Chemical Modification Strategies for Synthesis of Protein-Based Hydrogel

Der-Chyan Hwang and Srinivasan Damodaran*

Department of Food Science, University of Wisconsin—Madison, 1605 Linden Drive, Madison, Wisconsin 53706

A soy protein-based hydrogel capable of imbibing a large amount of water was synthesized by first modifying the lysyl residues of soy protein isolate (SPI) with ethylenediaminetetraacetic dianhydride (EDTAD) followed by cross-linking with glutaraldehyde. Modification of SPI with EDTAD at pH 9 resulted in polymerization involving intramolecular cross-linking of soy 11S and 7S globulin, which decreased the number of carboxyl moieties introduced per lysyl residue and impaired the swellability of the hydrogel. Pretreatment of SPI involving heating for 30 min at 65 °C and pH 12.0 followed by modification with EDTAD at 25 °C/pH 12 did not result in polymerization and, under these conditions, about 3.3 carboxyl moieties/lysyl residue were incorporated into the protein. The hydrogel prepared under these conditions was able to absorb more than 105 g of water/g of dry gel. The structural changes in SPI caused by the modification and its relevance to swelling properties of the gel are discussed.

Keywords: *Chemical modification; protein; hydrogel; soy protein*

INTRODUCTION

There is a growing awareness that the continued widespread use of nondegradable, petroleum-based polymeric materials (plastics) in industrial and consumer products may pose a serious environmental concern (Hamilton et al., 1995). Another concern is that the leaching of toxic monomers and oligomers from landfilled plastics will contaminate ground water, thereby causing health problems in humans and animals.

In addition to concerns regarding human health and the environment, worldwide depletion of petroleum reserves necessitates less dependence on petroleum-derived products. Because of these factual and/or perceived economic, environmental, and public health concerns accompanying nonbiodegradable, petroleum-based products, efforts to develop non-petroleum-based, environmentally safe, biodegradable materials from renewable agricultural biomass are needed (Jane et al., 1994; Narayan, 1994; Volesky, 1990; Chen, 1985).

Among biopolymers, proteins are perhaps the most under-rated and underutilized in terms of their industrial applications. They are primarily regarded solely as functional and nutritional ingredients in foodstuffs. Their enormous potential as structural elements in nonfood industrial applications is largely unrecognized and unrealized. This is unfortunate because proteins offer several distinct advantages over more conventional types of biomass. For example, unlike polyol-based natural polymers such as cellulose and other carbohydrates, proteins contain several reactive groups, including amino, hydroxyl, sulfhydryl, phenolic, and carboxyl groups. These reactive groups can be used as sites of chemical modification and cross-linking to produce novel polymeric structures. Since the polypeptide backbone is relatively rigid compared to those of polyol polymers, industrial polymers derived from proteins should exhibit structural and mechanical properties superior to those of cellulose-based or synthetic polymers. Several potential uses of plant proteins in industrial applications, such as paper coatings (Riley and Coco, 1986), adhesives (Bye, 1979; Kaleem et al., 1987), and cosmetic products (Marsh and Carlton, 1975), have been demonstrated. Hence, through appropriate chemical modification of proteins, it should be possible to produce a variety of novel industrial polymers with functionalities equal or superior to those of petroleum-based products.

One of the classes of cross-linked polymers that can find high volume use in industrial products (absorbents, controlled-release capsules), consumer products (diapers), and environmental applications is the hydrogel. A hydrogel is defined as "a polymeric material which exhibits the ability to swell in water and retain a significant amount of water within its structure, but which will not dissolve in water" (Ratner and Hoffman, 1976). Several synthetic hydrogel materials are currently in use. These include poly(hydroxyalkyl methacrylates), polyacrylate, poly(acrylamide), and poly(methacrylamide) and its derivatives poly(*N*-vinyl-2-pyrolidone) and poly(vinyl alcohol). Although these synthetic hydrogel polymers exhibit several interesting properties, their use in industrial, consumer, and environmental applications is less than desirable because of the toxicity of residual monomers that are usually present in these gels. Furthermore, poor biodegradability of these synthetic hydrogels also poses long-term environmental concerns. Clearly then, there exists the need for a biodegradable, superabsorbent, biomass-derived hydrogel.

The disadvantages encountered with synthetic hydrogel polymers can be overcome if hydrogels can be made from natural protein polymers. Most of the proteins form thermally induced gels (Ziegler and Foegeding, 1990). The most critical requirement for any biopolymer to function as a hydrogel is that it should have the capacity to absorb a large amount of water upon rehydration and resist dissolution. However, thermally induced protein gels do not swell to their original gel volume once they are dried. This inability to swell is related to increased protein—protein interac-

* Author to whom correspondence should be addressed [telephone (608) 263-2012; fax (608) 262-6872; e-mail sdamodar@facstaff.wisc.edu].

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tions via hydrogen bonding and electrostatic and hydrophobic interactions that occur as a result of dehydration. It follows then that if these attractive protein−protein interactions are sufficiently weakened and the polypeptide chain is allowed to assume a random coil conformation, it should be possible to enhance the swelling and water-absorbing properties of protein gels. This can, in principle, be achieved through appropriate chemical modification of protein side chains. In this paper we show that modification of lysyl residues of soy protein with a tetracarboxylic dianhydride, followed by cross-linking of protein chains with a bifunctional cross-linking agents, results in a polyanionic polymer gel that exhibits superabsorbent properties. The chemical modification strategies and optimum reaction conditions for producing protein-based superabsorbent hydrogels are described.

MATERIALS AND METHODS

Defatted soy flour was obtained from Central Soya (Fort Wayne, IN). Bovine serum albumin (BSA), 25% glutaraldehyde solution, and trinitrobenzenesulfonic acid (TNBS) were from Sigma Chemical Co. (St. Louis, MO). Ethylenediaminetetraacetic dianhydride (EDTAD) was from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of analytical grade.

Preparation of Soy Protein Isolate (SPI). Soy protein isolate was prepared essentially as described elsewhere (Thanh and Shibasaki, 1976). Briefly, the defatted soy flour was extracted with water at pH 8.0 at a meal to water ratio of 1:10. The solution was centrifuged and the supernatant adjusted to pH 4.5 with 2 M HCl. The precipitate was redissolved in water at pH 8, dialyzed against water (pH 8.0) overnight, and then lyophilized.

Protein Determination. Because the modifying groups used in this study interfered with all colorimetric methods for protein determination, the protein concentration was determined by the dry weight method. In this case, a weighed aliquot of a protein stock solution in deionized water was dried to constant weight at 105 °C in a vacuum oven. The protein concentration was expressed as percent w/v.

Chemical Modification. *Acylation*. Acylation of SPI was performed using a 1% protein solution at various pH values by stepwise addition of solid EDTAD. The pH of the protein solution during the reaction was kept constant by adding 1 N NaOH using a pH-Stat (Model 450, Fisher Scientific Instruments). The duration of the acylation reaction typically was 2−3 h. The protein solution was then dialyzed exhaustively against deionized water to remove salts, mainly the sodium salt of EDTA, and lyophilized.

Cross-Linking. To 10 mL of 15% (w/w) acylated protein solution at pH 9.0 was added 150 μL of a 25% aqueous solution of glutaraldehyde. The mixture was stirred well using a magnetic stirrer, and the gel was cured overnight at room temperature. The cured gel was then air-dried in an oven at 40 °C.

Determination of the Extent of Modification. The extent of acylation was expressed as the percent of total lysyl residues modified. The lysine content of unmodified and acylated proteins was determined by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method as described by Hall et al. (1973). To 1 mL of 4% $NaHCO_3$ was added 0.8 mL of a solution containing less than 5 mg of protein, followed by the addition of 0.2 mL of TNBS solution (12.5 mg/mL). The mixture was incubated at 40 °C for 2 h, and 3.5 mL of concentrated HCl was added. The tube was stoppered and kept at 110 °C for 3 h and then, after cooling, the volume was made up to 10 mL with deionized water. The solution was extracted twice with anhydrous diethyl ether. The tube was unstoppered and held at 40 °C to allow the residual ether to evaporate. The absorbance of the yellow (ϵ-TNP lysine) solution was measured at 415 nm against a blank. The amount of reactive lysyl residues in the acylated and unacylated soy protein was determined from the standard curve constructed using lysine.

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using a linear gradient slab gel of 5−20% was performed according to the method of Laemmli (1970). Gels were run at a constant voltage of 60 V for about 16 h. The molecular weight marker proteins were from Sigma.

Electrometric Titration. All electrometric titrations were performed at room temperature (25 ± 2 °C) using 0.1% protein in 6 M guanidine hydrochloride essentially as described elsewhere (Nozaki and Tanford, 1967). The titrant (0.01−0.5 N NaOH) was standardized against potassium acid phthalate. Typically, a 10 mL aliquot of a protein solution in 6 M guanidine hydrochloride at pH 2 was placed in a titration vessel. Nitrogen gas was continuously swept over the protein solution to prevent dissolution of atmospheric carbon dioxide. To this solution was added 25 μL aliquots of NaOH solution. After each addition, the pH of the protein solution was recorded using a titrimeter (Model 450, Fisher Scientific Instruments) equipped with a combination pH electrode with a silver/silver chloride reference electrode. A titration curve for 6 M guanidine hydrochloride (blank) also was performed under identical conditions. The titration curve of the blank was subtracted from the protein solution titration curve to obtain the titration curve of the protein alone. The number of moles of carboxyl groups per mole of protein was determined from the number of moles of NaOH consumed by 10^5 gmol of protein for titration from pH 2.0 to the isoionic point of the protein. The isoionic point was the pH of the protein solution after exhaustive dialysis against deionized water.

Fluorescence Measurements. The fluorescence spectra of proteins were measured by using a Perkin-Elmer Model LS-5B luminescence spectrometer. The emission spectrum was recorded in the range of 300−400 nm with excitation at 285 nm. The protein concentration was 0.02% in 20 mM phosphate buffer (pH 6.8). The fluorescence spectra were corrected for the fluorescence of the buffer blank.

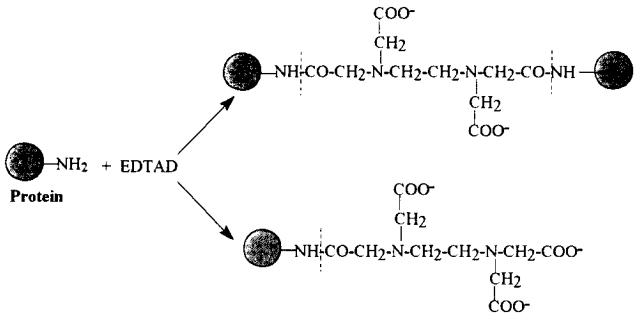
Circular Dichroic Measurement. Circular dichroic (CD) measurements were made in a computerized spectropolarimeter (On-Line Instruments Systems, Inc., Jefferson, GA). A cell path length of 1 mm and a protein concentration of 0.02% in 20 mM phosphate buffer (pH 6.8) were used. The instrument was calibrated using *d*-(+)-camphor-10-sulfonic acid. Ten scans of each sample were averaged, and the mean residue ellipticity, expressed as deg·cm^2 d-mol^{-1}, was calculated using a value of 115 for the mean residue molecular weight. All spectra were corrected for the appropriate buffer baselines. The secondary structures were estimated from CD spectra using computer software developed by Chang et al. (1978).

Swelling Properties. Swelling properties of hydrogels in water were studied as follows: A known amount of dry gel was placed in a preweighed filter pouch (similar to a tea bag), and the pouch was heat sealed. A control pouch of the same weight, without the gel, also was sealed (control). Both pouches were immersed in deionized water (pH 6.7) at room temperature for 24 h. The pouches were then centrifuged in a swinging-bucket-type clinical centrifuge for 5 min at 214*g* setting. The bottom of the centrifuge buckets was packed with a plastic wire mesh to facilitate free drainage of excess water from the swollen gel during centrifugation. After centrifugation, the sample and control pouches were weighed and the wet weight of the swollen gel was obtained by subtracting the wet weight of the control pouch from that of the sample pouch. The sample pouch was then dried in an oven at 105 °C to a constant weight. The weight of the dried gel was determined by subtracting the dry weight of the pouch. From the dry and wet weights of the gel, the grams of water absorbed per gram of dry gel at 214*g* was calculated.

RESULTS

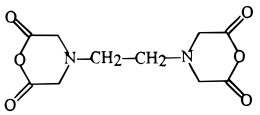
The basic premise of our approach is that through chemical modification of lysyl residues with a tetracarboxylic dianhydride, it is possible to introduce a large

Scheme 1



number of carboxyl groups into a protein molecule. In this approach, theoretically, for each lysyl residue modified, three carboxyl groups can be incorporated into the protein molecule. These added carboxyl groups, in addition to causing extensive unfolding of the protein molecule via intramolecular electrostatic repulsion, would impart a polyanionic character to the protein with numerous sites for water binding. Cross-linking of such a polyanionic protein with glutaraldehyde should produce a hydrogel with superabsorbent properties.

In preliminary experiments, we investigated modification of soy protein isolate (SPI) with several tetracarboxylic dianhydrides, such as benzenetetracarboxylic dianhydride, cyclobutane tetracarboxylic dianhydride, diethylenetriaminepentaacetic dianhydride, and ethylenediaminetetraacetic dianhydride (EDTAD). On the basis of several criteria, such as rate of reaction with lysyl residues, potential toxicity, and cost, EDTAD was selected as the best candidate. The structure of EDTAD is



EDTAD is a bifunctional reagent. Therefore, in aqueous solutions, reaction of EDTAD with protein lysyl residues can follow two different routes as shown in Scheme 1. In reaction I, one molecule of EDTAD reacts simultaneously with two lysyl residues from two protein molecules or two polypeptide chains of an oligomeric protein to form a linkage. When the reaction proceeds by reaction I, the result is the incorporation of only one carboxyl moiety per lysyl residue. Moreover, if reaction I occurs between subunits of a protein molecule, the intramolecular cross-linking may impair swelling of the modified protein. In reaction II, one molecule of EDTAD reacts with one lysyl residue and one water molecule. In this reaction, three carboxyl moieties per lysyl residue are incorporated into the protein, and no cross-linkages are formed. This greatly increases the net anionic charge of the modified protein, which may aid in unfolding the protein structure. Because no cross-linkages are formed, the swellability of the modified protein may not be impaired.

To increase the polyanionic character of soy proteins, the conditions that favor reaction II over reaction I were investigated. Figure 1 shows the SDS−PAGE of SPI modified to various extents with EDTAD at pH 9.0. The electrophoretic profiles of all EDTAD-modified SPI samples show significant amounts of high molecular weight protein polymers that could not penetrate the 3.75% stacking gel and the 5−20% separating gel. Since all SPI samples were treated with 5% β-mercaptoethanol prior to electrophoresis, these high molecular weight polymers cannot be disulfide cross-linked polymers. The possibility of lysinoalanine-type cross-links is also unlikely, since the occurrence of β-elimination reactions involving cysteine and cystine residues is negligible at pH 9.0 (Friedman et al., 1984). Therefore, these high molecular weight polymers must have formed as a result of protein cross-linking by the dianhydride. That is, at pH 9.0, modification of lysyl groups of SPI with EDTAD essentially follows reaction I.

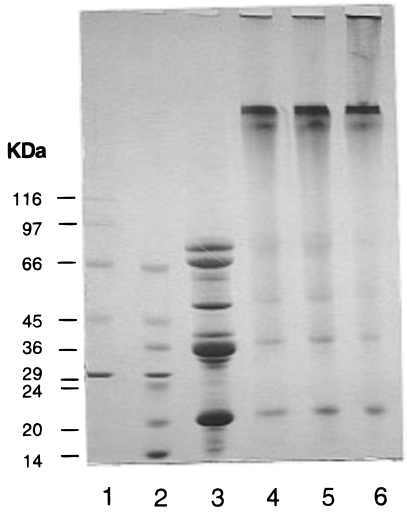


Figure 1. SDS−PAGE of unmodified and EDTAD-modified SPI. All samples were treated with 5% β-mercaptoethanol prior to electrophoresis. Samples 1 and 2 are high and low molecular weight markers; sample 3 is unmodified SPI; samples 4−6 were SPI samples modified at a SPI to EDTAD ratio of 1:0.08 (w/w). The protein concentrations during modification were 0.3, 0.5, and 1.0%, respectively. The extents of modification of samples 4, 5, and 6 were 60, 67, and 69%, respectively.

It should be noted that cross-linking occurs even when the protein concentration is as low as 0.3% (Figure 1). In molar concentration units, assuming an average molecular weight of 300 000 for soy proteins, 0.3% is about 10 μM. At this low protein concentration, the probability should be very low for two lysyl residues from two different protein molecules reacting with a EDTAD molecule. In other words, it is highly unlikely that the polymerization involves intermolecular cross-linking between 11S globulins, between 7S globulins, or between 11S and 7S globulins. On the other hand, the polymerization may involve intersubunit cross-linking within the oligomeric structures of soy 11S and 7S globulins. In such cases, the intramolecularly cross-linked 7S and 11S globulins will not dissociate to subunit level in SDS−PAGE and, consequently, these high molecular weight proteins may not be able to penetrate the separating gel.

To verify if intermolecular cross-linking of proteins by EDTAD indeed is unlikely at low protein concentrations, a 1% (~0.15 mM) BSA solution was modified with EDTAD under reaction conditions similar to that for SPI. The SDS−PAGE of the EDTAD-modified BSA (Figure 2) shows that it contains no high molecular weight polymers. The mobility of the modified BSA, however, is slower than that of the unmodified BSA. This might be because of an apparent increase in the molecular weight of EDTAD-modified BSA compared to that of unmodified BSA. Nevertheless, the data indicate that under similar reaction conditions, EDTAD does not cross-link monomeric BSA, whereas it cross-links the

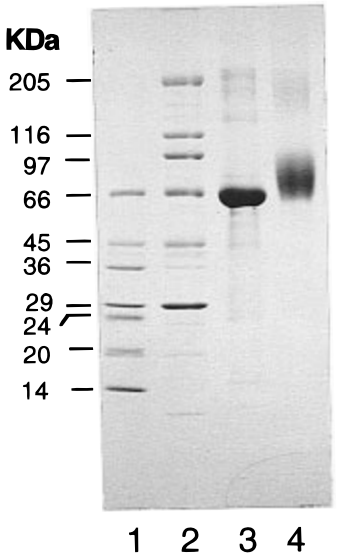


Figure 2. SDS−PAGE of unmodified and EDTAD-modified BSA. Samples 1 and 2 were high and low molecular weight markers; sample 3 is unmodified BSA; sample 4 is 45% EDTAD-modified BSA. All samples were treated with 5% β-mercaptoethanol prior to electrophoresis.

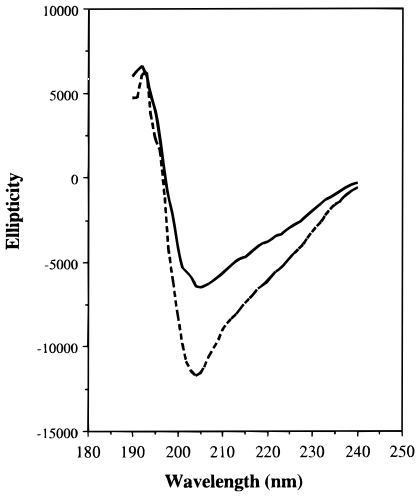


Figure 3. CD spectra of native (—) and 67% EDTAD-modified SPI (- - -) at pH 9.0. Modification was done at pH 9.0.

subunits of the oligomeric proteins of SPI. Therefore, the polymerization in soy proteins must occur intramolecularly, not intermolecularly. The fact that the molecular weights of polymers formed are greater than 180 000 suggests that the three subunits of 7S globulin are cross-linked to each other, and several acidic and basic subunits of 11S globulin also are cross-linked to each other.

Figure 3 shows circular dichroic spectra of native SPI and EDTAD-modified (67%) SPI at pH 9.0. Secondary structure estimates from these spectra indicated that native SPI contained 5% α-helix, 60% β-sheet, 35% aperiodic structures, and no β-turns, whereas the modified SPI contained 10% α-helix, 50% β-sheet, 40% aperiodic structure, and no β-turns. These values indicate that modification of SPI with EDTAD at pH 9.0 does not cause extensive structural changes in the SPI, presumably because of intramolecular cross-linking of the subunits within the oligomeric structures of 11S and 7S globulins.

Since intersubunit cross-linking during the reaction of EDTAD with SPI is undesirable as described earlier, several strategies were investigated to force the reaction

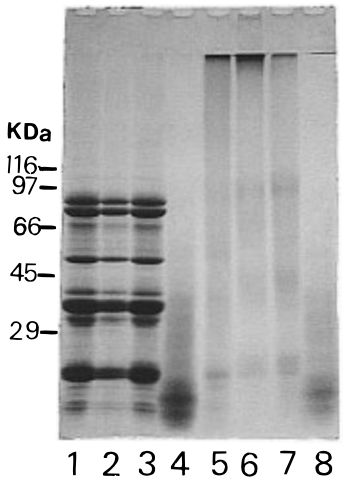


Figure 4. SDS−PAGE of unmodified and EDTAD-modified SPI. Samples 1−4 are unmodified SPI in pH 9, 11, 12, and 13 solutions, respectively; sample 5 is 67% EDTAD-SPI modified at pH 9; sample 6 is 60% EDTAD-SPI modified at pH 11; sample 7 is 88% EDTAD-SPI modified at pH 12; sample 8 is 2% EDTAD-SPI modified at pH 13. All samples were treated with 5% β-mercaptoethanol prior to electrophoresis.

to follow reaction II. These included inclusion of glycine (1−50 mM range) as a reactant and modification at various pH values. Addition of glycine as a second nucleophilic reactant did not prevent cross-linking of SPI by EDTAD at pH 9.0. Moreover, above 10 mM concentration, glycine completely prevented reaction of SPI with EDTAD at a SPI to EDTAD ratio of 1:0.08 (data not shown).

It seems that, to follow reaction II, the oligomeric structures of soy 11S and 7S globulins must first be dissociated. This may be achieved by exposing the protein to high pH. The effect of reaction pH on the extent of cross-linking of SPI by EDTAD is shown in Figure 4. The SDS−PAGE patterns of unmodified SPI exposed to corresponding pH values also are shown in Figure 4. The SPI samples modified at pH 11 and 12 show high molecular weight polymers (samples 6 and 7), indicating that the oligomeric structure of soy globulins is essentially intact even at pH 11−12 and EDTAD is still able to cross-link the subunits of 11S and 7S globulins. Both unmodified and modified SPI treated at pH 13 contain only low molecular weight peptides (samples 4 and 8), showing that the protein is extensively hydrolyzed at that pH. It should be noted, however, that no alkaline hydrolysis of either unmodified or modified SPI occurs at pH 11 and 12 (samples 2 and 3).

The effect of a combination of heat and high pH treatments on dissociation of soy globulins was investigated. Figure 5 shows that when SPI is first partially denatured/dissociated by incubating for 30 min at 65 °C and pH 12, followed by modification with EDTAD at room temperature and pH 12, the modified protein contains a negligible amount of high molecular weight polymers. This suggests that, under these modification conditions, reaction of EDTAD with SPI apparently follows reaction II (Scheme 1). Moreover, no detectable alkaline hydrolysis of SPI occurs under these modification conditions. The decrease in the intensities of protein bands with increasing extent of modification observed in SDS−PAGE (Figure 5) is not due to a loss of protein as a result of alkaline hydrolysis, but is attributable to the poor stainability of the modified protein. In fact, no small molecular weight peptides are found in either the unmodified or modified SPI samples.

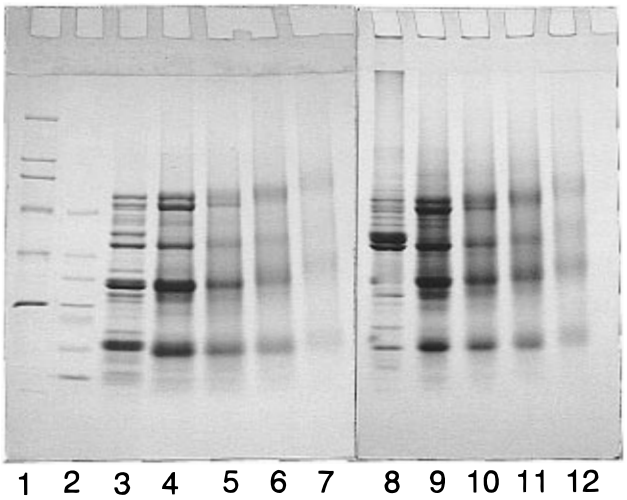


Figure 5. SDS−PAGE of unmodified and EDTAD-modified SPI. Samples 1−7 contained 5% β-mercaptoethanol in the sample buffer, and samples 8−12 were not treated with β-mercaptoethanol. Samples 1 and 2 are molecular weight markers; samples 3 and 8 are native SPI; samples 4 and 9 are unmodified SPI with pH 12/65 °C treatment; samples 5 and 10 are 38% EDTAD-modified SPI, respectively; samples 6 and 11 are 59% EDTAD-modified SPI, respectively; samples 7 and 12 are 91% EDTAD-modified SPI, respectively. Samples 1−7 and 8−12 were run on two different gels.

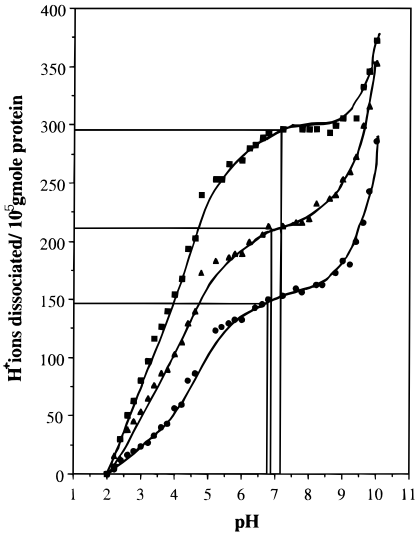


Figure 6. Electronic tritration curves of native and EDTAD-modified SPI samples: (●) native SPI; (▲) 49% EDTAD-modified SPI; (■) 91% EDTAD-modified SPI. The isoionic points of native and 49% and 91% EDTAD-modified SPI were pH 6.8, 6.9, and 7.2, respectively.

To determine if the reaction of EDTAD with SPI under the above reaction conditions in fact followed reaction II, the increase in the number of carboxyl moieties per modified lysyl residue was determined by electrometric titration. The electrometric titration curves of unmodified SPI and SPI modified to various extents with EDTAD at pH 12/65 °C are shown in Figure 6. The number of carboxyl groups per 10^5 gmol of soy protein was calculated from the number of moles of H^+ ion dissociated (or number of moles of NaOH consumed) by the protein for titration from pH 2.0 to the isoionic point of the protein. The vertical lines in Figure 6 denote the isoionic pH of the native and modified SPI samples. The titration curves of native SPI and the SPI control (i.e., unmodified SPI exposed to heat and pH conditions similar to that used for acylation) were very similar (Figure 7), showing that the heat treatment at pH 12

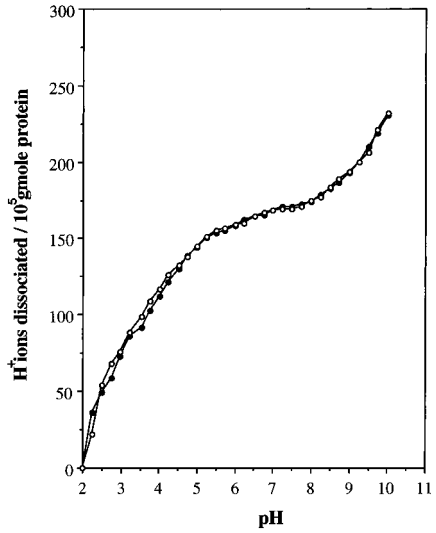


Figure 7. Electrometric titration curves of native SPI (○) and SPI heated for 30 min at 65 °C/pH 12.0 and incubated for 3 h at 25 °C/pH 12 (●).

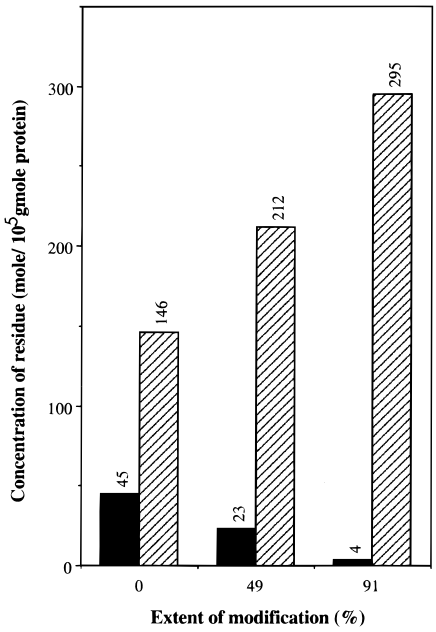


Figure 8. Relationship between lysyl residues and carboxyl group contents of EDTAD-modified SPI: (solid bar) lysyl residues; (slashed bar) carboxyl groups.

did not result in deamidation of glutamine and asparagine residues of SPI. Therefore, the increase in the carboxyl content of modified SPI samples is essentially due to incorporation of EDTA moieties at the lysyl residues of the protein.

The relationship between lysyl group content and carboxyl group content of EDTAD-modified SPI as a function of the extent of modification is shown in Figure 8. The native SPI contains about 146 carboxyl groups and about 45 lysyl residues per 10^5 gmol (about 860 total amino acid residues) of SPI (Barman et al., 1977). The 49% EDTAD-modified SPI is found to contain 212 carboxyl groups and 23 lysyl residues. Similarly, the 91% EDTAD-modified SPI is found to contain about 295 carboxyl groups and 4 lysyl residues per 10^5 gmol of SPI. The data show that for each lysyl group modified, an average of 3.3 carboxyl groups is incorporated into the protein, confirming that, under the above modification conditions, reaction of EDTAD with SPI in fact followed reaction II (Scheme 1).

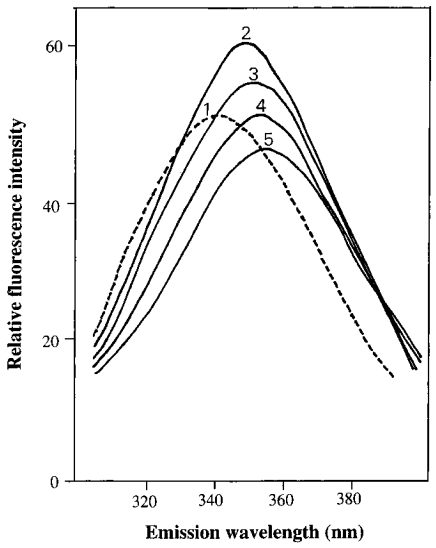


Figure 9. Fluorescence spectra of native and EDTAD-modified SPI: 1, native SPI; 2, SPI after pH 12/65 °C treatment; 3, 38% EDTAD-modified SPI; 4, 58% EDTAD-modified SPI; 5, 90% EDATAD-modified SPI.

As discussed earlier, one of the purposes of chemical modification of SPI with EDTAD is to convert the rigid globular structure of soy globulins into a random-coil-type polyanionic polymer. To determine if modification of SPI with EDTAD at pH 12/65 °C resulted in unfolding of soy globulins, circular dichroism and fluorescence properties were investigated. Figure 9 shows fluorescence spectra of native and EDTAD-modified SPI. The emission spectrum of native SPI showed maximum fluorescence at 340.8 nm. The wavelength of emission maximum (λ_{max}) of unmodified SPI control (i.e., the sample exposed to conditions similar to that employed for modification) shifted to 343.8 nm and the fluorescence intensity increased compared to that of native SPI, indicating that dissociation/denaturation of SPI occurred prior to modification. Upon modification, the λ_{max} of fluorescence emission shifted toward higher wavelength (red shift) with a decrease in fluorescence intensity. The greater the extent of modification, the greater was the red shift and the lower was the fluorescence intensity. These changes in fluorescence properties indicate that the tryptophan residues were increasingly exposed to the solvent as a result of unfolding of the protein upon modification with EDTAD.

Figure 10 shows far-UV CD spectra of native and EDTAD-modified SPI, and the secondary structure estimates are given in Table 1. The native SPI contains about 60% β-sheet and 5% α-helix, showing that it is predominantly a β-type protein. This is in good agreement with the structural data reported by Wang and Damodaran (1991). Upon exposure to pH 12 and heat, the random coil content of the unmodified SPI increases at the cost of reductions in α-helix and β-sheet contents. In the case of EDTAD-modified SPI, an increase in the extent of modification decreases the β-sheet content and increases both β-turn and random-coil contents. For example, for the 90% EDTAD-modified SPI, the β-sheet content decreases from 60 to 30% and β-turn and random-coil contents increase from 0 to 15% and from 35 to 55%, respectively. These results show that although the structure of SPI is significantly altered upon modification with EDTAD, a significant amount of β-sheet structure is retained. For example, the 90% EDTAD-modified SPI retains about 30% β-sheet structure, suggesting that complete randomization of conformation at the secondary structure level may be impossible. However, it is quite likely that the quaternary and tertiary structures of soy globulins might have been altered to a significant extent, as suggested by the fluorescence properties of modified SPI samples (Figure 9).

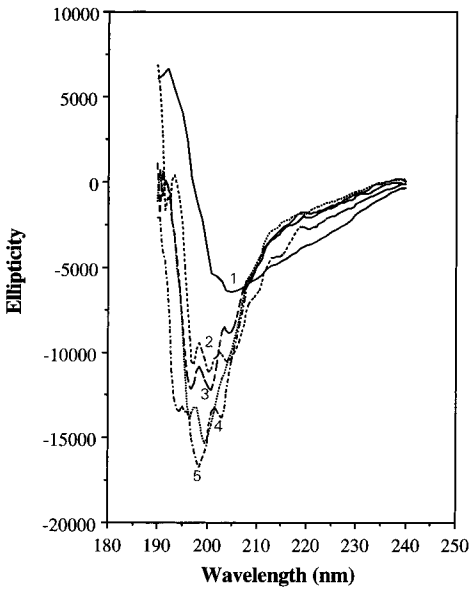


Figure 10. CD spectra of unmodified and EDTAD-modified SPI: 1, native SPI; 2, SPI after pH 12/65 °C treatment; 3, 38% EDTAD-modified SPI; 4, 58% EDTAD-modified SPI; 5, 90% EDATAD-modified SPI.

Table 1. Secondary Structure Content of Native and EDTAD-Modified SPI

	secondary structure content (%)			
protein	α-helix	β-sheet	β-turn	random coil
C1[a]	5	60	0	35
C2[a]	0	55	0	45
38% EDTAD-modified SPI	0	50	5	45
58% EDTAD-modified SPI	0	40	10	50
90% EDTAD-modified SPI	0	30	15	55

[a] C1 is native SPI; C2 is unmodified SPI subjected to pH 12/heat treatment.

Table 2. Water Uptake Capacities of EDTAD-Modified SPI

% modification	water uptake[a] (g of water/g of dry gel)
0	6.3 ± 0.4[b]
32	55.5 ± 6.1
47	76.5 ± 4.7
66	105.6 ± 4.0
67[c]	22.1 ± 1.0

[a] Hydrogels were prepared by cross-linking 15% solutions of modified SPI samples. Represents water retained by the swollen gel at 214*g*. [b] Values represent mean ± standard error (n = 3). [c] Modified at pH 9.0.

Cross-linked polymeric gels were prepared from EDTAD-modified SPI by mixing 150 μL of 25% aqueous glutaraldehyde with 10 mL of 15% EDTAD-modified SPI. The resulting gel was cured overnight and dried in an oven at 40 °C. The data presented in Table 2 show that an increase in the extent of modification increases the water uptake capacities of EDTA-modified SPI gels. The 66% EDTAD-modified SPI gel is able to take up about 105 g of water/g of gel at 214*g*. A photograph of the 66% EDTAD-modified gel before and after swelling in deionized water is presented in Figure 11. The water uptake of the gel can be increased further by increasing the extent of modification of lysyl groups. However,

Figure 11. Photograph of a 0.1 g sample of 66% EDTAD-modified SPI in the dry and swollen (after 3 h in deionized water) states.

since lysyl residues are also required for cross-linking the protein molecules with glutaraldehyde, a certain percentage of the lysyl groups should be left unmodified. Theoretically, at least three lysyl residues per protein chain would be needed to create a cross-linked continuous polymer network. Therefore, the optimum extent of modification (percent) of lysyl residues depends on the total number of lysyl residues in a protein.

DISCUSSION

The results presented here show that through appropriate chemical modification and cross-linking, superabsorbent hydrogels can be prepared from proteins. Although soy protein isolate has been used in this study as a model protein, the approach described here can be applied to any kind of protein, including other oilseed proteins, leaf (alfalfa) proteins, microbial proteins, animal proteins, and proteins recovered from food-processing wastes. Protein concentrates, instead of protein isolates, also may be used as starting materials. Since the hydrogel is not intended for food uses, the starting material need not be of food grade.

The synthesis of a protein-based hydrogel essentially involves three steps: (1) dissociation and partial denaturation of the protein; (2) modification of lysyl residues with ethylenediaminetetraacetic dianhydride or with any other tetracarboxylic dianhydrides to cause unfolding and to enhance the polyanionic character of the protein; and (3) cross-linking of the modified protein with a bifunctional reagent to obtain an insoluble polymer network capable of absorbing a large amount of water. A process flow chart for commercial preparation of protein-based hydrogels is shown in Figure 12. Proteins modified with EDTAD are essentially nontoxic, because no reactive group, other than carboxyl groups, is introduced into the protein. Furthermore, since EDTAD readily reacts with water and is converted to sodium salt of EDTA during the reaction, it is unlikely that any amount of unreacted dianhydride would be present in the final modified protein. Since the sodium salt of EDTA is a "generally regarded as safe" food additive, one need not be concerned with the environmental safety or toxicity of the residual amount, if any, of NaEDTA in the modified protein. Therefore, unlike poly(acrylate)- or poly(acrylamide)-based hydrogels, which may contain residual toxic monomers, the EDTA protein

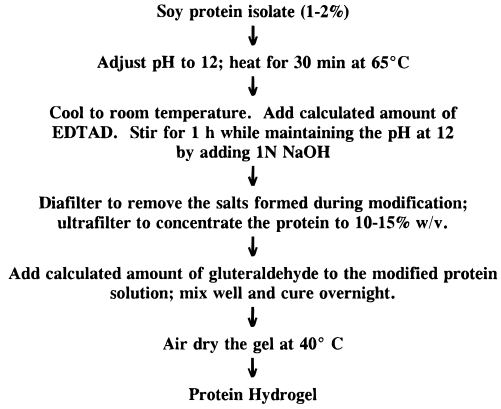


Figure 12. Process flow chart for preparation of soy protein-based hydrogel.

hydrogel should be safe. The only step in the chemical modification scheme (Figure 12) that might pose safety concerns is the use of glutaraldehyde in the cross-linking step. The residual amount of unreacted glutaraldehyde in the final gel depends on the ratio of glutaraldehyde to protein used. Optimizing this ratio can minimize the residual amount of unreacted glutaraldehyde in the gel. Moreover, the residual glutaraldehyde in the gel can be "neutralized" by treating the gel with low molecular weight amino compounds, such as ethanolamine or glycine.

Hydrogels may be used in several industrial processes, such as dewatering (Huang et al., 1989; Wang et al., 1993), in ion exchange processes, in environmental applications, such as remediation of heavy metal contaminated soil (Qin, 1993; Mitani et al., 1992), biodegradable encapsulating media for pesticides and herbicides (Weinhold et al., 1993; Schreiber et al., 1993), and in consumer products such as diapers. A protein-based hydrogel that is biodegradable will have an enormous advantage over synthetic hydrogels (e.g., poly(acrylate)-based hydrogels) in all of these applications. Some of these properties of protein-based hydrogels will be discussed elsewhere.

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