 5 and above (Figure 1). Nearly stoichiometric modification (to approximately 50%) occurred at ratios below one, indicating that about one-half of the lysine residues are at, or very near, the surface of the proteins in SPI, while the remaining residues are probably sterically hindered or buried within the protein structure.

The 7S globulin also underwent 95% lysine acylation at a fivefold molar excess of anhydride to lysine, whereas lipoxygenase revealed only 80% lysine modification at this level of anhydride and required a molar excess of 10 to achieve 93% modification.

Effect of Incubation Conditions on the Extent of SPI Lysine Decitraconylation. A significant dependence of lysine deacylation on pH was demonstrated when citraconylated SPI (with 95% lysine residues modified) was incubated for 2 h and a temperature of 35°C at various pH values (Figure 2). As the pH was decreased from 7.0 to 3.0 the amount of free lysine (i.e., deacylation) increased from 20% to almost 100%. This observation is consistent with the acid-catalyzed mechanism of deacylation proposed by Butler et al. (1969) for the structurally similar amino derivatives of maleic anhydride.

Although the extent of deacylation was greater at low pH, when deacylation was carried out below pH 3.8, the deacylated protein precipitated and could not be resolubilized even at pH values as high as 10. The deacylated protein demonstrated better solubility after pH 3.8 incubation if acidification of the citraconylated protein was done with 1.0 N HCl rather than with glacial acetic acid.

The decitraconylation of SPI was also very dependent on the temperatures of incubation. At pH 3.8 for 2 h the degree of lysine deacylation varied widely in the temperature range of 5–50 °C (Figure 3). Above 20 °C the extent

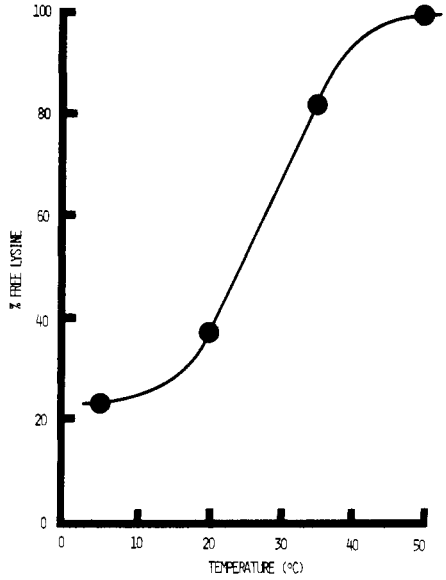

Figure 3. Extent of lysine decitraconylation in citraconylated soy protein isolate after 2 h at pH 3.8 as a function of temperature. Decitraconylation was performed as described under Materials and Methods.

of regeneration of free lysine was enhanced compared to that obtained at lower temperatures. Incubation above 40 °C was required to deacylate all of the lysine residues under these conditions.

Complete decitraconylation of lysine at 35 and 50 °C required approximately 1 and 3 h, respectively, at pH 3.8 (Figure 4). Thermal denaturation which occurred at 50 °C substantially reduced the solubility of the deacylated protein at pH 10, even after only 20 min at pH 3.8. However, when deacylation was done at 35 °C resolubilization was complete within a minute at pH 9–10.

Due to the insolubility of decitraconylated SPI after incubation at low pH or high temperature, the optimal conditions chosen for complete lysine deacylation were pH 3.8, 3 h, and 35 °C.

Effects of Reversible Citraconylation of Lysine on the Structure of 7S Soy Globulin. Citraconylated 7S soy globulin (β-conglycinin) was also completely deacylated

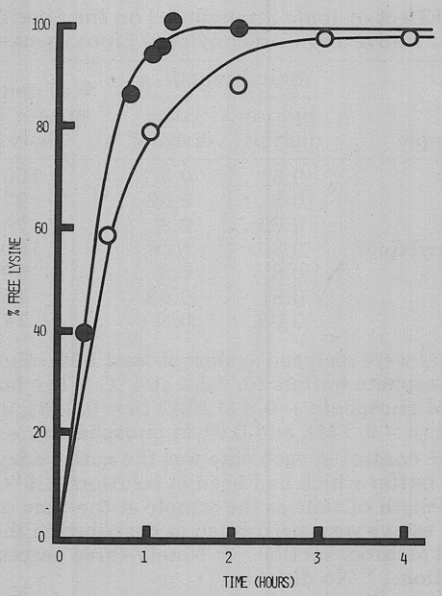

Figure 4. Rate of decitraconylation of lysine in citraconylated soy protein isolate at 35 °C (○) and 50 °C (●) at pH 3.8. Decitraconylation was performed as described under Materials and Methods.

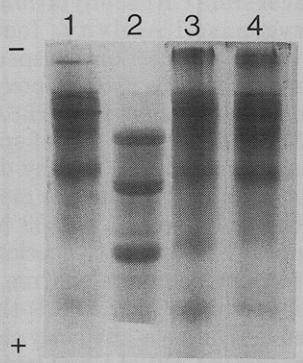

Figure 5. Polyacrylamide gel electrophoresis patterns of (1) native, (2) citraconylated, (3) decitraconylated, and (4) control 7S soy globulin. Seven percent slab gel and samples were prepared and run as described under Materials and Methods.

by using the optimal deacylating conditions determined from the SPI studies. There was no reduction in solubility at pH 8.0 following decitraconylation.

Polyacrylamide gel electrophoresis (PAGE) was used to study the effects of the reversible citraconylation of lysine on the charge characteristics of the 7S globulin (Figure 5). The electrophoretic heterogeneity of the native 7S globulin (lane 1) due to the association of its three major subunits into six trimeric species has been well established (Thanh et al., 1975; Thanh and Shibasaki, 1976b). After citraconylation of 95% of the lysine residues, a shift in mobility of the subunits toward the anode was observed with three distinct sets of bands having two very closely spaced bands discernible within each set (lane 2). Complete decitraconylation of the lysine residues regenerated the electrophoretic pattern of the native protein to a large extent (lane 3) though the bands were slightly more diffuse and a low mobility component appeared near the top of the separating gel. The control sample of 7S subjected to the deacylating treatment showed a pattern (lane 4) almost identical with that obtained with the decitraconylated protein.

Treatment of the deacylated sample with 1.0 M hydroxylamine for 1 h at pH 10 and 40 °C (Habeeb and Atassi, 1970) to deesterify any citraconylated hydroxyl amino acids gave no change in the electrophoretic pattern

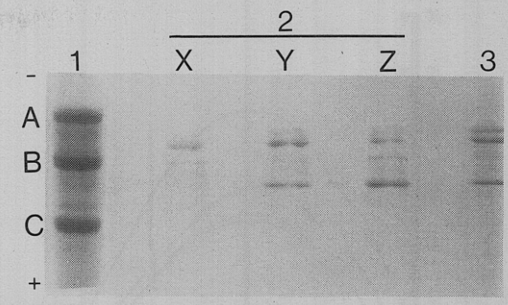

Figure 6. Fractionation of the three electrophoretically separated citraconylated 7S soy globulin groups. Bands (1A, 1B, and 1C) of the citraconylated 7S soy globulin from the PAGE gel were excised from the gel and run individually on an $NaDodSO_4$–polyacrylamide (5–20%) gradient slab gel where lanes 2X, 2Y, and 2Z correspond to bands 1A, 1B, and 1C, respectively. Native 7S was run as a subunit marker (lane 3). Electrophoresis and sample preparation were performed as described under Materials and Methods.

(not shown), indicating that only lysine derivatives were formed.

The appearance and similarity of the low mobility band in the decitraconylated and control 7S patterns suggested that some aggregation may have been caused by the thermal or acidic conditions of decitraconylation. The increased mobility of the citraconylated 7S sample was obviously due to the higher net negative charge caused by the introduction of several citraconyl carboxyl groups into the derivatized 7S globulins.

Further fractionation of the three bands obtained on PAGE for the citraconylated 7S protein by subsequent $NaDodSO_4$–PAGE electrophoresis allowed the subunit composition of each to be examined (Figure 6). When the mobilities of the subunits of the three PAGE bands were compared to those of α', α, and β subunits (in order of increasing mobility) of the native 7S protein, band A (lane 2X) contained primarily the α subunit, band B (lane 2Y) contained mostly α but also some β subunit, and band C (lane 2Z) contained mostly β with some α subunit. Small amounts of α' subunit were detected in each PAGE band. Although citraconylation would be expected to slightly increase the molecular weights of the 7S subunits and thereby decrease their $NaDodSO_4$–PAGE mobility, no differences in subunit mobility were observed because most likely the proteins in the polyacrylamide gel were deacylated by the acidic fixing and destaining solutions prior to analysis by $NaDodSO_4$–PAGE.

It is possible that the three citraconylated 7S PAGE bands correspond to the three classes of 7S isomers proposed by Thanh and Shikasaki (1978), which they grouped according to the number of β subunits (0, 1, or 2) in the trimeric structure. The observation that two components appear within each set of PAGE bands is further evidence for this conclusion since two isomers were assigned to each of the three classes (Thanh and Shibasaki, 1978).

The fluorescence spectra of the 7S samples (native, control, citraconylated, and decitraconylated) were compared to determine structural changes accompanying the various treatments as indicated by alterations in the environments of tyrosine and especially tryptophan (Figure 7). Although the tryptophan content of the 7S globulin is reported as only 0.3% (Koshiyama, 1968), the native 7S sample exhibited an emission maximum near 340 nm (characteristic of tryptophan fluorescence) and a shoulder near 310 nm (tyrosine). In comparison to the native protein a 25% reduction in fluorescent intensity was observed for the control, i.e., unmodified protein sample treated under the same conditions of deacylation. The

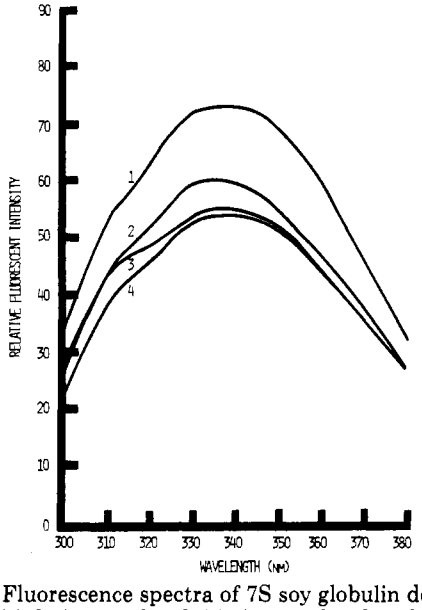

Figure 7. Fluorescence spectra of 7S soy globulin derivatives. (1) Native, (2) decitraconylated, (3) citraconylated, and (4) control 7S samples. Sample preparation and fluorescence measurements were performed as described under Materials and Methods.

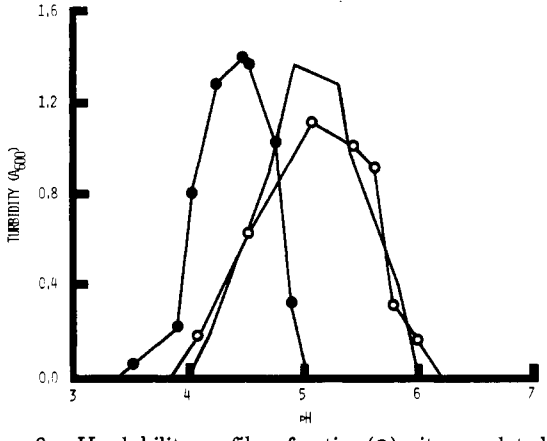

Figure 8. pH solubility profiles of native (○), citraconylated (●), and decitraconylated (—) 7S soy globulin. Measurements and sample preparation were performed as described under Materials and Methods.

spectrum of the citraconylated protein was nearly identical with the control sample; however, decitraconylation caused an increase in fluorescence accompanied by a shift in the emission maximum to 334 nm.

Quenching of fluorescence by exposure of the aromatic groups to the solvents was likely caused by slight acidic and thermal denaturation in the control and decitraconylated samples and by intramolecular Coulombic repulsion in the modified protein due to the negatively charged citraconyl groups. The increase in fluorescence of the deacylated sample over that of the control can be attributed to a partial refolding of the protein upon removal of the citraconyl groups. In addition, the blue shift suggests a change in the electronic environment of tryptophan after this refolding (Chen et al., 1969).

The pH solubility profiles of the various 7S derivatives (Figure 8) showed that citraconylation decreased the apparent p*I* of the native protein from 5.2 to 4.3 and also reduced the range of insolubility from 2.2 to approximately 1.5 pH units. The turbidity of the modified protein was measured within minutes after acidification to the various pH values, so negligible acid induced deacylation occurred, assuring that the profile reflected the properties of the fully modified sample. Complete deacylation, i.e., removal of the citraconyl carboxyl groups, resulted in a solubility profile similar to that of the native protein.

Table I. Effect of Ionic Strength (μ) on the Specific Activity of Native and Citraconylated Lipoxygenase-1

sample	ionic strength, μ: before dialysis	ionic strength, μ: after dialysis[a]	% of control[b] sp act.[c] after dialysis
native	0.8	0.3	103
	0.8	0.03	97
	0.03	0.8	75
citraconylated[d]	0.8	ND[e]	100
	0.8	0.3	99
	0.8	0.03	15
	0.03	0.8	14

[a] Samples were dialyzed against at least 500 volumes of pH 8.0 phosphate buffers for 12 h at 4 °C. The buffers were 0.1 M phosphate + 0.5 M NaCl (μ = 0.8 M), 0.1 M phosphate (μ = 0.3 M), and 0.01 M phosphate (μ = 0.03 M). [b] The control in each case was the native enzyme in μ = 0.8 M buffer which had been in solution at 4 °C for the same length of time as the sample at the time of assay. [c] Activity assays were performed as described in the Materials and Methods section. [d] Ninety-three percent lysine acylation. [e] No dialysis.

Effects of Reversible Citraconylation of Lysine on Soybean Lipoxygenase-1 Activity. Ionic strength (μ) was found to have a large effect on the specific activity of the citraconylated, but not the native, lipoxygenase (Table I). Modification of lipoxygenase-1 at pH 8.0 in phosphate buffer (μ = 0.8 M) resulted in no loss of specific activity. Reduction in ionic strength to 0.3 M by dialysis had no significant effects on either the native or modified enzyme; however, a further decrease to μ = 0.03 M reduced the specific activity of the citraconylated species to 15%, while there was no effect on the native enzyme.

At higher ionic strengths ($\mu \geq 0.3$ M) the structure of the citraconylated derivative is obviously stabilized by the electrostatic environment, but at lower ionic strengths the intermolecular charge repulsion caused by the negatively charged citraconyl groups is greater than the stabilizing effects of the buffer ions, resulting in structural changes which affect the catalytic site. Activity could not be restored by dialysis against the higher ionic strength buffer, indicating that irreversible changes occurred at low ionic strength. Further evidence is implied by the low activity of the sample at μ = 0.03 M when assayed in the relatively high ionic strength borate buffer. In an analogous study, Nakagawa et al. (1972) showed that immunoglobulin G underwent expansion and loss of β structure when citraconylated at low ionic strength, but regained its native form when dialyzed against a high ionic strength buffer.

Prior to the determination of the effects of decitraconylation on the specific activity of lipoxygenase-1, it was found that incubation of the native enzyme at acidic pH values caused significant irreversible inactivation, and the precipitated protein could not be resolubilized completely at pH 10. Treatment at pH 4.8 and 35 °C for 2 h resulted in a loss of 85% of specific activity when compared to a control at pH 8.0, but less than 40% was lost at pH 6.5 and 35 °C for 8 h. Therefore, as a compromise among enzyme activity, solubility, and rate of deacylation, incubation of citraconylated lipoxygenase-1 was done at pH 6.0 and 35 °C in μ = 0.8 M buffer (Figure 9). Within 8 h under these conditions the specific activity fell to approximately 50% of its original value and was constant thereafter although deacylation continued through 32 h to a maximum of 85%. Even at pH 8.0 there was a gradual reduction in specific activity to 65% of its original value.

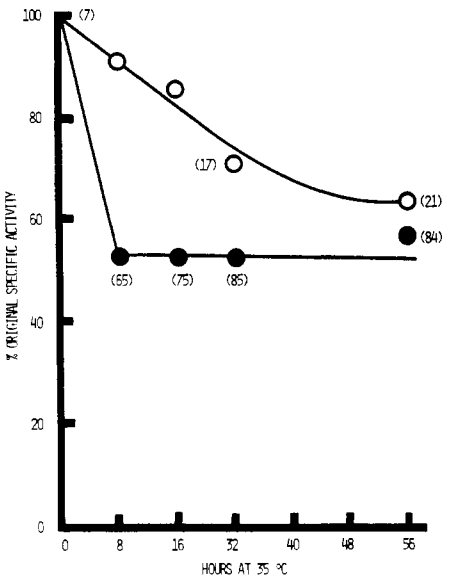

Figure 9. Effects of incubation (35 °C) at pH 6.0 (●) and pH 8.0 (○) on the specific activity of citraconylated lipoxygenase-1. Numbers in parentheses represent the percentage of free lysine. Specific activity and lysine assays were performed as described under Materials and Methods.

Decitraconylation of the modified enzyme at pH 8.0 was not unexpected since it was also observed with citraconylated lysozyme under similar conditions (Habeeb and Atassi, 1970). The observation that only about 85% of the lysine was deacylated at pH 6.0 suggests that the remaining residues may be more buried than the others and therefore require more rigorous conditions of deacylation. Similarly, the leveling off of deacylation at 20% free lysine at pH 8.0 indicates that more extreme conditions are necessary for further unblocking. Therefore, these are apparently different "types" of lysine residues in the enzyme with varying susceptibilities to deacylation.

SUMMARY AND CONCLUSIONS

Citraconylation of the lysine residues of SPI and the 7S globulin was shown to be a completely reversible chemical modification. Data on the pH, temperature, and time dependence of SPI lysine decitraconylation indicated that a variety of deacylating conditions can be employed although incubation at pH 3.8 and 35 °C for 3 h appears to minimize acidic and thermal denaturation without making the incubation time impractical. Under these conditions, electrophoretic and fluorescence data indicated that slight aggregation or unfolding occurred in the deacylated 7S globulin, but the pH solubility was nearly identical with that of the native protein.

Citraconylation of the 7S globulin caused a significant change in its electrophoretic pattern which was attributed to the fractionation of the six 7S isomers into three distinct groups. Since citraconylation magnifies the difference in their charge properties, this method may have potential in simplifying the isolation of the three 7S classes by anion exchange chromatography, which is known to give partial resolution of the native isomers (Thanh and Shibasaki, 1976a). Since the 7S globulin has no detectable cysteine or cystine residues (Thanh and Shibasaki, 1977), the interaction between citraconic anhydride and these residues was not investigated in this study; however, other results indicate that citraconylation of some proteins may irreversibly block sulfhydryl groups and/or induce disulfide bonding (Brinegar and Kinsella, 1980).

Since a hydrophobic binding site for fatty acid substrates is essential for the catalytic activity of lipoxygenase (Mitsuda et al., 1967), it is not surprising that blocking of hydrophilic residues such as lysine might have little effect on activity; however, at low ionic strength where the charge repulsion of the citraconyl groups cannot be stabilized, the resulting unfolding of the polypeptide could indirectly disrupt the active and/or binding sites. The data on lipoxygenase-1 decitraconylation were partially obscured by the pH inactivation but demonstrated that deacylation can proceed under alkaline conditions and that the lysine residues of the enzyme are probably deacylated in a stepwise manner, depending on the incubation conditions.

From a practical point of view, the ability of citraconylation to induce large changes in pH solubility (as shown with the 7S globulin) may be of use when a soluble protein ingredient is required in processed foods at a pH where the native protein is insoluble. The rate of deacylation could be minimized by low temperature. In addition, citraconylation could protect lysine residues from Maillard browning products or lysinoalanine formation during processing under conditions of high temperature, pH, and moisture. It can also be speculated that citraconylated food proteins could be deacylated at the temperature and low pH environment of the stomach, thereby making the previously blocked lysine residues nutritionally available.

Hence, citraconylation of food proteins may have potential as a method of improving the functionality of food proteins without permanently affecting their nutritional quality. In view of this, future studies will examine the functional, nutritional, and toxicological properties of citraconylated food proteins.

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LITERATURE CITED

Arai, S., Yamashita, M., Fujimaki, M., *Cereal Foods World* **20**, 107 (1975).
Association of Official Analytical Chemists, "Official Methods of Analysis", 11th ed., Washington, DC, 1970, p 858.
Bjarnason, J., Carpenter, K. I., *Br. J. Nutr.* **23**, 859 (1969).
Brinegar, C., Kinsella, J. E., unpublished material, 1980.
Butler, P. J. G., Harris, J. L., Hartley, B. S., Leberman, R., *Biochem. J.* **112**, 679 (1969).
Chan, H. W.-S., *Biochim. Biophys. Acta* **327**, 32 (1973).
Chen, L., Richardson, T., Ammundson, C., *J. Food Milk Technol.* **38**, 89 (1975).
Chen, R. F., Edelhoch, H., Steiner, R. F., in "Physical Principles and Techniques of Protein Chemistry", Part A, Leach, S. J., Ed., Academic Press, New York, 1969, p 171.
Cleveland, D. W., Fisher, S. G., Kirschner, M. W., Laemmli, U. K., *J. Biol. Chem.* **252**, 1102 (1977).
Creamer, L., Roeper, J., Lohrey, E., *N.Z. J. Dairy Sci. Technol.* **6**, 107 (1971).
Davis, B. J., *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).
Dixon, H. B. F., Perham, R. N., *Biochem. J.* **109**, 312 (1968).
Feeney, R. E., in "Evaluation of Proteins for Humans", Bodwell, C. E., Ed., Avi Publishing Co., Westport, CT, 1977, p 233.
Franzen, K. L., Kinsella, J. E., *J. Agric. Food Chem.* **24**, 788 (1976a).
Franzen, K. L., Kinsella, J. E., *J. Agric. Food Chem.* **24**, 914 (1976b).
Friedman, M., in "Food Proteins", Whitaker, J. R., Tannenbaum, S. R., Eds., Avi Publishing Co., Westport, CT, 1977, p 446.
Gibbons, I., Perham, R. N., *Biochem. J.* **116**, 843 (1970).
Grant, D., *Cereal Chem.* **50**, 417 (1973).
Groninger, H. S., *J. Agric. Food Chem.* **21**, 978 (1973).
Groninger, H., Miller, R., *J. Food Sci.* **40**, 327 (1975).
Habeeb, A. F. S. A., Atassi, M. Z., *Biochemistry* **9**, 4939 (1970).
Hall, R. J., Trinder, N., Givens, D. I., *Analyst (London)* **98**, 673 (1973).
Kinsella, J. E., *CRC Crit. Rev. Food Sci. Nutr.* **7**, 219 (1976).
Kinsella, J. E., *J. Am. Oil Chem. Soc.* **56**, 242 (1979).

Kinsella, J. E., Shetty, K. J., in "Nutritional Improvements of Food and Feed Proteins", Friedman, M., Ed., Plenum Press, New York, 1978, p 797.
Koshiyama, I., *Cereal Chem.* **45**, 394 (1968).
Laemmli, U. K., *Nature (London)* **227**, 680 (1970).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193**, 265 (1951).
Melnychyn, P., Stapley, R. (to Carnation Co.), Acylated Protein for Coffee Whitener Formulations, U.S. Patent 3 764 711, Oct. 9, 1973.
Mitsuda, H., Yasumoto, K., Yamamoto, A., *Arch. Biochem. Biophys.* **118**, 664 (1967).
Nakagawa, Y., Capetillo, S., Jirgensons, B., *J. Biol. Chem.* **247**, 5703 (1972).
Nakagawa, Y., Perlmann, G. E., *Arch. Biochem. Biophys.* **149**, 476 (1972).
Provansal, M. M. P., Cuq, J.-L. A., Cheftel, J.-C., *J. Agric. Food Chem.* **23**, 938 (1975).
Puski, G., Melnychyn, P., *Cereal Chem.* **45**, 192 (1968).
Rackis, J. J., Sessa, D. J., Honig, D. H., *J. Am. Oil Chem. Soc.* **56**, 262 (1979).
Shetty, K. J., Kinsella, J. E., *J. Food Sci.* **44**, 633 (1979).
Singhal, R. P., Atassi, M. Z., *Biochemistry* **10**, 1756 (1971).
Thanh, V. H., Shibasaki, K., *J. Agric. Food Chem.* **24**, 1117 (1976a).
Thanh, V. H., Shibasaki, K., *Biochim. Biophys. Acta* **439**, 326 (1976b).
Thanh, V. H., Shibasaki, K., *Biochim. Biophys. Acta* **490**, 371 (1977).
Thanh, V. H., Shibasaki, K., *J. Agric. Food Chem.* **26**, 692 (1978).
Thanh, V. H., Okubo, K., Shibasaki, K., *Agric. Biol. Chem.* **39**, 1501 (1975).
Wolf, W. J., Cowan, J. C., "Soybean as a Food Source", CRC Press, Cleveland, OH, 1975, pp 58–60.

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Tannin Deactivation and Nutritional Improvement of Sorghum by Anaerobic Storage of H_2O-, HCl-, or NaOH-Treated Grain

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Extractable tannin content of sorghum grain was markedly reduced by imbibing H_2O, HCl, or NaOH solutions into whole seeds and storing them under CO_2 atmosphere. The extent of the reduction was dependent on storage time and temperature, quantity of liquid imbibed, and concentration of acid or base. Typically, tannin content was reduced from 3.63% to 2.2, 0.6, and 0.1% by imbibing 25% by weight of water, 0.8 N HCl, or 0.8 N NaOH, respectively, and storing for 2 days at 25 °C. Increasing the storage time of the water-treated sample to 9 days decreased the tannin content to 0.3%. Extractable tannin was also markedly reduced by germination. Rat feeding studies showed that these treatments produced weight gains and feed/gain ratios which were equivalent to those obtained on a low tannin sorghum (LTS) diet. The apparent digestibility of protein and total dry matter and the PER were improved but not rendered equal to the values obtained for the LTS diet in most cases. It appears that the vanillin–HCl assay for tannin content is an adequate predictor of the nutritional quality of high tannin sorghum treated by these methods.

High tannin sorghums (HTS) with an open panicle structure appear to prevent or at least decrease bird depredation (McMillian et al., 1972; Tipton et al., 1970; Niehaus and Schmidt, 1970). Another desirable characteristic associated with tannins in sorghum is weather resistance, in particular retardation of preharvest seed germination and seed molding (Harris and Burns, 1973; Harris and Burns, 1970). Sorghum tannins, however, also have deleterious effects due to their strong interaction with proteins. The resulting complexes are not readily digested by monogastrics and this leads to lower protein digestibility, lower PER's, and weight gains (Featherston and Rogler, 1975; Maxson et al., 1973; Chang and Fuller, 1964). Sorghum tannins also inhibit enzymic reactions and microbial activity which are required during the brewing of beer (Watson, 1975).

A number of methods have been used to try to overcome problems associated with HTS. Mechanical abrasion of the seed coat layers has been shown to reduce tannin content (Chibber et al., 1978). However, this method results in low yields and large protein losses. Supplementation with methionine or the addition of polyvinylpyrrolidone to the diet improved performance of chicks (Armstrong et al., 1973; Fuller et al., 1967). Extraction of tannins with aqueous alkali resulted in marked improvements in weight gain and feed efficiency of rats (Armstrong et al., 1974) and improved in vitro protein digestibility (Chavan et al., 1979). Soaking seeds in dilute formaldehyde solution has been shown to decrease tannin content, presumably by cross-linking with some constituent in the seed (Daiber, 1975a). Imbibing dilute NH_4OH into whole seeds or mixing dilute K_2CO_3 with ground grain reduced tannin content and increased chick weight gain and feed efficiency (Price et al., 1979; Price and Butler, 1978).

Except for mechanical abrasion these treatments involve chemicals and therefore there is some question regarding the economic feasibility of the processes. In addition, a certain degree of expertise, which may or may not be available in a developing country, is required in the handling of chemicals. In this investigation we report the marked reduction in tannin content caused by anaerobic

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