Swelling Behavior of Protein-Based Superabsorbent Hydrogels Treated with Ethanol

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Received 13 July 2000; accepted 10 September 2000

ABSTRACT: A fish protein-based superabsorbent hydrogel has been synthesized by modifying 80% of the ϵ-amino groups of the protein with ethylenediaminetetraacetic dianhydride followed by crosslinking the remaining ϵ-amino groups with glutaraldehyde. The hydrogel thus prepared was capable of absorbing about 200 g water per g dry gel after centrifuging at 214*g*. Denaturation of the gel by treatment with absolute ethanol increased the equilibrium water uptake of the gel from 200 to 425 g water per g of dry gel. The saline uptake of the gel also increased from 24 g saline (0.1*M* NaCl) for the control gel to 35 g saline per g dry gel for the ethanol-treated gel. Analysis of the structural state of protein chains in the crosslinked swollen gel network using circular dichroism spectroscopy showed that, whereas the protein chains in the control gel were predominantly in β-turn form, those in the ethanol-treated sample were mainly in the α-helical form. In addition to improving the swelling properties, the ethanol treatment offered the following advantages: 1. It dehydrated the gel and thereby eliminated the need for drying the gel. 2. It extracted low molecular weight off-odor compounds from the protein gel. 3. It could also extract any residual unreacted glutaraldehyde (believed to be carcinogenic) that might be present in the gel. The ethanol used in the process can be easily recovered and recycled in the process. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 81: 2190–2196, 2001

Key words: super absorbent; hydrogel; fish protein; swelling; biodegradability

INTRODUCTION

Hydrogels are crosslinked hydrophilic polymers that can absorb a large amount of water. Several hydrogel materials made from synthetic polymers, such as polymethacrylates, polyacrylate, poly-acrylamide, poly-vinyl pyrrolidone, and poly-vinyl alcohol,[1–3] are currently being used in several industrial,[4–15] pharmaceutical,[16–21] biomedical,[22,23] and biotechnology[24] applications. Although the hydrogels made from synthetic polymers exhibit excellent water-absorbing properties, the toxicity and carcinogenicity of residual monomers in these gels might pose problems with their use in drug delivery systems and consumer products, such as diapers. Furthermore, because these synthetic polymers are non-biodegradable, they might also pose long-term environmental problems.

Recently, it has been shown that chemical modification of natural proteins, such as soy protein and fish proteins (FPs), with a tetracarboxylic dianhydride followed by crosslinking of the protein with glutaraldehyde resulted in a polyanionic hydrogel with superabsorbent properties[25–27] and divalent metal chelating properties.[28] These protein-based hydrogels were capable of imbibing 80–300 g water per g dry gel at

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Contract grant sponsor: University of Wisconsin Sea Grant Institute; contract grant number: R/AQ-32.

Journal of Applied Polymer Science, Vol. 81, 2190–2196 (2001)

equilibrium, depending on the extent of modification, crosslinking density, and protein concentration used during the crosslinking step.[26] Although the equilibrium swelling properties of these protein-based hydrogels were comparable to those of synthetic hydrogels, their rate of swelling was slower than that of synthetic hydrogels. This was because of the presence of a significant amount of residual folded structures, such as α-helix and β-sheet structures, in the crosslinked protein polymers. It has been shown[26,28] that, even after exposure to severe denaturing conditions (such as incubation at pH 12 and 60°C for 1 h), soy and FPs regained a significant amount of β-sheet structure when the conditions were reversed back to pH 9 and room temperature. It is likely that, unlike the random-coil structure present in synthetic hydrogels, these folded secondary structures in the crosslinked protein network might oppose the rate of relaxation of protein chains as water diffuses into the network. Conversely, if the structure of protein chains in the crosslinked polymer network can be completely converted to a random-coil state, it would greatly improve the rate of swelling of protein-based hydrogels. If the maximum swelling capacity of protein-based hydrogels could be achieved in 30 min, it would greatly increase its potential as a superabsorbent in diapers and other consumer products.

In this report, we show that treatment of crosslinked FP hydrogel with organic solvents decreases the amount of folded secondary structures in the gel network and greatly improves both the rate and extent of swelling of the hydrogel.

EXPERIMENTAL

Materials

Walleye pike (fish) was obtained fresh from a local fish farm. Ethylenediaminetetraacetic dianhydride (EDTAD) and butanol were from Aldrich Chemical Co. (Milwaukee, WI). Glutaraldehyde (50% aqueous solution) and propanol were obtained from Sigma Chemical Co. (St. Louis, MO). Absolute ethyl alcohol was purchased from Apper Alcohol and Chemical Co. (Shelbyville, KY). Heat sealable, water wettable paper was procured from Bolmet Inc. (Dayville, CT). Dialysis tubing (m.w. cut off 6000–8000), acetone, and ether were obtained from Fisher Scientific (Pittsburgh, PA). All other chemicals were of analytical grade. Deionized water was used for the swelling studies.

Methods

Isolation of Crude Protein FP

Isolation of protein from fresh fish was performed as described elsewhere.[28] Briefly, the fresh fish upon arrival was filleted, chopped, and blended with chilled deionized water at a meat-to-water ratio of 1:9 (w/w). The suspension was adjusted to pH 12 and stirred using a magnetic stirrer for 30min. The suspension was filtered through a 0.5-mm sieve, and the filtrate was dialyzed against water and lyophilized.

Protein Determination

Because the modifying groups used in this study interfered with all colorimetric methods for determination of protein concentration, the protein concentration was determined by the dry weight method.[28] A weighed aliquot of a protein stock solution in deionized water was dried to constant weight at 105°C in an oven. The protein concentration was expressed as % w/v.

Modification of FP

Chemical modification of the lysyl residues of the protein with EDTAD was performed as reported elsewhere.[26,28] One percent protein solution in water was prepared at pH 12 and incubated for 30 min at 65°C. The solution was cooled to room temperature and a calculated amount of EDTAD was added in incremental amounts over a period of 2 h with continuous stirring. After complete addition of EDTAD, the reaction mixture was stirred constantly for 1 h while maintaining the pH at 12.0. At the end of the reaction, the pH of the protein solution was adjusted to 4.5 to precipitate the protein. The suspension was centrifuged at 10,000 rpm for 15 min. The protein sediment was washed with water at pH 4.5 and centrifuged. The final protein precipitate was then redissolved in water at pH 7.0 and lyophilized. The extent of acylation, i.e., the percentage of lysyl residues modified with EDTAD, was determined by the trinitrobenzenesulfonic acid method.[29]

Preparation of Crosslinked Hydrogel

A 10% dispersion of the EDTAD-modified fish protein was prepared by dissolving the required amount of protein in deionized water at pH 10

and mixed homogeneously with an egg beater for 15 to 20 min. Because of high viscosity, the 10% protein dispersion looked like a thick paste. To this was added a known amount of 50% glutaraldehyde solution (which was also preadjusted to pH 10) so that the ratio of protein to glutaraldehyde in the final mixture was about 1:0.035 (w/w). The mixture was mixed uniformly for about 15 min using an eggbeater and allowed to cure overnight at room temperature. The cured gel was divided into two equal parts. One part was dried in an oven at 40°C. The other portion was suspended in ethanol for 3 h, during which time ethanol was changed at least twice. The ethanol treatment caused both denaturation of protein and dehydration of the crosslinked gel. At the end of the ethanol treatment, the gel was in the form of dried particles. The particles were further dried in an oven at 35°C for 2 h to remove ethanol and any residual moisture. Unmodified FP control gels were prepared in the same manner. After complete drying, the gels were ground to particle size less than 1.0 mm and used for swelling studies.

Swelling Kinetics

Swelling studies for all of the above gels were done gravimetrically at 36°C. A weighed amount of dried gel was taken in triplicate in heat-sealable pouches and allowed to swell in deionized water. At specific time intervals, the bags were removed and centrifuged at 214*g* in a clinical centrifuge equipped with sample holders containing plastic wire mesh for proper drainage of the expelled water to the bottom of the holder. The weight of the swollen gel was determined immediately. Appropriate controls for the wet weight of the pouch were included. The wet pouch with swollen gel was dried in a oven at 104°C to constant weight. The final dry weight of the gel was determined by subtracting the dry weight of an equivalent empty pouch treated in the same manner. The water uptake was determined as g water absorbed per g dry gel.

The effect of ionic strength on water uptake was studied in a manner similar to that described above by immersing the gel samples in 0.1*M* and 0.15*M* NaCl solutions. The influence of temperature on water uptake was studied in the range of 5–40°C in temperature-controlled water baths.

Circular Dichroic (CD) Measurements

Qualitative CD measurements were made in a computerized spectropolarimeter (On-Line Instruments Systems, Inc., Jefferson, GA). The gels, swollen in water, were placed between two quartz plates (2.5 × 2.5 cm) separated by 0.8-mm-thick spacers. The cell was sealed using parafilm. The far UV CD spectrum of the sandwiched gel was recorded in the 190–240-nm range. Twenty scans of each sample were averaged and all spectra were corrected for the appropriate water baseline. Because the samples were in the form of gel, the spectra were recorded in the millidegree mode, instead of the ellipticity mode which would require the exact concentration of protein in the gel.

RESULTS AND DISCUSSION

The lysine content of the crude protein isolated from the fish muscle contained about nine residues per 10,000 molecular weight. Reaction of the crude protein with EDTAD at a protein-to-EDTAD weight ratio of 1:0.2 resulted in acylation of about 80% of the lysyl residues in the fish protein (80% EDTAD-FP). At a protein-to-EDTAD ratio of 1:0.25 (w/w), about 90% of the lysyl residues were acylated (90% EDTAD-FP). Previously, it was reported that, under the reaction conditions used in this study, reaction of EDTAD with the protein lysyl groups results in introduction of about three carboxyl groups for each lysyl residue modified.[25] Figure 1 shows water uptake of unmodified FP and 80% EDTAD-FP hydrogels. The equilibrium water uptake of the unmodified FP was only about 6 g/g gel, whereas the water uptake of the 80% EDTAD-FP hydrogel reached an equilibrium value of about 200 g/g after 24 h of swelling. Clearly, introduction of three carboxyl groups at each lysyl residue in the protein enabled the protein network to imbibe a large amount of water. The rate and extent of swelling of hydrogels are governed by the rate of diffusion of water and the rate and extent of relaxation of the polymer network in response to water diffusion.[30–32] The data in Figure 1 show that the rate of water uptake by the dry (glassy) gel increased rapidly during the first hour and slowed down thereafter. The initial rapid phase might be related to diffusion of water into, and hydration of, the charged groups in the polymer network. During this phase, in addition to hydrating the ionic groups, water may tend to disrupt polar protein–protein (i.e., inter-chain hydrogen bonding) interactions in the gel network. This should enhance the relaxation rate of the polymer network. However, the decrease in the rate of swelling of the hydrogel after the first hour

indicates that, although the protein was denatured by exposing it at pH 12 and 65°C before crosslinking with glutaraldehyde, its rate of structural relaxation in the gel network does not seem to be comparable to a truly random-coil polymer. Previously,[26,28] it was shown that, even after exposure of soy and fish proteins to the above denaturing conditions, the proteins regained a significant amount (>40%) of β-sheet structure when the conditions were reversed back to pH 9 and room temperature. These intra-chain hydrogen-bonded secondary structures in the crosslinked protein chains might oppose relaxation and expansion of the gel network as water diffuses into the network.

It is probable that if protein chains in a crosslinked protein network are subjected to denaturing conditions, they may remain in a disordered state when the denaturant is removed because of steric constraints imposed by the crosslinks. To elucidate this, after crosslinking with glutaraldehyde (and before drying), the gel was suspended in ethanol. Because of osmosis, ethanol penetrated into the gel and water diffused out into ethanol. After 3 h of exposure, the gel lost most of its water and collapsed into a dry solid. The dry gel was removed by filtration, dried in an oven at 35°C to remove ethanol, and its swelling properties were studied. Figure 2 shows the rate of water uptake of the gels of unmodified-FP and 80% EDTAD-FP prepared with ethanol treatment. In the case of unmodified-FP, the equilibrium water uptake was about 15 g/g, which is at least twofold greater than without the ethanol treatment (Fig. 1). Similarly, the equilibrium water uptake of the 80% EDTAD-FP was 425 g/g, which is about twofold greater than without the ethanol treatment (Fig. 1).

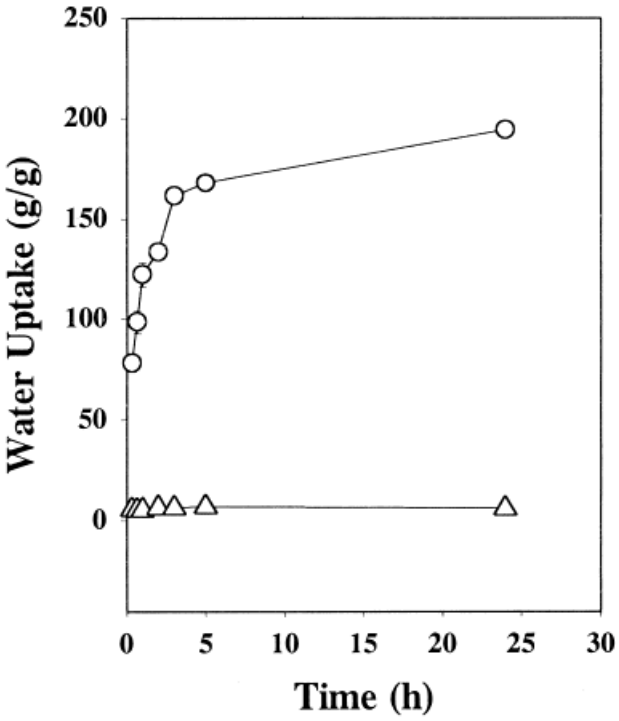


Figure 1 Rate of swelling of unmodified (△) and 80% EDTAD-modified (○) FP-based hydrogel at 36°C without the ethanol treatment. The concentration of protein was 10% at the time of crosslinking with glutaraldehyde.

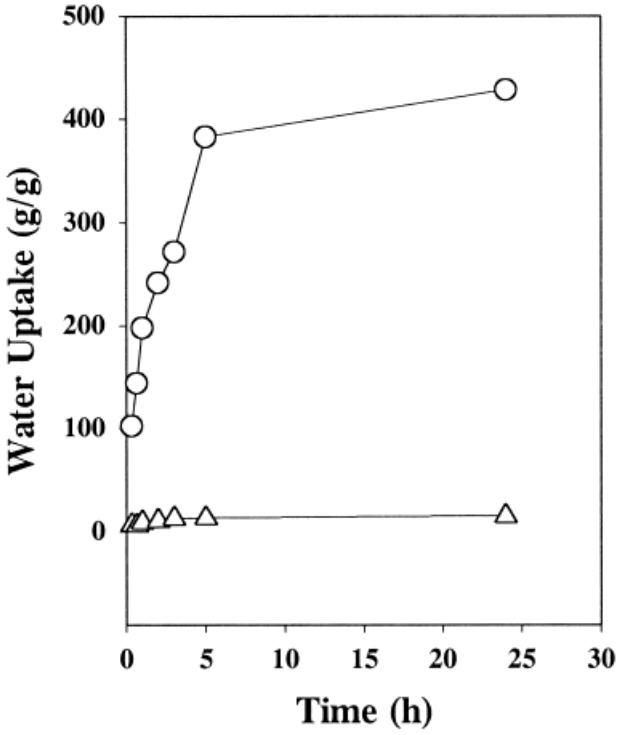


Figure 2 Rate of swelling of unmodified (△) and 80% EDTAD-modified (○) FP-based hydrogel after treatment with ethanol. The concentration of protein was 10% at the time of crosslinking with glutaraldehyde.

A comparison of the data in Figures 1 and 2 suggests that both the initial rate and the extent of swelling of the gels are markedly improved by the ethanol treatment. This must be due to ethanol-induced denaturation of protein in the gel network. To determine whether ethanol treatment caused structural changes in proteins in the gel network, the CD spectra of swollen gels were analyzed. Figure 3 shows far UV-CD spectra of swollen gels of 80% EDTAD-FP prepared with and without the ethanol treatment. Qualitatively, the

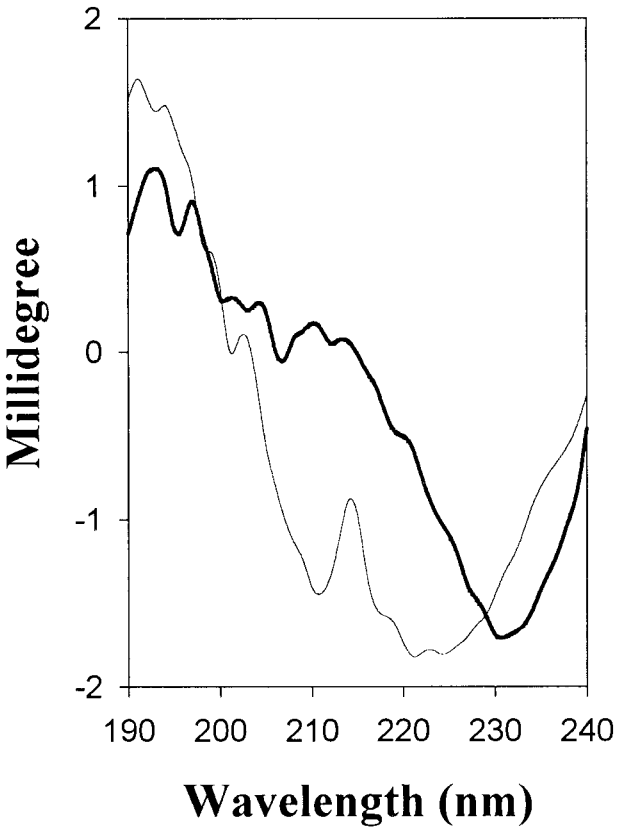
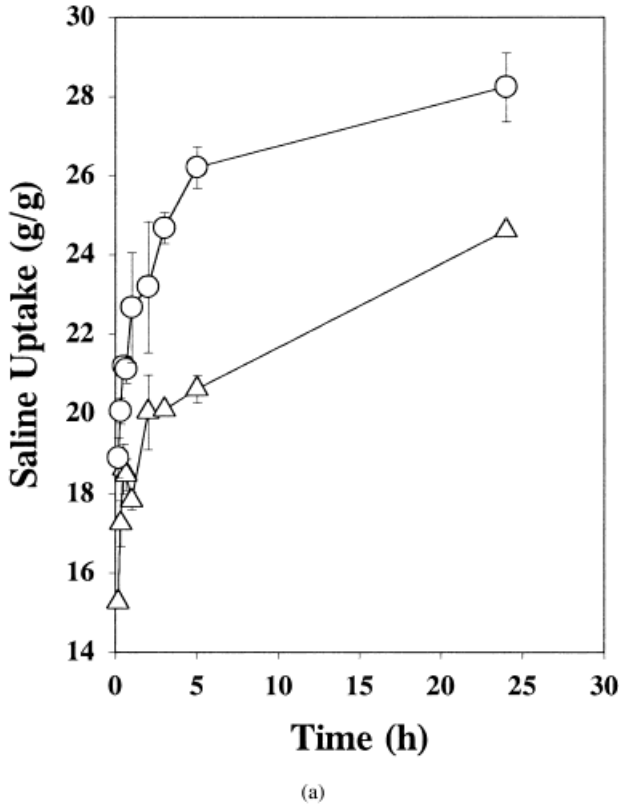


Figure 3 Circular dichroic spectra of control (thick line) and ethanol-treated (thin line) 80% EDTAD-FP hydrogel in the swollen state.

CD spectrum of the gel that was not subjected to ethanol treatment showed a major negative trough at 230 nm and a positive peak at 200 nm. This type of CD spectrum has been ascribed to proteins rich in type-I β-turns.[33–35] In contrast, the CD spectrum of the ethanol-treated gel showed two major negative troughs at 209–210 nm and 221–223 nm regions, which are typical of α-helix structure. Although it is difficult to quantitatively interpret the relationship between water uptake properties and the CD spectra of swollen gels, the data in Figure 3 do highlight that ethanol treatment alters conformational properties of proteins in the gel network, and this in turn significantly impacts the water uptake properties of the gels.

The effect of propanol, butanol, and acetone treatment on the swelling properties of 80% EDTAD-FP gels were also investigated. The extent of equilibrium swelling of the gel treated with these solvents was slightly lower than that treated with ethanol.

Figure 4(a) shows the effect of ethanol treatment on swelling properties of 80% EDTAD-FP in



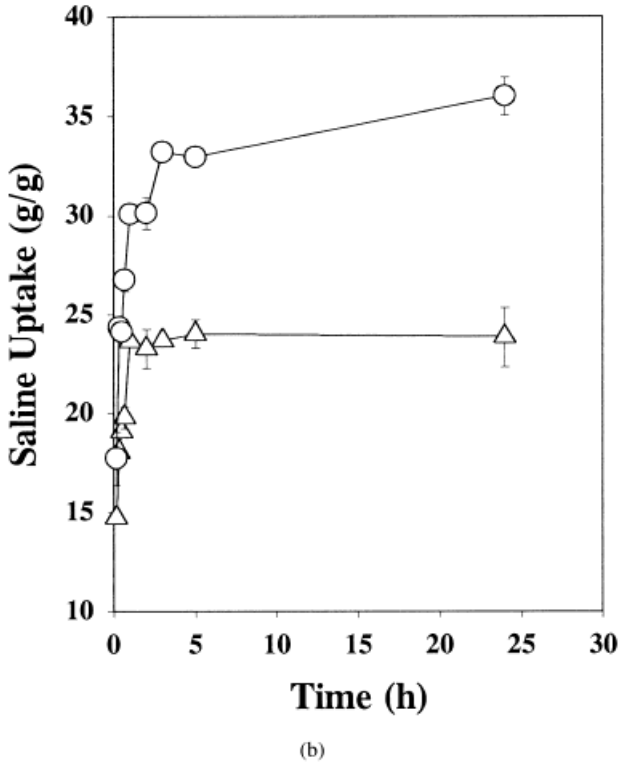


Figure 4 Saline [(a) 0.1*M* NaCl; (b) 0.15*M* NaCl] uptake of 80% EDTAD-FP hydrogel before (△) and after (○) treatment with ethanol.

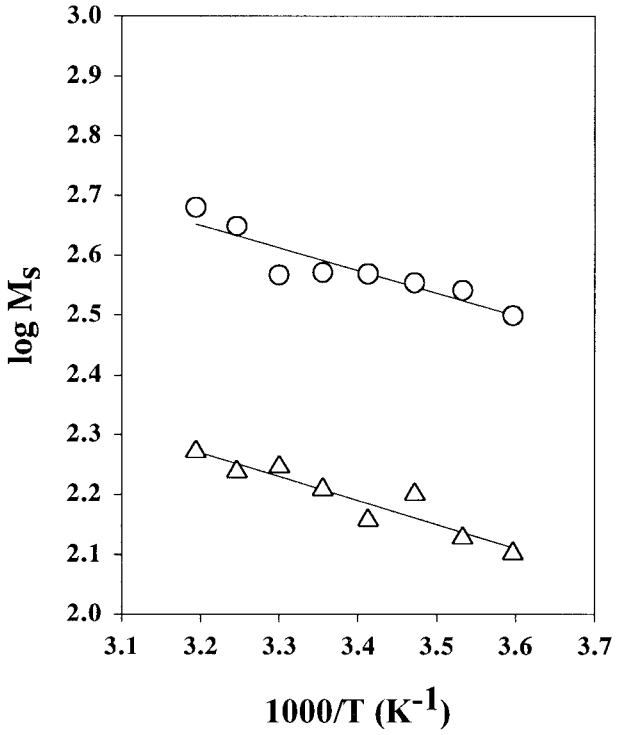


Figure 5 Effect of temperature on the maximum water uptake of 80% EDTAD-FP hydrogel before (△) and after (○) treatment with ethanol.

0.1*M* NaCl at 36°C. The saline uptake of the gel that was not treated with ethanol was about 24 g/g, whereas that of the gel treated with ethanol was about 35 g/g. The rate of saline uptake was also apparently higher in the ethanol-treated gel than that without ethanol treatment. Similar behavior is also observed with uptake of 0.15*M* saline [Fig. 4(b)]. The improvement in the rate and extent of saline uptake of the ethanol-treated gel must be related to an increase in the rate and extent of relaxation of the protein chains in the network.

Figure 5 shows the effect of temperature on equilibrium water uptake by 80% EDTDA-FP gels. The logarithm of equilibrium water uptake versus reciprocal temperature plots for both the ethanol-treated and ethanol-untreated gels showed a linear behavior in the temperature range 5–40°C. The slope of these plots were the same, suggesting that the enthalpy change (ΔH) for water uptake is the same for both these gels. Thus, the net difference in the absolute amount of water uptake at any given temperature must arise from differences in structural flexibility of the network (i.e., entropy related).

CONCLUSIONS

The results of this study clearly indicate that the swelling properties of protein-based hydrogels can be dramatically improved by treating the crosslinked gel with ethanol. This improvement occurs apparently because of ethanol-induced conformational reorganization in protein chains in the gel network. This rearrangement apparently increases flexibility of protein chains and hence the rate and extent of relaxation of the polymer network as water diffuses into the network. In addition to improving the swelling properties, the ethanol treatment offers the following advantages: 1. It dehydrates the gel and thereby eliminates the need for drying the gel. 2. It can extract low molecular weight off-odor compounds from the protein gel, especially from the fish protein-based gel, and thereby improve its acceptability in several consumer products. The ethanol-treated FP hydrogel was found to be completely free of fishy off-odor compared with that made without ethanol treatment. 3. It can also extract any residual unreacted glutaraldehyde (believed to be carcinogenic) that might be present in the gel. The ethanol used in the process can be easily recovered and recycled in the process. Although other polar organic solvents, such as propanol, butanol, acetone, etc., also can be used, ethanol is the solvent of choice because it is cheap and safe.

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