# Synthesis and Properties of Fish Protein-Based Hydrogel

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**ABSTRACT:** The novel use of fish protein (FP) in the manufacture of a superabsorbent hydrogel was investigated. Following the introduction of a large number of hydrophilic groups into fish protein by modification with ethylenediaminetetraacetic dianhydride (EDTAD), proteins were cross-linked by sulfhydryl-disulfide interchange reaction between the endogenous sulfhydryl groups and disulfide bonds to produce an insoluble gel structure. The water uptake of 76% EDTAD-modified FP hydrogel was 540 g water/g dry gel at 214 × *g,* and it depended on pH and ionic strength of the solution. The structural changes in proteins consequent to modification were also investigated. EDTAD-FP hydrogels had increased rigidity after glutaraldehyde treatment but displayed diminished water uptake characteristics.
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**KEY WORDS:** Chemical modification, fish protein, hydrogel, water absorption.

The total world fishery catch was about one million metric tons in 1993 (1), of which approximately 30% can be considered underutilized and unexploited by-catch (2). Typically, most of these low-cost fish resources are used in the manufacture of fish meal. Besides the underutilized trash fish, the waste generated from fish processing operations contains 27–49% protein on a dry weight basis and constitutes another valuable resource (3). Since the 1940s, a variety of chemical modifications have been employed to modify the functional properties (water absorption, emulsifying activity, emulsifying capacity, gelation and aeration capacity) of fish protein (FP) concentrate (4–6). However, these chemically modified protein products have never been introduced in the market owing to safety concerns. In the present study, a new application for FP as a superabsorbent hydrogel for nonfood uses has been investigated. The process to synthesize FP hydrogel and the swelling properties of the gel are described.

## EXPERIMENTAL PROCEDURES

*Materials.* Ethylenediaminetetraacetic dianhydride (EDTAD) and *N*-acetylhomocysteine thiolactone (NAHCTL) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Picrylsulfonic acid (TNBS), 25% glutaraldehyde solution, and 5,5′-dithio-bis(2-nitrobenzoic acid) were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade. Heat-sealable, water-wettable paper was purchased from Bolmet Inc. (Dayville, CT).

*Preparation of crude FP.* The fish used in this study was walleye pike *(Stizostedion vitreum),* obtained from a Wisconsin fish farm. The fish were filleted immediately upon arrival, and whole muscle was chopped and blended in chilled deionized water at a meat-to-water ratio of 1:10. The pH of the fish suspension was adjusted to 12 by the addition of 2.5 N NaOH solution, and the suspension was mixed for 30 min to totally solubilize the fish protein (FP) from muscle cells. The suspension was filtered through a 0.5-mm sieve to remove insoluble material. The filtrate was dialyzed against water (the molecular weight cut-off of the membrane was 6,000–8,000 Daltons) and lyophilized.

*Protein determination.* The protein content of the lyophilized sample was determined by subtracting the lipid and moisture content of the sample. The moisture content was determined by drying a preweighed sample to a constant weight at 105˚C. The lipid content of the lyophilized sample was determined using a solvent extraction method described below. Because the modifying groups used in this study interfered with all colorimetric methods for protein concentration determination, the protein concentration was determined by the dry weight method. 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 a percentage (wt/vol) after subtracting the lipid content of the sample.

*Determination of lipid content.* The amount of lipid present in FP and EDTAD-modified FP was determined by a solvent extraction method. To 5 mL of 5% (wt/vol) protein solution, 24 mL of mixed solvent was added to extract the total lipid. The solvent system used was ethanol-diethyl ether-petroleum ether (2:5:5, vol/vol/vol). The extraction was repeated three times, and the combined solvent fractions were evaporated in aluminum weighing pans, then dried in an oven at 100°C until an equilibrium weight was obtained.

*Modification of FP.* EDTAD-modified FP (EDTAD-FP) were prepared as follows. A 1% protein solution was ad-

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justed to pH 12 with 2.5 N NaOH solution, heated at 65°C for 30 min, and then cooled in an ice bath to room temperature. Pretreated protein was reacted with solid EDTAD. During the 2–3-h reaction period, the protein solution was stirred constantly, incremental amounts of anhydride were added during the first 30–90 min, the reaction was held at room temperature, and the pH of the protein solution during the reaction was kept constant by adding 1 N NaOH with a pH-Stat (Model 450; Fisher Scientific Instruments, Fairlawn, NJ). The reaction was terminated when the pH had remained constant for 30 min. The pH of the protein solution was then adjusted back to 7, and the solution was dialyzed exhaustively against deionized water to remove salts and lyophilized. The extent of acylation was determined by the trinitrobenzenesulfonic acid (TNBS) method (7).

*Determination of sulfhydryl group content.* The sulfhydryl group content was determined by the method of Buttkus (8). To 1.0 mL of FP solution (5–10 mg/mL) was added 9 mL of chilled potassium phosphate buffer (50 mM, pH 8.0) that contained 6 mM EDTA and 8 M urea; the resultant mixture was stirred for 30 min at 25°C. To 3 mL of the mixture was added 20 mL of 10 mM 5,5′-dithio-bis(2-nitrobenzoic acid) solution, and the resultant mixure was incubated at 40°C for 15 min. The absorbance at 412 nm was measured to calculate the total sulfhydryl group content according to Ellman (9).

*Electrophoresis.* Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a linear-gradient slab gel of 5–15% was performed according to the method of Laemmli (10). Gels were run at a constant voltage of 60 V for about 16 h. The molecular weight marker proteins were purchased from Sigma Chemical Co.

*Circular dichroic (CD) measurement.* CD measurements were made in a computerized spectropolarimeter (On-Line Instruments Systems, Inc., Jefferson, GA). A cell pathlength of 1 mm and a protein concentration of 0.02% in a 20 mM phosphate buffer, pH 6.8, were used. The instrument was calibrated with d(+)-10-camphorsulfonic acid. Ten scans of each sample were averaged, and the mean residue ellipticity, expressed as deg·cm$^2$ d-mol$^{-1}$where d = deci, was calculated by using a value of 115 for the mean residue molecular weight. All spectra were corrected for the appropriate buffer baseline. The secondary structures were estimated from CD spectra with a computer software developed by Chang *et al.* (11).

*Fluorescence measurement.* The fluorescence spectra of proteins were measured with a Perkin-Elmer Model LS-5B luminescence spectrometer (Norwalk, CT). The emission spectra were 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.

*Cross-linking by endogenous sulfhydryl–disulfide interchange reaction.* EDTAD-FP powder, enough to make a 15% (wt/vol) solution, was weighed and gradually dissolved in water and mixed well. The solution was cured at room temperature overnight.

*Cross-linking by glutaraldehyde.* Ten percent (wt/vol) of 76% EDTAD-modified FP solution was adjusted to pH 9.0 by adding 1 N NaOH solution, and glutaraldehyde solution was added at ratios of 0.025–0.01:1 (dianhydride/protein, w/w). After the addition of the cross-linking agent, the solution was mixed vigorously to homogenize it, and the solution was allowed to cure at room temperature overnight.

*Determination of water uptake.* Water uptake of gels was determined by the Tea Bag method. A gel sample of 20–30 mg was placed in a heat-sealable pouch (4 cm × 6 cm) made from a nylon and paper complex (Bolmet Inc.). The bag was dipped in water for 24 h, centrifuged at 214 × *g* for 5 min in a clinical centrifuge, and the increase in weight was measured immediately. All swelling studies were carried out at room temperature (25 ± 2°C). The water uptake of gel was indicated by the weight of water absorbed divided by the weight of dried gel. Each data point represents an average value of three gel samples.

*Effect of salt and pH on water uptake of gel.* The influence of pH and ionic strength on water uptake by gels was studied as follows. The effect of ionic strength on the swelling properties of gels was tested by immersing gel samples in various concentrations of NaCl, from 0.01 to 0.15 M, for 24 h. A control, without salt added, was also used. The influence of pH on swelling behavior of the hydrogels was determined by placing the dry gel samples in buffers of different pH, ranging from 3.0 to 10.0, at room temperature until equilibrium was attained. Buffers used for the swelling studies were pH 3.0, formic acid–KOH; pH 4.0, succinic acid–KOH; pH 5.0, acetic acid–KOH; pH 6.0, succinic acid; pH 7.0, phosphate buffer; pH 8.0, tris(hydroxymethyl)-aminoethane buffer; pH 9.0, boric acid; pH 10, carbonic acid. All buffer solutions were kept at the same ionic strength of 0.01.

*Swelling kinetics and reversibility of swelling.* To study the kinetics of swelling, gel samples were immersed in deionized water. At regular time intervals, gel samples were taken out, centrifuged at 214 × *g* for 5 min, and weighed. The reversibility of swelling and deswelling of the gel was determined by using the same samples for sequential swelling in deionized water and deswelling in 0.15 M NaCl solution.

## RESULTS AND DISCUSSION

The crude FP was extracted by using an alkali treatment (pH 12.0) to solubilize the protein from fish muscle. The protein isolate thus prepared contained both myofibrillar and sarcoplasmic proteins. The protein and lipid contents of the lyophilized protein samples were 90 and 4% on a dry weight basis, respectively. Based on the comparison of such composition with reported values for this species of fish (12), we realized that some lipid was removed from the protein sample (from 5 to 4%) during extraction, and also a part of the water-soluble sarcoplasmic proteins (MW< 6,000) must have diffused out during dialysis against water.

*Extent of chemical modification.* Figure 1 shows percentage modification of lysyl residues of FP as a function of the weight ratio of EDTAD to protein. About 90% of EDTAD

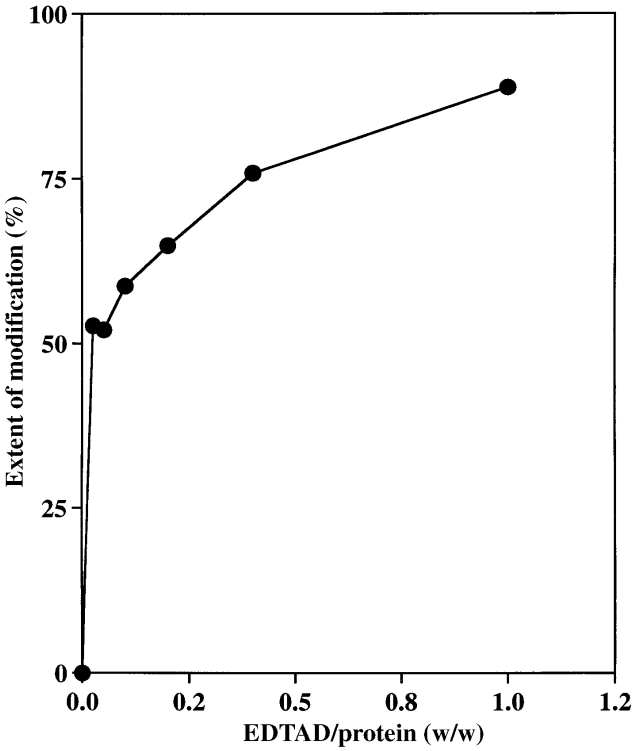


**FIG. 1.** The extent of modification of lysyl residues of fish protein as a function of the ratio of ethylenediaminetetraacetic acid dianhydride (EDTAD) to protein (w/w).

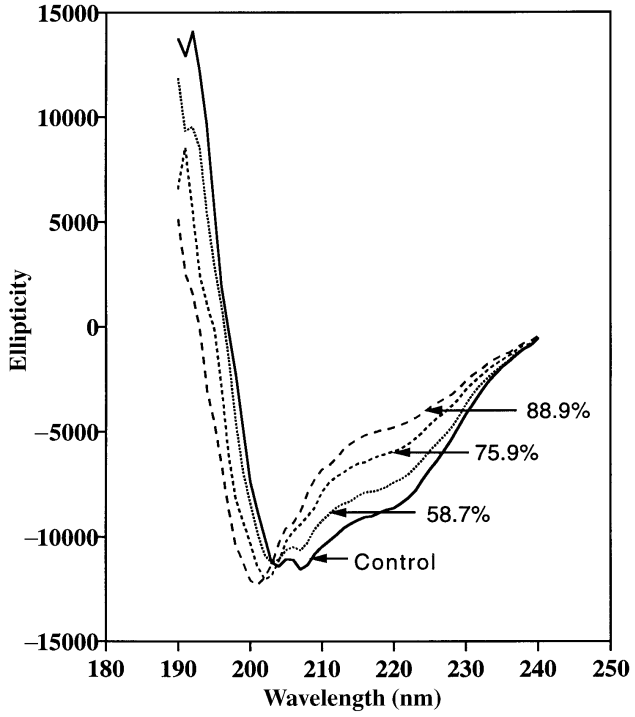


**FIG. 2.** Circular dichroic spectra of unmodified and EDTAD-modified fish proteins. The numbers represent the percentage of lysyl residues modified with EDTAD. For abbreviation see Figure 1.

modification was achieved at an EDTAD-to-protein ratio of 1:1. For soy protein, an EDTAD-to-protein ratio of 0.5 was sufficient to modify 90% of the lysyl residues (13). The higher amount of EDTAD required for FP may be related to the higher lysyl content of FP, which is about 2.5-fold greater than that of the soy protein isolate.

*Effect of modification on the structure of FP.* To determine if modification of FP with EDTAD resulted in structural changes in the protein, CD and fluorescence properties were investigated. Figure 2 shows the CD spectra of control FP and EDTAD-modified FP. The control FP, which was pretreated at pH 12, 65°C for 30 min, contained 15.5% α-helix, 43% β-sheet, and 41.5% aperiodic structure (Table 1). Slight secondary structural changes occurred in the 58.7 and 75.9% EDTAD-modified FP samples, and the major change was the transformation of α-helix to β-sheet structure. The α-helix structure, which is typical of myosin and tropomyosin, seems to have been disrupted by the alkali treatment at pH 12 because a high content of β-sheet (43.0%) instead of α-helix (15.5%) was observed in the control FP. For the 88.9% EDTAD-FP, a lower content of β-sheet (23.5%) and random coil structure and a higher amount of β-turn and α-helix structure were obtained (Table 1).

The conformational changes in EDTAD-modified FP samples were also examined by measuring the fluorescence emission spectra in the range of 300 to 400 nm with excitation at 285 nm. Figure 3 shows the fluorescence spectra of the control and EDTAD-modified FP. The results show a single peak at 341 nm (characteristic of tryptophan fluorescence) in the control sample and a progressive shift of the peak to longer wavelengths with increasing extent of modification. A similar red shift was caused in the denaturation of other proteins (14–16), indicating that EDTAD modification induced unfolding of the protein and exposure of tryptophan residues to a more polar environment.

Figure 4 shows that SDS-PAGE analysis of FP indicated that polymerization occurred during modification with EDTAD. An increasing amount of soluble high-molecular-weight polymers formed with increasing extent of modification. The polymerization must be due primarily to the formation of intermolecular cross-linking by the bifunctional EDTAD. It is also possible that other covalent bonds, such as lysinoalanine and lanthionine cross-links, might form under

**TABLE 1**
**Secondary Structure Content of Unmodified and EDTAD-Modified Fish Protein in 10 mM Phosphate Buffer, pH 7.0[a]**

| | Secondary structure (%) | | | |
|---|---|---|---|---|
| Protein | α-Helix | β-Sheet | β-Turn | Aperiodic |
| FP-C[b] | 15.5 | 43.0 | 0 | 41.5 |
| 58.7%[c] | 11.0 | 46.5 | 0 | 42.5 |
| 75.9% | 4.5 | 51.5 | 0 | 44.0 |
| 88.9% | 16.0 | 23.5 | 26.5 | 34.0 |

[a]Abbreviations: EDTAD, ethylenediaminetetraacetic dianhydride.
[b]Fish protein (FP) heated for 30 min at pH 12, 65°C.
[c]Fish protein heated for 30 min at pH 12, 65°C and then modified with EDTAD at pH 12, 25°C.

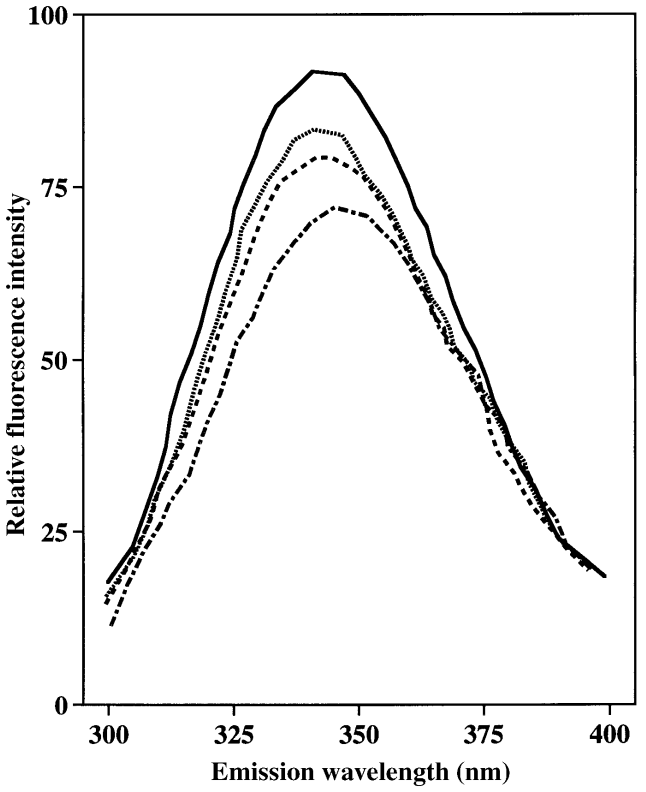


**FIG. 3.** Fluorescence spectra of unmodified and EDTAD-modified fish proteins (FP). _____, unmodified; . . . . . , 58.7% EDTAD-FP; - - - - -, 75.9% EDTAD-FP, - • - • - , 88.9%. For other abbreviation see Figure 1.

the alkaline reaction conditions used. Because these samples were treated with 5% β-mercaptoethanol and 2% SDS, the high-molecular-weight polymers present in Figure 4 cannot be due to disulfide cross-linking. They must be due to protein cross-linking by the bifunctional EDTAD. Previously it has been demonstrated that protein cross-linking by EDTAD at low protein concentration occurs only when the protein molecules are in an oligomeric or aggregated state (16). It appears that a pH 12/65°C pretreatment is not sufficiently drastic to dissociate the myofibrils and to prevent protein–protein cross-linking by the bifunctional EDTAD.

*Changes in free sulfhydryl group content of FP.* Figure 5 shows changes in the free sulfhydryl group content of fish protein during modification with EDTAD. The free SH group content increased from 6 mol per $5 \times 10^5$ g protein in the control sample to a maximum of 21 mol per $5 \times 10^5$ g in the 75% EDTAD-FP sample. Further increase of the extent of modification decreased the free SH group content, for example, 14.5 mol per $5 \times 10^5$ in the 89% EDTAD-FP sample. It appears that the EDTAD-modification of FP at pH 12 caused cleavage of protein disulfide bonds, probably via conformational changes and partial β-elimination of the newly exposed cystine residues.

*Water swelling properties of EDTAD-FP hydrogel.* When a weighed amount of EDTAD-FP, sufficient to form 15% solution, was dissolved in water and left to stand at room temperature overnight, it formed a gel without the addition of a

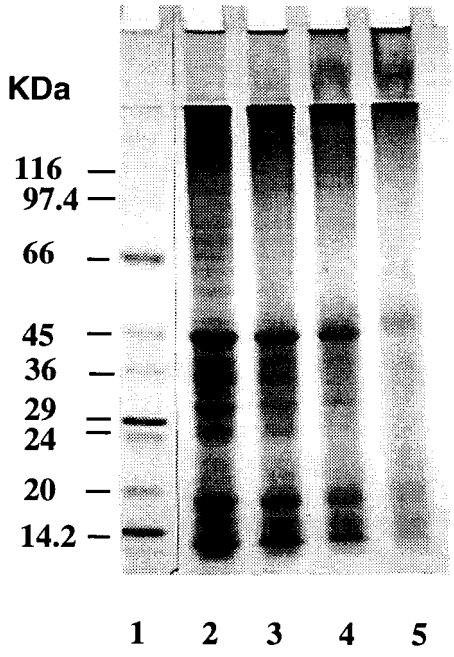


**FIG. 4.** Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis of unmodified and EDTAD-modified FP. All samples were treated with 5% β-mercaptoethanol and 2% SDS. Lane 1, molecular weight markers; 2, unmodified FP; 3, 58.7% EDTAD-FP; 4, 75.9% EDTAD-FP; 5, 88.9% EDTAD-FP. See Figures 1 and 3 for abbreviations.

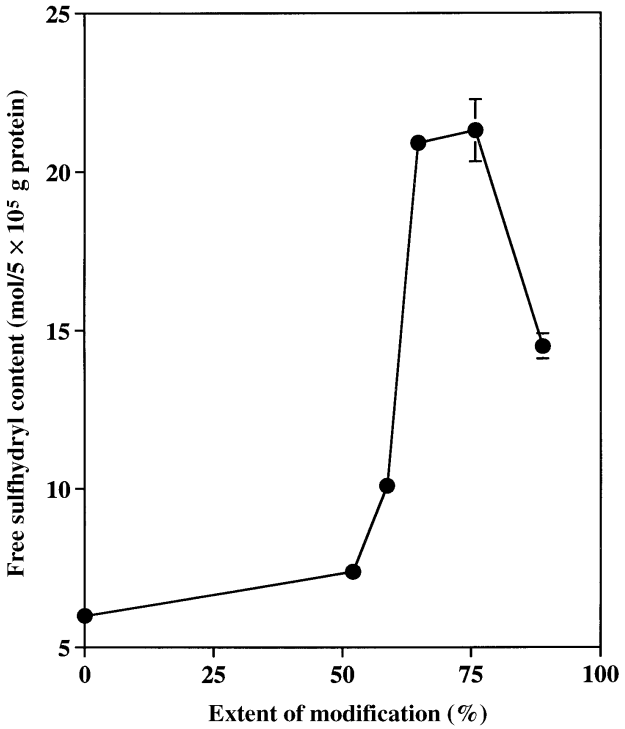


**FIG. 5.** Free sulfhydryl group content of FP as a function of extent of modification of lysyl residues with EDTAD. See Figures 1 and 3 for abbreviations. The bars represent standard error.

cross-linking agent, e.g., glutaraldehyde. When the dried gel was suspended in water, it swelled and absorbed a large quantity of water but did not dissolve. The formation of this insoluble gel network appeared to be due to both intermolecular SH oxidation and SH-S-S interchange reaction. When the gel was immersed in 5% β-mercaptoethanol solution, it disintegrated with time and finally became a solution, confirming that disulfide linkages contributed mainly to the formation of the gel structure. To elucidate the advantages of S-S-cross-linked versus glutaraldehyde-cross-linked hydrogels, the water uptake properties of these gels were examined.

*Swelling kinetics.* Figure 6 shows the swelling kinetics of the 76% EDTAD-FP hydrogel in deionized water. Water uptake by the gel occurred rapidly during the first 5 h of immersion and reached a maximum water uptake of 580 g water/g of dry gel after 24 h. Comparison of the water uptake property of the 76% EDTAD-FP hydrogel with that of the control gel (which absorbed only a maximum of 30 g water/g dry gel under identical conditions) shows that incorporation of EDTA carboxyl groups into the protein caused significant unfolding and expansion of the polypeptide gel network *via* electrostatic repulsion and thus facilitated greater imbibition of water.

*Effect of ionic strength on water uptake.* The effect of salt on water uptake by the control and EDTAD-modified FP hydrogels is shown in Table 2. The water uptake of the EDTAD-FP hydrogel was sensitive to ionic strength, which is typical of polyanionic hydrogels. For example, the 76% EDTAD-FP hydrogel showed a steep decrease in water uptake from 540 to 12.3 g water/g dry gel when it was exposed to a 0.15 M NaCl solution.

**TABLE 2**
**Effect of Salt Concentration on Water Uptake of EDTAD-FP Hydrogels**

| NaCl concentration (M) | FP[a] | EDTAD-FP65 | EDTAD-FP76 |
|---|---|---|---|
| 0 | 33.3 ± 2.0[b] | 386.6 ± 45.2 | 526.0 ± 57.2 |
| 0.01 | 9.0 ± 1.5 | 35.2 ± 0.2 | 52.6 ± 1.2 |
| 0.05 | 6.7 ± 0.2 | 18.2 ± 5.7 | 20.8 ± 6.2 |
| 0.10 | 5.4 ± 1.0 | 12.9 ± 3.1 | 20.6 ± 6.3 |
| 0.15 | 4.9 ± 1.3 | 10.7 ± 0.6 | 12.3 ± 3.9 |

[a]FP, unmodified fish protein hydrogel; EDTAD-FP65, 65% EDTAD-modified FP hydrogel; EDTAD-FP76, 76% EDTAD-modified FP hydrogel. For other abbreviations see Table 1.
[b]Values represent mean ± standard error ($n$ = 3).

*Reversible swelling–deswelling.* Given that the EDTAD-FP hydrogels were salt-sensitive, the effects of sequential exposure to water and 0.15 M NaCl solution on their water absorbency were investigated. As shown in Figure 7, the 76% EDTAD-FP gel exhibited a faster rate of water absorption after one cycle of swelling–deswelling, and the recycled gel imbibed more water (700 g water/g dry gel) than the original gel. This might be due to an increase in the flexibility of polypeptide chains in the gel network after one cycle of the swelling–deswelling process. This result indicates that the EDTAD-FP hydrogel can be used repeatedly in dewatering processes.

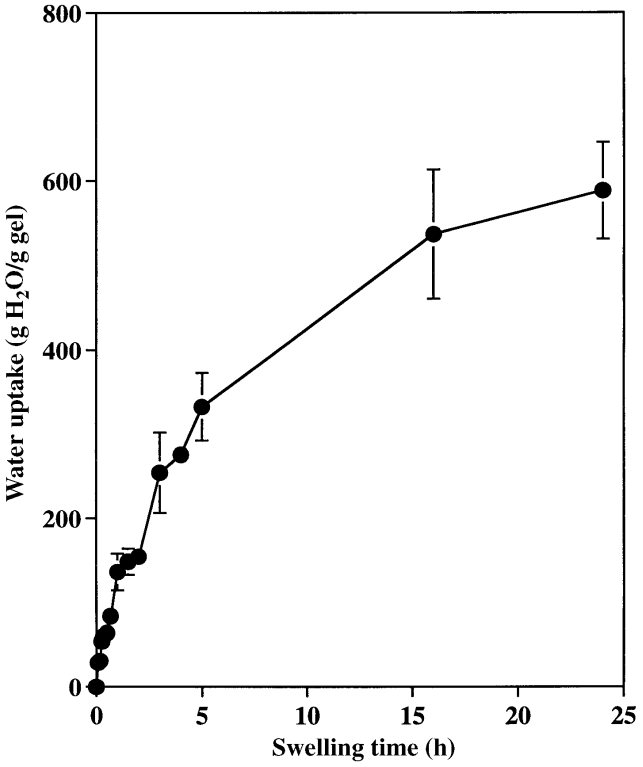


**FIG. 6.** Kinetics of swelling of 76% EDTAD-FP hydrogel. See Figures 1 and 3 for abbreviations. The bars represent standard error.

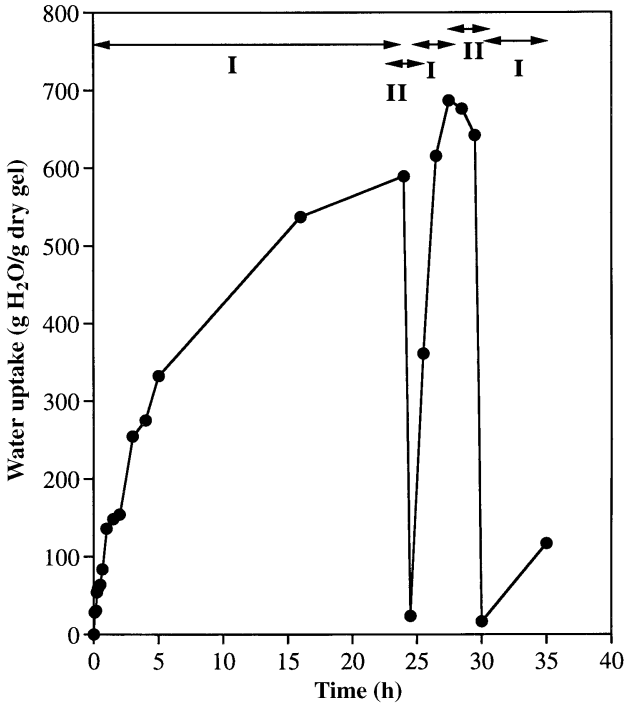


**FIG. 7.** Reversible swelling of 76% EDTAD-FP hydrogel. **I** represents swelling in deionized water, and **II** represents deswelling in 0.15 M NaCl. See Figures 1 and 3 for abbreviations.

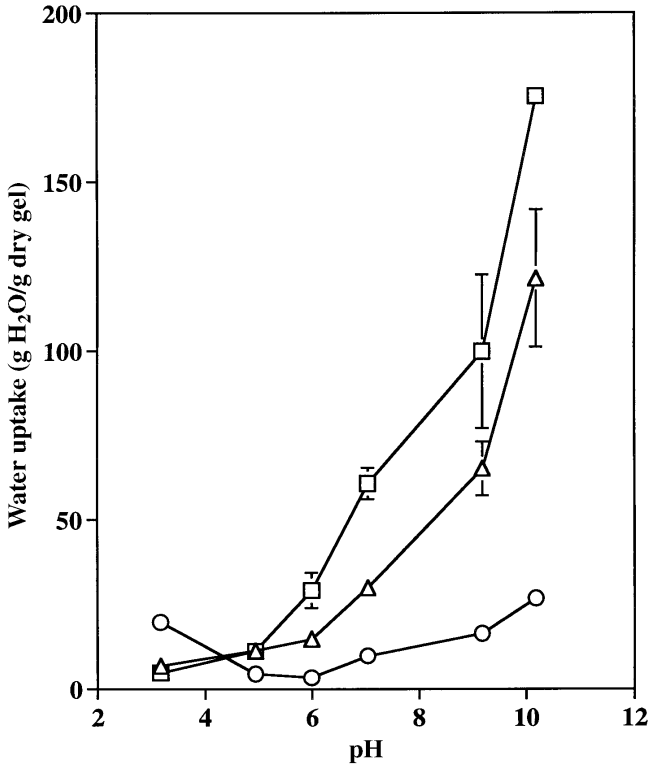


**FIG. 8.** Effect of pH on the water uptake of EDTAD-FP hydrogel. ○, unmodified control; △, 65% EDTAD-FP; □, 76% EDTAD-FP. See Figures 1 and 3 for abbreviations. The bars represent standard error.

*Effect of pH.* Because the solution pH affects the ionization of carboxyl and other ionizable groups (17,18), one would expect that changing the solution pH would affect water uptake of protein gels. The effect of pH on the water uptake by EDTAD-FP hydrogels was more significant than its effect on the control gel (Fig. 8). For example, water uptake of the 76% EDTAD-FP hydrogel increased from 5 g water/g dry gel at pH 3.2 to about 180 g water/g dry gel at pH 10. This might be related to ionization of acidic groups, e.g., carboxyl groups of the protein at high pH, resulting in an increase of electrostatic repulsion between polypeptide chains and expansion of the gel network. The $pK_1$, $pK_2$, and $pK_3$ values of the carboxyl groups of EDTA are 2.0, 2.6, and 6.2, respectively. Thus, in theory, all carboxyl groups should fully ionize, and the water uptake should reach a maximum value at about pH 8. The increase in water uptake to pH 10 therefore must have been due to a further increase in the net charge of the protein, resulting from the ionization of tyrosine residues ($pK_3 = 9.6$). Interestingly, the control gels showed an increase in water uptake at pH 3.0, which was not so for the EDTAD-FP gels. This is probably due to the higher amount of lysyl residues in control FP than in EDTAD-FP, which are protonated at pH 3.0. A net increase of positive charge, owing to protonation of the carboxyl groups below pH 4, may cause electrostatic repulsion within the gel matrix and thus allow water uptake at pH 3.0. The ionic strength of all buffers was 0.01, which may account for the lower water uptake of the gel obtained in this pH-effect study. For example, the 76% EDTAD-FP gel took up 540 g water/g dry gel in deionized water at pH 7.0, while it absorbed about 60 g water/g dry gel in pH 7.0 phosphate buffer at $I = 0.01$.

*Effect of cross-linking density on water uptake.* The three-dimensional network of the gels already described was mainly supported by the endogenous disulfide linkages. However, although the 76% EDTAD-FP contained 21 mol of SH group per $5 \times 10^5$ g FP (Fig. 5), all these SH groups may not have participated in the formation of disulfide linkages in the gel structure. Thus, it is understandable that EDTAD-FP hydrogels were relatively weaker than other protein-based hydrogels (13) because of the low cross-linking density. Consequently, another strategy, as described next, to increase the cross-linking density of EDTAD-FP hydrogel was investigated.

*Cross-linking with glutaraldehyde.* Glutaraldehyde, a frequently used bifunctional agent, was employed to cross-link the EDTAD-FP. The influence of three ratios of glutaraldehyde to protein, i.e., 0.0025, 0.005, and 0.01:1, on the water uptake of gels is presented in Figure 9. The addition of glutaraldehyde did slightly increase the rigidity of 76% EDTAD-FP gel, and it was unaffected by the amount of added glutaraldehyde. However, the increase in cross-linking density impaired the water uptake of gels. Figure 9 shows a dramatic decrease in water uptake by the gel, even at a low glutaraldehyde-to-protein ratio of 0.0025:1. Thus, if the gels were to be used to absorb water under certain applied pressure, the glutaraldehyde cross-linked hydrogel is preferable to the S-S-

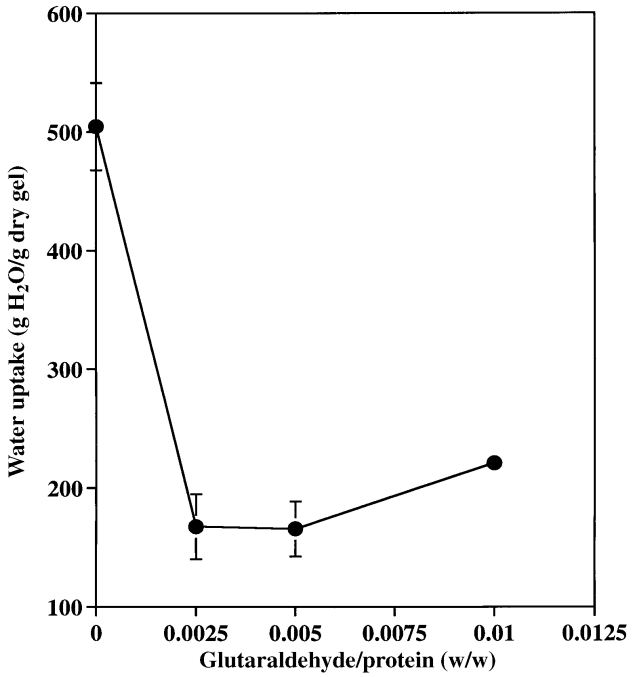


**FIG. 9.** Effect of cross-linking with glutaraldehyde on the water uptake of 76% EDTAD-FP hydrogel. See Figures 1 and 3 for abbreviations. The bars represent standard error.

only cross-linked gel.

Hydrogels may be used in several industrial processes, such as dewatering (19,20) and ion exchange processes; in environmental applications, such as remediation of heavy metal-contaminated soil (21,22) and biodegradable controlled-release media for pesticides and herbicides (23,24); and in consumer products, such as disposable diapers. A FP-based hydrogel that is biodegradable will have an enormous advantage over synthetic hydrogels (e.g., polyacrylate-based hydrogels) in all these applications.

## ACKNOWLEDGMENT

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