Equilibrium Swelling Properties of a Novel Ethylenediaminetetraacetic Dianhydride (EDTAD)-Modified Soy Protein Hydrogel

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SYNOPSIS

A novel pH and ionic strength-sensitive protein-based hydrogel was synthesized via crosslinking ethylenediaminetetraacetic dianhydride-modified soy protein isolate (EDTAD–SPI) with glutaraldehyde. Incorporation of ionizable carboxyl groups into soy proteins increased the net negative charge of the protein and caused extensive unfolding of the protein structure. The EDTAD–SPI hydrogel was capable of imbibing 80–300 g water per g dry gel after centrifuging at 214*g*, depending on the extent of modification, protein structure, crosslinking density, protein concentration during the crosslinking step, gel particle size, and environmental conditions, such as temperature, pH, and ionic strength. The protein concentration used during the crosslinking step was found to be the most important factor affecting the water uptake of the gel. The lower the protein concentration, the higher was the water uptake at 214*g*. The hydrogel was highly sensitive to pH and exhibited reversible swelling when sequentially exposed to water and 0.15*M* NaCl. © 1996 John Wiley & Sons, Inc.

INTRODUCTION

Hydrogels are crosslinked, water-soluble, or hydrophilic polymers which can absorb and retain a large amount of water in their polymer network.[1] Considerable amount of research has been reported on the properties of hydrogels, especially on the pH- and temperature-sensitive gels, made from a variety of polymeric materials.[1-7] A variety of novel applications of hydrogels also have been explored. These include separation agents,[2-7] matrices for drug delivery,[8] water retention in soil,[9] immobilized enzyme reactors,[10] contact lens materials,[11] and many other applications.[12,13] Two types of polymers, viz., biodegradable and nonbiodegradable polymers, are used in the preparation of hydrogels. The biodegradable hydrogels are usually made from natural polymers, such as gelatin, chitin, starch, polylactic acid, and hyaluronic acid.[8,13,14] The nonbiodegradable hydrogels are made from synthetic materials, such as polymers of acrylic acid, vinyl–alcohol, and acrylamide.[11-13] Although the nonbiodegradable synthetic hydrogels exhibit several interesting properties, their use in industrial, consumer, and environmental applications has been less than desirable because of the toxicity of residual monomers that are usually present in these gels. On the other hand, the properties of hydrogels made of natural polymers often do not meet the requirements of different applications. In recent years, there has been a growing interest in the development of biodegradable and environmentally safe hydrogels which can be used in a variety of applications, including biomedical, consumer products, and environmental applications. In this article, we describe the synthesis of a protein-based hydrogel using soy protein isolate (SPI). The swelling properties of the SPI hydrogel under various environmental conditions are also described.

EXPERIMENTAL

Materials

Defatted soy flour was purchased from Central Soya Co. (Fort Wayne, IN). Ethylene-diaminetetraacetic dianhydride (EDTAD) was from Aldrich Co. (Mil-

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waukee, WI), and a 25% glutaraldehyde solution (Grade II, Sigma Chemical Co., St. Louis, MO) was used as received. All other chemicals were of analytical grade. Heat-sealable and water-wettable filter paper was purchased from Bolmet Inc. (Dayville, CT).

Methods

Preparation of Soy Protein Isolate (SPI)

SPI was prepared as described elsewhere.[15] Briefly, the deffated soy flour was extracted with water at pH 8.0 at a meal-to-water ratio of 1 : 10. The insoluble material was removed by centrifugation, and the protein was precipitated at pH 4.5. This isoelectric curd was redispersed and dissolved at pH 8.0 by adding sodium hydroxide and dialyzed overnight against water and then lyophilized.

Modification of SPI

Acylation of lysyl residues of SPI with EDTAD was carried out as follows: The pH of a 1% solution of SPI was adjusted to 12 by adding 2.5*N* NaOH solution and heated for 30 min at 65°C. To the heat- and alkali-denatured soy protein solution cooled to room temperature, pH 12, was added a calculated amount of EDTAD. The mixture was constantly stirred and the pH of the protein solution during the reaction was maintained at 12.0 by adding 1*N* NaOH using a pH-stat apparatus (Model 450, Fisher Scientific Instruments). After a reaction time of 2–3 h, the solution was dialyzed against deionized water overnight and then lyophilized. The extent of the modification of lysyl groups was determined by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method, as described by Hall et al.[16] The carboxyl group content of the unmodified and EDTAD-modified SPI was estimated by electrometric titration as described by Nozaki and Tanford[17] and expressed as mol carboxyl groups per 10^5 g protein.

Preparation of Hydrogel

Hydrogels were prepared as follows: A protein solution (8.5–15%, w/v) was made in deionized water and the pH was adjusted to 9.0 by adding 1*N* NaOH. To this was added a known aliquot of 25% glutaraldehyde to crosslink the protein. In all cases, the protein-to-glutaraldehyde ratio was 40 on a weight basis, unless otherwise indicated. After the addition of glutaraldehyde, the solution was mixed vigorously and allowed to cure overnight at room temperature. The gels were oven dried at 40°C, ground with a pestle and mortar, and passed through 32 and 16 mesh steel sieves to get two particle sizes. The moisture content of the gels was about 5%.

Determination of Water Uptake

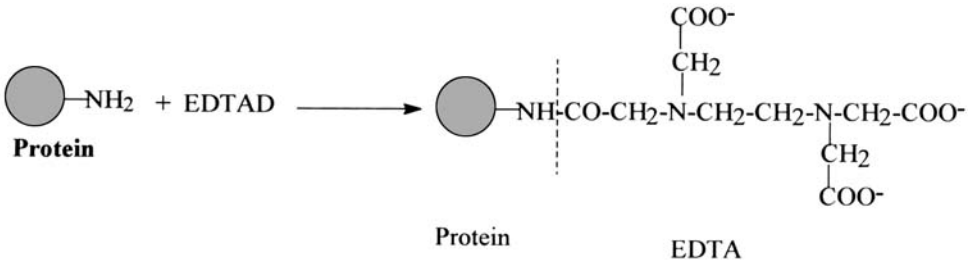
The water uptake of gels was determined as follows: A 20–30 mg dry gel sample was taken in a heat-sealable filter pouch (similar to a tea bag). The sealed bag was immersed in water (or salt solution) for 24 h. A control pouch of the same weight but with no gel was also immersed in the same solution for 24 h. The pouches were then centrifuged in a clinical centrifuge for 5 min at 214*g*. The bottom of the centrifuge bucket was packed with a plastic wire mesh to facilitate free drainage of excess water from the swollen gel/pouch during centrifugation. The sample and control pouches were weighed and the wet weight of the swollen gel was determined. The control and sample pouches were then dried in an oven at 105°C to a constant weight. The difference in weight was taken as the dry weight of the gel. From these determinations, the weight of water bound per gram of dry gel at 214*g* was calculated. All swelling studies were carried out at room temperature (25 ± 2°C). Triplicate measurements were made for each gel sample.

pH and Ionic Strength Effects

The effect of ionic strength on water uptake was studied by immersing dry gels in 0.01–0.15*M* NaCl solutions for 24 h and the amount of saline solution absorbed per gram of dry gel was determined as described above. Different buffer solutions with pH ranging from 3.0 to 10.0 were used to determine the influence of pH on the water uptake of the hydrogels. The following buffers were used: pH 3, formic acid–KOH solution; pH 4–6, succinic acid–KOH; pH 7, phosphate buffer; pH 8, tris(hydroxymethyl)-aminoethane buffer; pH 9, borate buffer; and pH 10, carbonate buffer. The ionic strength of all buffers was 0.010.

Circular Dichroic Measurement

Circular dichroic (CD) measurements were carried out with a computerized spectropolarimeter (On-Line Instrument Systems, Jefferson, GA). A cell pathlength of 1 mm and a protein concentration of 0.02% (in water, pH 9.0) were used. The instrument was calibrated using *d*(+)-10-camphorsulfonic acid. All spectra were determined by taking an average of 10 scans and the mean residue ellipticity, expressed as deg cm^2 $dmol^{-1}$, were calculated by using a value of 115 for the mean residue molecular weight.



Scheme 1

The secondary structures were estimated using a computer program based on a procedure described by Chang et al.[18]

Nomenclature

For the sake of convenience, various EDTAD–SPI hydrogel samples will be denoted as EDTADxG, where x is the number of mol of carboxyl groups introduced (as a result of modification with EDTAD) per 10^5 g of SPI. For example, EDTAD41G would refer to a EDTAD–SPI hydrogel with 41 mol of added carboxyl groups per 10^5 g of SPI.

RESULTS AND DISCUSSION

The basic premise of our approach is that through chemical modification of lysyl residues with a tetracarboxylic dianhydride a large number of carboxyl groups can be introduced into a protein molecule. In this approach, theoretically, for each lysyl residue modified, three carboxyl groups can be incorporated into the protein molecule as shown in Scheme 1. 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. Crosslinking of such a polyanionic protein with a crosslinking agent should produce a hydrogel with superabsorbent properties.

In preliminary studies, it was noted that modification of the lysyl residues of SPI with EDTAD in the pH range 7–10 resulted in incorporation of only about 1–1.5, instead of 3, carboxyl groups per lysyl residue. This was found to be because of the reaction of the dianhydride simultaneously with two lysyl residues, resulting in intramolecular crosslinking. The gels prepared from such samples did not exhibit superabsorbent characteristics. Preliminary studies indicated that, in order for the reaction to follow Scheme 1, the oligomeric structures of the 11S and 7S globulins of SPI must be dissociated and denatured. To achieve this, the SPI solution was first heated for 30 min at 65°C, pH 12.0, followed by modification with EDTAD at room temperature at pH 12.0. Under these conditions, for each lysyl group modified, about 3.3 carboxyl groups were incorporated into the protein. A ratio of more than 3 indicated that some glutamine and asparagine residues were deamidated under the alkaline reaction conditions.

Extent of Modification

Figure 1 shows the effect of the ratio of EDTAD to protein (w/w) in the reaction mixture on the extent of modification of lysyl residues. The extent of modification increased with increasing ratio of EDTAD

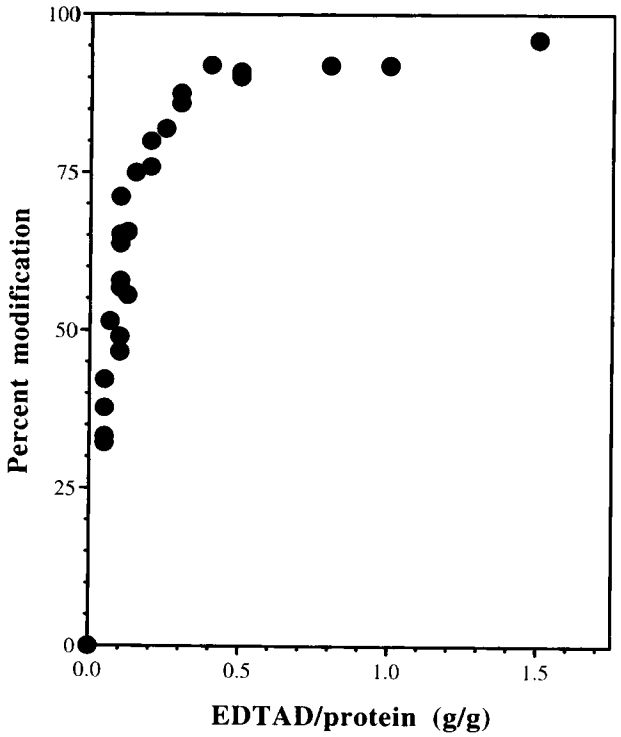


Figure 1 Effect of EDTAD to SPI ratio (g/g) on the percentage of lysyl residues modified in SPI.

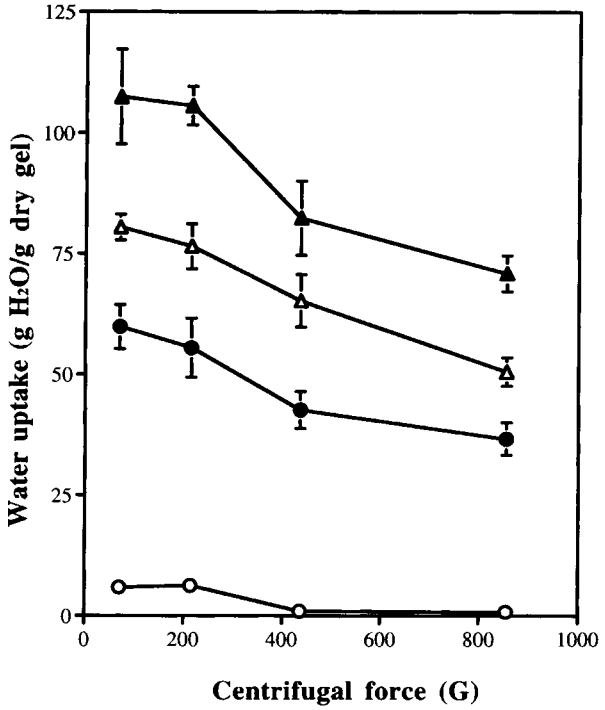


Figure 2 Effect of centrifugal force on the water uptake of EDTAD-SPI hydrogel: (○) SPIG; (●) EDTAD21G; (△) EDTAD40G; (▲) EDTAD63G. Each data point represents an average of three measurements.

to protein; about 90% of the lysyl residues were modified at an EDTAD to protein ratio of 0.4. The data in Figure 1 was used to prepare SPI samples with different extents of modification. Since lysyl residues were also required for crosslinking of the modified proteins with glutaraldehyde, we set a limit of 70% as the maximum extent of modification of the SPI in these studies.

Effect of Centrifugal Force

The water-uptake properties of three EDTAD-modified SPI gels are shown in Figure 2. Since there are no universally accepted test procedures or applicable ASTM/ISO methods for measuring the absorbency of hydrogels, we chose the tea bag method as described here, because it provided reproducible results. As shown in Figure 2, the measured water uptake decreased as the centrifugal force was increased. However, the decrease was negligible from 17*g* to 214*g* compared to at higher centrifugal force. It appears that, in the range of 17–214*g*, the water expelled from the sample was primarily loosely absorbed free water and the additional amount expelled in the range of 214–855*g* was the water entrapped within the gel matrix. Since the water uptake of the gel should reflect the total amount of water including the bound water and entrapped water in the gel, a centrifugal force of 214*g* was chosen as the reference condition to compare the water-uptake properties of EDTAD-SPI hydrogels. This centrifugation condition is similar to that reported by Nagorski.[19] The data in Figure 2 also show that EDTAD63G absorbed more than 100 g water/g dry gel at 214*g* and the water uptake increased with increase of the extent of modification.

Effect of Carboxyl Group Content

Figure 3 shows the relationship between the carboxyl group content and the water uptake of EDTAD-SPI hydrogels. Although the water uptake was positively correlated to the carboxyl group content, the water uptake of the unmodified SPI gel did not follow the slope of the modified proteins. This indicated that the water-binding capacities of the EDTA carboxyl groups introduced into the protein were quite different from those of the endogenous ionic groups of the protein. It is quite likely that, in addition to increasing the total negative charges of SPI or because of it, the added EDTA carboxyl groups may cause extensive unfolding of the protein and expose

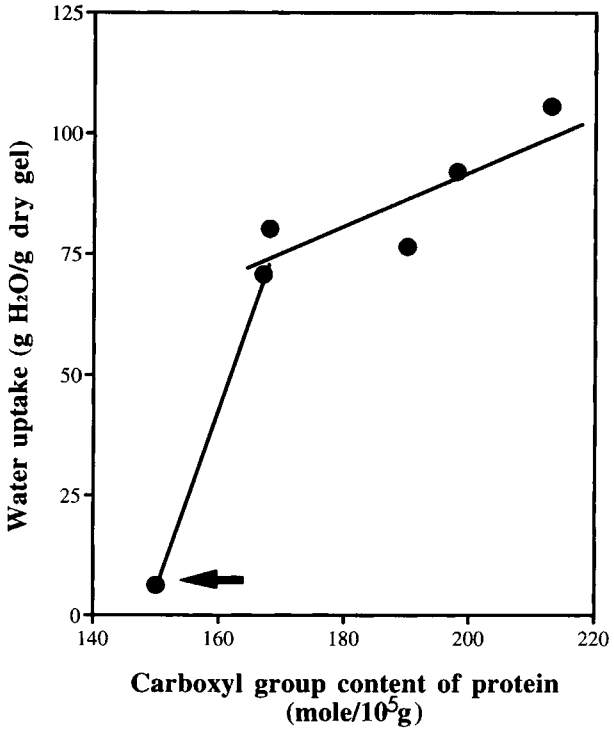


Figure 3 Relationship between the carboxyl group content and the water uptake of EDTAD-SPI hydrogels. Arrow indicates the data of unmodified SPI gel.

additional polar groups (peptide groups) for water binding. In other words, the structural changes in the protein (from a compact folded state to a flexible random state) as a result of modification may have a greater impact than the number of carboxyl groups per se on the water uptake by the gel.

Conformational Changes

Figure 4 shows the far-UV circular dichroic spectra of native and EDTAD-modified SPI samples at pH 9.0, and the secondary structure contents estimated by using the method of Chang et al.[18] are given in Table I. The β-sheet content decreased and the aperiodic structure increased with increasing extent of modification of SPI with EDTAD. The data indicated that chemical modification with EDTAD caused extensive conformational changes in SPI. Since proteins with loose structures have better water-binding capacity,[20] the dependence of water-uptake properties on the extent of modification might be related to the degree of randomization of the protein structure. The randomization of the protein structure is due primarily to intramolecular electrostatic repulsion and also possibly via hydration repulsion.

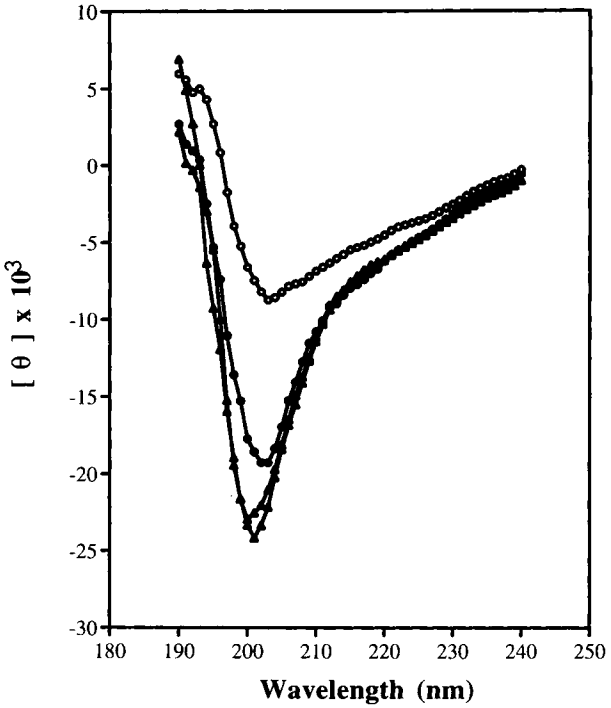


Figure 4 Circular dichroic spectra of native and EDTAD-modified SPI: (○) SPIG; (●) EDTAD21G; (△) EDTAD40G; (▲) EDTAD63G.

Effect of Protein Concentration

Figure 5 shows the water uptake of EDTA–SPI hydrogels as a function of protein concentration used in the crosslinking step. The water-uptake capacity of hydrogels decreased with increase of protein concentration. For instance, the water-uptake capacity of EDTAD65G was about 300 g water/g dry gel when the concentration of the protein solution during the crosslinking step was 8.5% (w/v). However, when crosslinking was done with a 15% (w/v) protein solution, the equilibrium water uptake of the EDTAD65G hydrogel was only about 90 g water/g dry gel. This is attributable to a higher crosslinking density which may decrease the size of the water entrapment cells in the polymeric network. This behavior also has been observed in other hydrogels made from synthetic polymers.[3,21] Since the mechanical stability of the gel is related to polymer concentration in the swollen gel,[22] the EDTAD–SPI hydrogel prepared with lower protein concentration, i.e., 8.5%, was much softer than was the hydrogel made with higher protein concentration. Thus, depending on the final application, gels with a wide range of water-uptake capacity and mechanical strength can be prepared by controlling the protein concentration during the crosslinking step.

Rate of Swelling

Figure 6 shows the water uptake of EDTAD–SPI hydrogels at room temperature as a function of time. The hydrogel made from EDTAD63G showed a water-uptake capacity of about 105 g water/g gel, whereas the hydrogels made with EDTAD21G and EDTAD40G took up about 40 and 53 g water/g gel, respectively, after a 24 h absorption period. The native SPI gel (unmodified) took up only about 10 g water/g gel. The water uptake for all the hydrogels, except the EDTAD63G, reached an equilibrium value after about 3 h. The water uptake of EDTAD63G apparently did not attain equilibrium swelling even after a 24 h period.

The rate of swelling of hydrogels is influenced by a number of factors, including the rate of ionic exchange,[23] rate of water diffusion, and the rate of polymer relaxation.[23–25] Initially, water uptake is governed only by the diffusion of water molecules into the gel matrix. The data in Figure 6 shows that swelling from the glassy (dry) state increased dramatically in the first hour of swelling. At this stage, the protein molecules became hydrated and protein–protein interactions were disrupted by the absorbed water. Since the polymer relaxation rate during hydration is generally slower than is the rate of dif-

Table I Secondary Structures of SPI and EDTAD–SPI in pH 9.0 Solution

	% Secondary Structure			
EDTAD–SPI	α-helix	β-sheet	β-turns	Aperiodic
0[a]	5	60	0	35
21	0	40	0	60
40	0	32	0	68
63	0	29	3	68

[a] The number of moles of carboxyl groups incorporated into 10^5 g of SPI.

fusion of water into the gel, the polymer-relaxation process is often the rate-limiting step in the swelling. The EDTAD63G exhibited a higher rate of swelling than did the others, presumably because of its highly random coil structure, which may permit a higher rate of relaxation. Moreover, since the stress developed during the swelling of ionic polymers is proportional to the total amount of ionized groups in the network,[26] ionization of a greater number of carboxyl groups in the EDTAD63G may, in turn, increase the relaxation rate of the protein chain in the gel network.

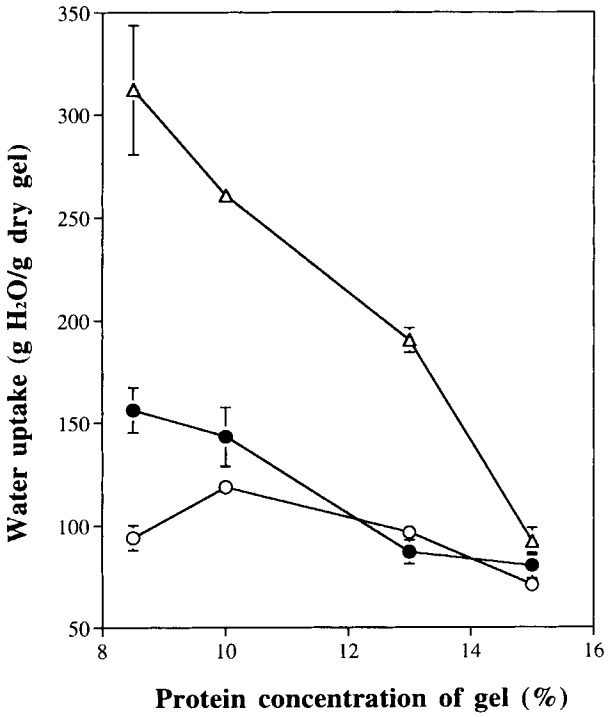


Figure 5 Effect of protein concentration employed during the glutaraldehyde crosslinking step on the water uptake of EDTAD–SPI hydrogel: (○) SPI; (●) EDTAD41G; (△) EDTAD65G.

Effect of pH

The pH affected the water-uptake capacity of the SPI hydrogels (Fig. 7). *The effect of pH on the EDTAD63G was more dramatic than that with the other gels.* In the case of EDTAD63G, the water-uptake capacity increased from 10 g water/g dry gel at pH 3.2 to about 140 g water/g dry gel at pH 10. This was essentially due to ionization of carboxyl groups as the pH was increased from the acidic to the alkaline side. However, since the pK_1, pK_2, and pK_3 values of carboxyl groups of EDTA are 2.0, 2.6, and 6.2, respectively, one might expect that the swelling capacity of the gels should reach a maximum value at about pH 8.0, wherein all carboxyl

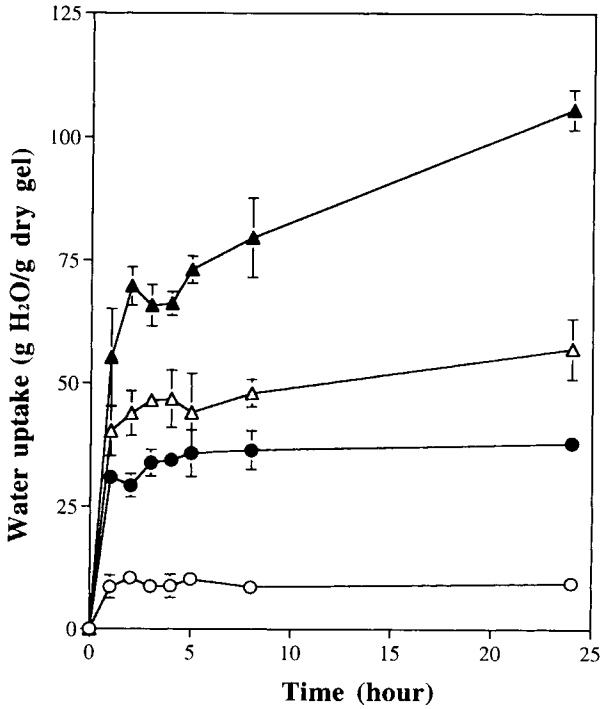


Figure 6 Rate of swelling of EDTAD–SPI hydrogels. Legends are same as in Figure 2.

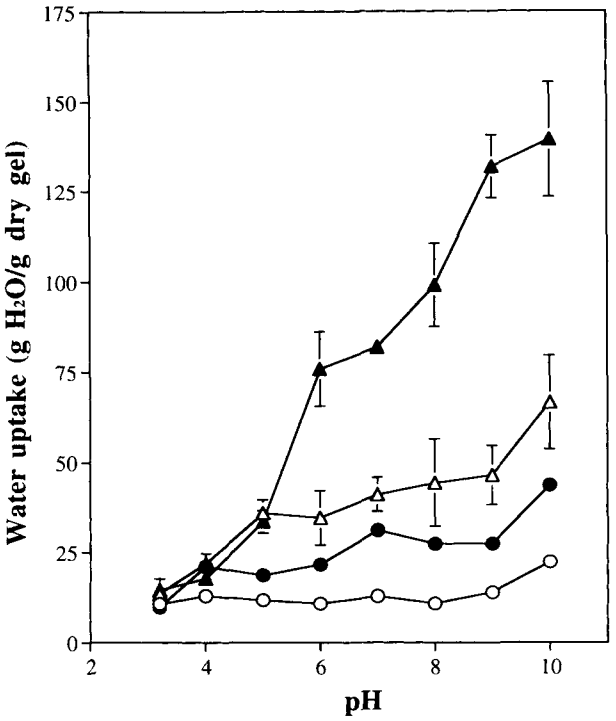


Figure 7 Effect of pH on equilibrium water uptake of EDTAD–SPI hydrogels. Legends are same as in Figure 2.

groups would have been fully ionized. Contrary to this expectation, the water-uptake capacity of all EDTAD–SPI hydrogels increased continuously up to pH 10 (Fig. 7). The increase in water uptake at pH values above 8.0 might be due to a further increase in the net charge of the protein due to ionization of tyrosine residues. It should be emphasized that the increase in water-uptake capacity is not simply related to the binding of water to ionized carboxyl groups; it is related to unraveling of the protein chain due to electrostatic repulsion within the gel network. Since the electrostatic repulsive force is directly proportional to the square of the net charge of the molecule, even a small increase in the net charge due to ionization of tyrosine residues (pK_3 = 9.6) will cause a large increase in the electrostatic repulsion within the gel network, resulting in a large expansion of the gel and imbibition of water.

Ionic Strength Effect

Figure 8 shows the effect of NaCl on the water-uptake capacity of EDTAD–SPI hydrogels. The water-uptake capacity decreased with increase of NaCl concentration. In a 0.1*M* NaCl solution, the EDTAD63G took up only about 17 g saline/g gel (at 214*g*) compared to about 105 g/g in deionized water. When a simple salt, such as NaCl, is added to a polyelectrolyte solution, some of the mobile electrolyte diffuses into the polyelectrolyte network. The electrostatic screening of carboxyl groups by sodium ions causes a marked reduction in the repulsive potential between charged segments in the gel network, which leads to network contraction and a decrease in the porosity of the gel network.

Figure 9 shows reversible swelling and deswelling behavior of the EDTAD63G when sequentially exposed to water and 0.15*M* NaCl at room temperature. When the swollen gel (in deionized water) was exposed to 0.15*M* NaCl, the water uptake decreased from 105 g/g to about 20 g/g over a period of about 1 h. When the shrunken gel was transferred to a large pool of deionized water, it regained its water-uptake capacity. Whereas the rate of water release in 0.15*M* NaCl was very rapid, the rate of water uptake in deionized water was considerably slow. This might be attributable to a slow rate of dissociation of bound ions from the gel network. It was noted that when the gel was repeatedly swelled and deswelled in deionized water and 0.15*M* NaCl, respectively, both the rate of reswelling and the extent of water uptake increased after a couple of cycles. This behavior could be due, in part, to an increase

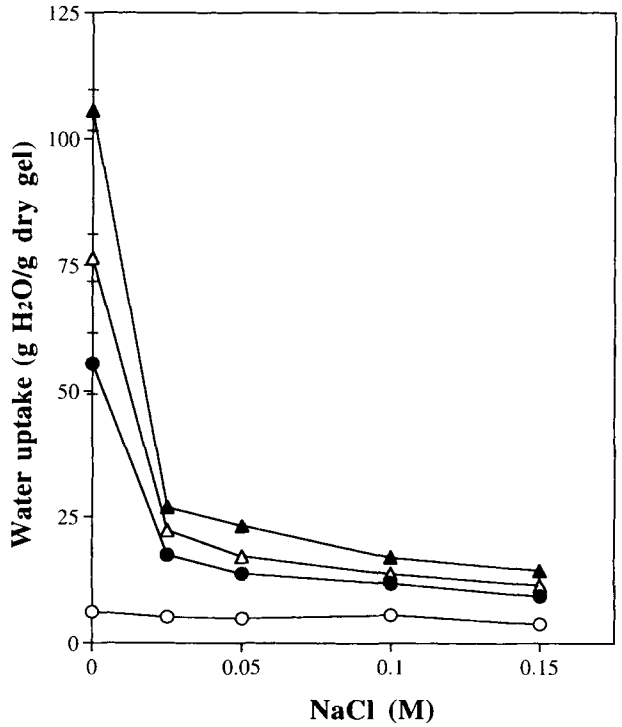


Figure 8 Effect of NaCl on water uptake of EDTAD–SPI hydrogel. Legends are same as in Figure 2.

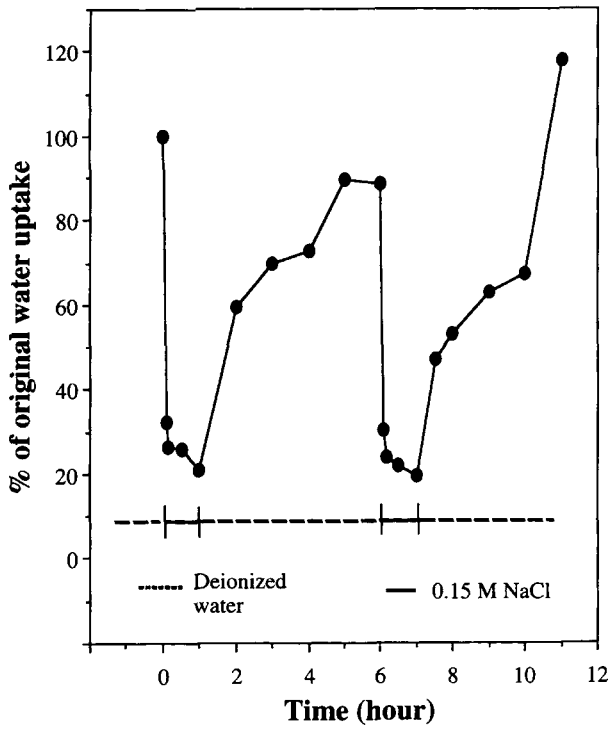


Figure 9 Reversible swelling and dewelling of EDTAD66G hydrogel in response to sequential exposure to deionized water and 0.15*M* NaCl.

in the flexibility of the gel matrix (i.e., polymer relaxation rate) after repeated swelling and deswelling. These results indicated that the SPI hydrogels can be repeatedly used (recycled) in industrial dewatering processes.

Effect of Temperature

Figure 10 shows the effect of temperature (at pH 6.0) on water uptake of 15% (w/v) EDTAD–SPI hydrogels. The water-uptake capacity of the EDTAD–SPI gels dramatically increased with temperature between 5 and 45°C, whereas that of native SPI gel did not show any change. This positive effect of temperature on water-uptake capacity must be related to an increase in the degree of relaxation and expansion of the polymer network at high temperatures. Similar behavior also has been observed in gelatin–polyacrylamide interpenetrating hydrogels.[27]

Effect of Gel Particle Size

The effect of the gel particle size on the water uptake of EDTA–SPI hydrogels is shown in Figure 11. The smaller the particle (powder) size, the lesser was the water-uptake capacity. It appears that, in a given mass, the large gel particles contain more entrapment regions than do small gel particles for absorbing a large amount of water.

CONCLUSIONS

The results presented here show that protein-based superabsorbent hydrogels can be prepared by using appropriate chemical modification and crosslinking strategies. Although soy protein isolate has been used in this study as a model, a similar approach can be used on other proteins, such as leaf (alfalfa) protein, microbial proteins, animal proteins, and proteins recovered from food-processing wastes. Hydrogels can be used in several industrial processes, such as dewatering, ion exchange, environmental applications including remediation of heavy metal contaminated soil and biodegradable encapsulating media for pesticides and herbicides, and in consumer products such as diapers. Protein-based hydrogels, which are biodegradable, will have an enormous advantage over synthetic hydrogels in all these applications. Since the EDTA group in the EDTAD–protein hydrogel is an excellent divalent

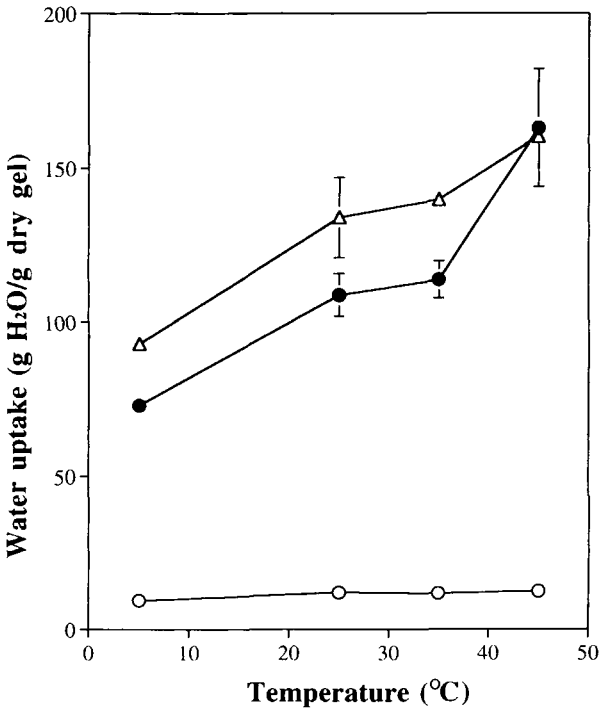


Figure 10 Effect of temperature on the water uptake of EDTAD–SPI hydrogels: (○) SPIG; (●) EDTAD41G; (△) EDTAD65G.

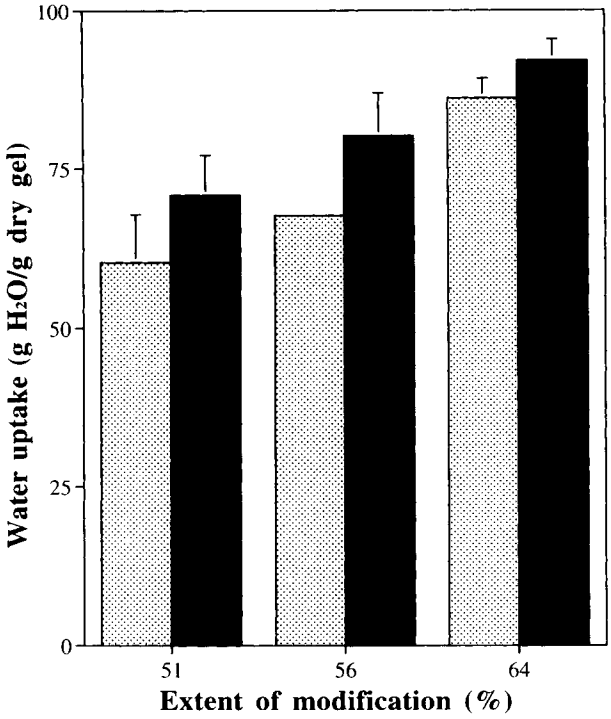


Figure 11 Effect of gel particle size on the water uptake of hydrogel: (□) powder; (■) 0.5 mm.

metal ion chelator, such gels can be successfully used for removing heavy metals from industrial effluents.

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