Metal-Chelating Properties and Biodegradability of an Ethylenediaminetetraacetic Acid Dianhydride Modified Soy Protein Hydrogel

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Received 27 August 1996; accepted 12 September 1996

ABSTRACT: The heavy-metal chelating properties of a soy protein based hydrogel, prepared by crosslinking an ethylenediaminetetraacetic acid dianhydride (EDTAD) modified soy protein isolate (SPI), have been studied. The equilibrium binding capacities of the divalent calcium, zinc, mercury, and lead ions by the gel were 0.70, 0.65, 0.95, and 0.70 mmol per gram of dry gel, respectively. The distribution ratio of metal ions between the gel and the solution was in the range of 370 to 15,000 mL/g, depending on the initial metal concentration. A positive relationship between the carboxyl group content of EDTAD-modified SPI and the metal-binding ability of the gel was observed; the optimum metal binding occurred at 25°C. The metal-binding ability increased with increasing pH, in the range where the solubility of the metal ions was not affected by the pH. In binary metal ion solutions, the metal ions adsorbed to the gel in a competitive fashion, influenced by the initial ion concentration. The EDTAD-modified protein hydrogel was readily degraded by proteolytic enzymes and was biodegraded in a fungal overgrowth test. The EDTAD-SPI hydrogel was completely degraded after a 28-day incubation with fungal spores. © 1997 John Wiley & Sons, Inc. J Appl Polym Sci **64:** 891–901, 1997

Key words: hydrogel; soy protein; biodegradability; metal chelation; chemical modification

INTRODUCTION

In technologically developed societies, the widespread use of heavy metals in a diverse number of applications, including pigments, coatings, electronic equipment, etc., has led to a surfeit of heavy metal-contaminated materials in municipal disposal sites and groundwater. Societal concerns over the toxicity of heavy metals is intense and has prompted the vigorous investigation of biopolymers as heavy-metal adsorbing agents. However, few of these biopolymers are protein based. In previous reports,[1–2] the synthesis and water-swelling properties of a biodegradable ethylenediaminetetraacetic dianhydride (EDTAD) modified soy protein based hydrogel were described. It has been shown that the introduction of a number of carboxyl groups (from EDTAD) to the protein backbone of the glutaraldehyde-crosslinked gel resulted in products that imbibed large volumes of water, i.e., 100 to 300 grams of water per gram of dry gel. Proteins contain an electric array of chemical functions, i.e., carboxyl, amino, sulfhydryl, hydroxyl, and imidazole groups with thoroughly described heavy-metal binding properties.[3–10] Thus, in addition to the endogenous heavy metal-binding groups of proteins, it is expected that protein gels with an excess of incorporated carboxyl groups will have enhanced heavy-metal chelating properties.

In the present study, the binding affinity of this

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Contract grant sponsor: USDA-NRICGP; contract grant number: 93-37500-9242.

gel for calcium ions and environmentally relevant heavy-metal ions, including lead, zinc, and mercury, was investigated. The effects of environmental pH, carboxyl group content of the protein, and competitive adsorption between ions, as well as the biodegradability of this EDTAD-modified soy protein isolate (SPI) hydrogel have also been investigated.

EXPERIMENTAL

Materials

Defatted soy flour was purchased from Central Soya Co. (Fort Wayne, IN). EDTAD, $CaCl_2$, $PbCl_2$, $HgCl_2$, and $ZnCl_2$ (>98% pure) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Calcium, lead, mercury, and zinc reference standard solutions for atomic absorption spectrophotometry were obtained from Fisher Scientific (Pittsburgh, PA). Three enzymes—α-chymotrypsin (from bovine pancreas, Type II, 42 units/mg), protease (from *Stretomyces griseus,* type V, 1.1 units/mg), and trypsin (from porcine pancreas, Type II, 1800 BAEE/mg)—and 25% glutaraldehyde solution were purchased from Sigma Chemical Co. (St. Louis, MO). Five fungi—*Aspergillus niger, Penicillium pinophilum, Chaetomium globosum, Gliocladium virus,* and *Aureobasidium pullulans*—were obtained from American Type Culture Collection (Rockville, MD). All other chemicals were of analytical grade.

Preparation of SPI

Soy isolates were prepared essentially as described elsewhere.[11] Briefly, the defatted soy flour was extracted with water at pH 8.0 using a meal-to-water ratio of 1 : 10. The insoluble materials were removed by centrifugation, and the supernatant was adjusted to pH 4.5 with 2*N* HCl. This isoelectric precipitate was neutralized (to pH 8.0) with sodium hydroxide, dialyzed against water overnight, and then lyophilized.

Modification of SPI

EDTAD-modified soy protein isolates (EDTAD-SPI), were prepared according to the method described in a previous report.[1] A 1% SPI solution at pH 12 was heated at 65°C for 30 min, cooled to room temperature, and modified by the stepwise addition of solid EDTAD to a protein-to-EDTAD ratio of 10 : 1. The pH of the protein solution during the reaction was kept constant by the addition of 1*N* NaOH using a pH-Stat (Model 450, Fisher Scientific Instruments). After a 3-h reaction period, the pH of the protein solution was adjusted to 7 and the solution was dialyzed exhaustively against deionized water to remove salts and then lyophilized. The extent of acylation of gels was determined using the 2,4,6-trinitrobenzenesulphonic acid method as described by Hall and colleagues.[12] The extent of modification of the sample gel used throughout the study was 64%. The carboxyl group contents of samples were estimated by electrometric titration and results are presented as mole carboxyl group per 10^5 g protein (assuming the molecular weight of SPI is 10^5 Daltons).

Preparation of Hydrogel

A 150-μL portion of a 25% aqueous solution of glutaraldehyde was added to 10 mL of 15% acylated protein solution, pH 9.0. Following mixing, the gel was cured overnight at room temperature and subsequently dried in an oven at 40°C. A 0.5-mm gel particle size was obtained by passing the ground gel through 32- and 16-mesh steel sieves.

Equilibrium Adsorption Studies

Equilibrium adsorption of metal ions at 25°C was studied using an equilibrium dialysis cell (Fisher Scientific) containing a 2000-Dalton molecular weight cutoff membrane. The metal ion solutions studied covered the concentration range 1–500 ppm and were made by dissolving $CaCl_2$, $HgCl_2$, $PbCl_2$, and $ZnCl_2$ in deionized water. The pH of each solution was adjusted to 6.0 by adding 1*N* NaOH. For equilibrium dialysis experiments, 20 mg of dry sample was immersed in 7.5 mL of the pertinent metal ion solution and dialyzed against 7.5 mL of the same metal ion solution for 48 h on a shaker in an incubator. Two reference tests were used for each dialysis experiment and consisted of dialyzing the gel against water or not placing the gel in the ion solution. After 48-h equilibrium dialysis, the metal ion concentration of the solution in the non-gel side was determined. Uptake values (mmol/g) were calculated as the difference in metal ion content of the pre- and postdialyzed solutions divided by the weight of dry gel. To eliminate contamination, all glassware and plasticware were cleaned with RBS detergent (Pierce, Rockford, IL) and rinsed with deionized water.

Distribution Ratio of Metals

Required data were extracted from the equilibrium adsorption study, and the distribution ratio of metal ions between the gel and the solution was calculated according to the formula of Parija and associates[13]:

$$D(\text{mL/g}) = \frac{\text{mmol of metal bound/dry weight of sample}}{\text{mmol of metal in solution/volume of solution}}$$

Effect of pH and Temperature on the Metal Binding of Gel

The pH range investigated in the present study was 3.6 to 7.0, and the buffer systems used were succinic acid-KOH for the pH range 3.6 to 6.5, and phosphate buffer for pH 6.5 and 7.0. The ionic strength of all buffer systems was 0.01. Three different temperatures were studied by incubating at 5, 25, or 45°C.

Competitive Adsorption Study

The selectivity of the gel for various metals was determined using the following procedure. A known weight of the sample (0.02 g) was equilibrated for 48 h with 15.0 mL of metal ion solution containing various same molar concentrations of ion pairs. The pH of these solutions was adjusted to 6.0 because all of the metal ions investigated were soluble at this pH and the functional groups, e.g., the carboxyl groups in proteins, would also be ionized at this pH. The procedure to measure the concentration of metal ion remaining in solution was as described earlier.

Metal Ion Analysis

Metal ion concentrations were determined by flame atomic absorption spectrophotometry (AAS) (Perkin-Elmer Model 2380 atomic absorption spectrophotometer). All instrumental conditions were optimized for maximum sensitivity as described by the manufacturer. For each sample, the mean of 10 AAS measurements was recorded.

Enzyme Hydrolysis of EDTAD-SPI Hydrogel

Dried EDTAD-SPI granules (10 mg, 0.5 mm diameter) were added to 4 mL of 0.05 mg/mL enzyme solution. Chymotrypsin and protease were dissolved in 10 mM phosphate buffer, pH 7.4; trypsin was dissolved in 20 m*M* phosphate buffer, pH 7.0. The mixtures of enzyme solution and gel were shaken gently in an incubator at 25°C for chymotrypsin and trypsin digestion and at 37°C for protease digestion, followed by centrifugation in a swinging-bucket-type clinical centrifuge for 5 min at 1744 $\times$ g. Aliquots were drawn from the supernatant at different time intervals and the absorbance at 280 nm was measured after proper dilution with buffer. The control was prepared by placing exactly the same amount of the sample in deionized water. The absorbance of the control was subtracted from that of the enzyme–gel mixture to obtain the net absorbance resulting from enzyme hydrolysis. The samples for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis were prepared by drawing aliquots of enzyme–gel mixture and mixing with the same volume of sample buffer after 24-h hydrolysis.

Fungal Overgrowth Test of EDTAD-SPI Hydrogel

The biodegradability of this hydrogel by fungi was investigated by using ASTM method G21-90.[14] EDTAD-SPI samples (0.5-mm size) were placed in sterile Petri dishes containing solidified nutrient salt agar. A mixed fungus spore solution was prepared from spores of five fungi (see Materials section) as described by the ASTM method. The samples were then incubated at 28°C with the spore suspension and the covered dishes were sealed with a paraffin film. The only source for the growth of the fungi was from the samples. The fungus growth was followed by visual observations and photography at 0, 4, 7, 14, 21, and 28 days of incubation.

Electrophoresis

SDS-PAGE using a 12.5% gel was performed according to the method of Lammeli.[15] Gels were run at a constant current of 25 mA for about 5 h. The molecular weight marker proteins were from Sigma Chemical Co.

RESULTS AND DISCUSSION

Based on the pH titration data (not shown), the carboxyl group content of the 64% EDTAD-modified SPI was calculated to be 237 moles per 10^5 grams of protein, i.e., 94 extra carboxyl groups

were added to each mole of protein (the carboxyl group content of unmodified protein was 143 mol/10^5 g). The efficiency of modification, i.e., the number of carboxyl groups introduced per modified lysyl group, was about 3. Thus, three of the four carboxyl groups on each EDTA molecule attached to the lysyl groups were presumably still in the free ionized form. As shown in Figure 1, the geometric characteristics of these carboxyl groups were favorable to form a stable complex with metal ions, and the complex was expected to have a better heavy-metal chelating property. Another anticipated benefit to the incorporation of EDTA functional groups into SPI is the possibility of causing protein unfolding as a result of increased electrostatic repulsion between the newly incorporated carboxyl groups. Such unfolding may expose more endogenous functional groups and possibly improve the surface activity and increase water- and ion-binding capacities. The structural changes of soy protein molecules after EDTAD modification have been discussed in detail in previous reports.[1–2]

Adsorption Isotherm

Figure 2 shows the Pb^{2+}, Zn^{2+}, Hg^{2+}, and Ca^{2+} adsorption isotherms of EDTAD-SPI gel. In order to compare the adsorption phenomena of various metals by this gel, we chose molar concentration instead of ppm as the *x*-axis. Figure 2 indicates that the binding of metal ions by this gel was a function of the equilibrium concentration of metal ions. The binding capacity of the gel for Ca^{2+}, Zn^{2+}, Hg^{2+}, and Pb^{2+} ions was about 0.7, 0.65, 0.95, and 0.70 mmol per gram of dry gel, respectively, after 48-h equilibrium dialysis. It appears that the gel had the strongest affinity for Hg^{2+} and Pb^{2+} ions, because adsorption of these ions

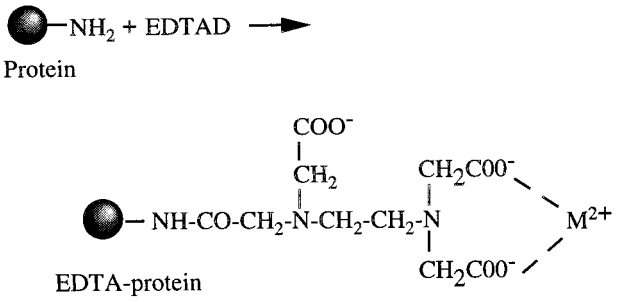

Figure 1 Schematic representation of metal ion binding to the EDTAD moieties attached to protein molecules.

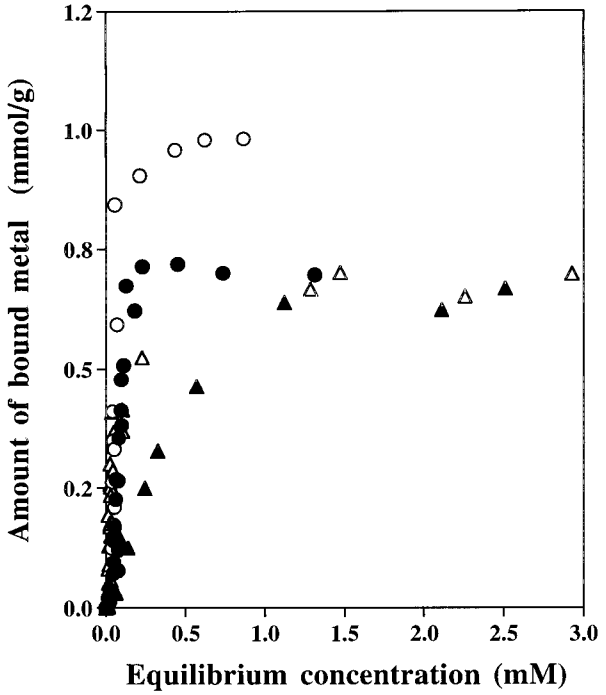

Figure 2 Adsorption isotherms of divalent metal ions to EDTAD-SPI hydrogel at pH 6.0 and 25°C. (○) Hg^{2+}; (●) Pb^{2+}; (△) Ca^{2+}; (▲) Zn^{2+}.

reached saturation at a lower equilibrium bulk concentration, i.e., at about 0.2 mM, whereas Ca^{2+} and Zn^{2+} adsorption reached saturation at higher equilibrium concentrations, i.e., about 0.3, 0.7, and 1.0 m*M*, respectively. Conversely, the binding capacity of the gel for the Zn^{2+} ion was much lower than for the other three ions and required a 5-fold higher equilibrium ion concentration than for Pb^{2+} to achieve saturation binding.

Among the divalent ions studied, mercury exhibited the highest extent of binding. It is known that the sulfhydryl group of cysteine residues form a covalent bond with mercury during adsorption.[3,6] In addition to the binding of Hg^{2+} by sulfhydryl groups, histidine residues may also play a role in chelating Hg^{2+} above pH 6.0.[3] Furthermore, the two nitrogen atoms of EDTA may also function as Hg^{2+} chelators.[16] It is these combined chemical functions that may account for the higher extent of binding of Hg^{2+} to the gel. Moreover, it should be noted that the adsorption studies were conducted using dry gel as the adsorbent, and therefore the metal-binding process consists of at least two steps, i.e., water-swelling of the gel and the binding of metal ions. Apparently the adsorption of metals by this protein gel did not follow an ideal behavior, and it was not surprising

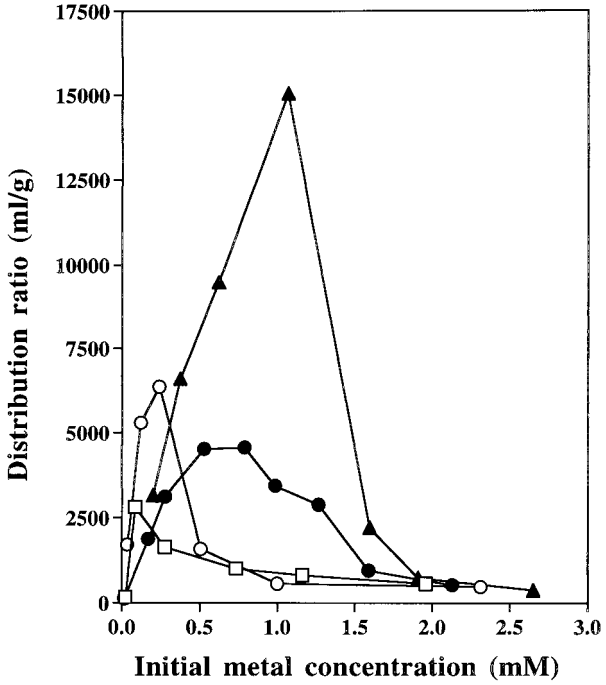

Figure 3 Effect of initial metal concentration on the distribution ratio of metal ions between EDTAD-SPI hydrogel and the aqueous solution at 25°C. (○) Ca^{2+}; (▲) Hg^{2+}; (□) Zn^{2+}; (●) Pb^{2+}.

that the Langmuir and Freundich adsorption models could not be fitted to present data.

Distribution Ratio of Metal Ions

The effect of initial metal concentration on the distribution ratio of the metal ions between the gel and solution is shown in Figure 3. The distribution ratio indicates the strength of metal-binding by absorbent. Two phases on each curve were observed in Figure 3. Below a critical initial metal concentration the metal-binding ability of this gel was enhanced by increasing the initial concentration of metals in solution. Conversely, a lower distribution ratio was observed as the initial metal concentration was increased above the critical concentration. The critical concentrations where a maximum occurred in the distribution ratio for Zn^{2+}, Ca^{2+}, Pb^{2+}, and Hg^{2+} binding were 0.08, 0.24, 0.78, and 1.07 mM, respectively, and the maximum distribution ratios were 2,821, 6,364, 4,565, and 15,044 mL/g in the order designated. Others have reported single-phase curves and a decreasing metal-ion distribution ratio with increasing metal concentration in solution.[17–18] According to the present study, the EDTAD-SPI gel showed a different distribution ratio pattern from those of plant tissues such as tomato and tobacco roots[17] or synthetic polymers such as crosslinked copolymers of 1-(β-acryl amidoethyl)-3-hydroxy-2-methyl-4(1H) pyridione and *N,N*-dimethylacrylamide.[18] The existence of such break points might indicate a change in the equilibrium behavior of the adsorption system. As mentioned in the previous report,[2] EDTAD-SPI hydrogel was ionic strength-sensitive and it showed a lower water uptake in the presence of salt. Thus, the lower distribution ratio at higher initial metal concentrations might be due to poor swelling of the dry gel in high salt solutions. Within the critical concentration, the gel can swell normally, expose metal-binding sites on protein molecules, and adsorb metal ions from the aqueous solution. In contrast, the gel does not swell well at the higher initial metal-ion concentrations, e.g., 2.0 m*M* Hg^{2+}, and consequently fewer metal-ion binding sites on the protein are exposed. Thus the critical concentration for metal binding as shown in Figure 3 can be interpreted as the maximum allowable salt concentration at which the gel can freely swell and adsorb metal ions.

The results shown in Figure 3 provide very useful information for optimizing the metal-binding efficiency of the EDTAD-SPI, e.g., knowledge of the critical concentrations of various metal ions at which a maximum distribution ratio occurs will ensure optimal metal binding by the gel.

Effect of Carboxyl Group Content on Metal Binding

In order to evaluate the relationship between the carboxyl group content of the protein and the metal-binding capacity of the gel, we compared the metal adsorption capacity of the gel with different extents of EDTAD modification. The results shown in Figure 4 indicate that the metal ion binding was positively correlated with the carboxyl group content of the protein. The first data point on each curve represents the metal binding of control gel (which contained 140 moles of carboxyl groups per 10^5 grams of protein). Generally speaking, the metal binding increased with increasing carboxyl group content except for the lead binding, and the most significant effect was observed on Ca^{2+} binding. The metal ion binding by the hydrogel is a multifactorial phenomenon and is related to the number of binding sites as well as some other factors, e.g., swelling properties, the geometric arrangement of the acceptor groups, temperature, and pH. The results

also indicate that the added carboxyl groups behave differently from the endogenous carboxyl groups (amino acids such as aspartic and glutamic acid), and it seems that the pendant carboxyl groups contributed to better metal-binding capacity. For example, the mercury-binding capacity of the gel with 65 moles extra carboxyl groups per mole protein (about a 40% increase of carboxyl group content) was almost double that of the control protein gel.

Structural modification of the protein also has to be considered since the incorporation of extra carboxyl groups caused protein unfolding due to electrostatic repulsion and resulted in the exposure of more binding sites on the surface of the protein.

Effect of Temperature on Metal Binding

The effect of temperature on the Pb^{2+} binding capacity of the gel is shown in Figure 5. Optimum Pb^{2+} binding (0.75 mmol/g) by the EDTAD-SPI gel occurred at 25°C and the lowest level of Pb^{2+} adsorption occurred at 5°C, i.e., 0.5 mmol/g; at 45°C, the extent of Pb^{2+} was lower than that at 25°C. As reported in the previous paper,[2] the swelling rate of EDTAD-SPI gels was found to be

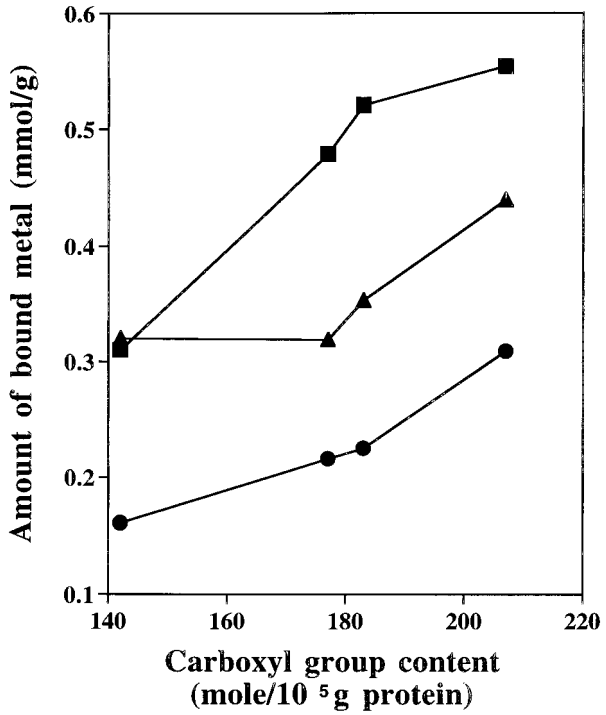

Figure 4 Effect of carboxyl group content on the metal-binding capacity of EDTAD-SPI hydrogel at 25°C. (■) Ca^{2+}; (▲) Pb^{2+}; (●) Hg^{2+}.

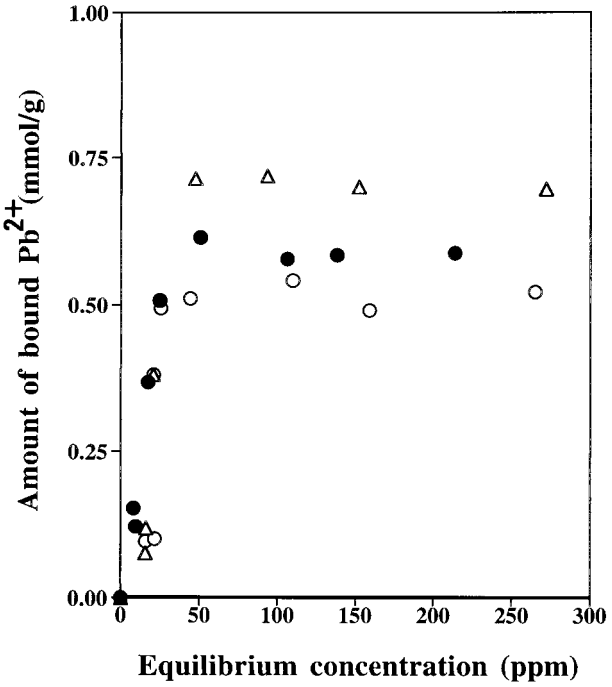

Figure 5 Effect of temperature on the Pb^{2+} binding capacity of EDTAD-SPI hydrogel. (○) 5°C; (△) 25°C; (●) 45°C.

temperature-dependent. The gel had the fastest swelling and best water uptake at 45°C, and a slow swelling and low water uptake at 5°C. Apparently, the swelling properties of the gel are not the only factor affecting metal binding, and it appears that the major force (i.e., electrostatic interactions) involved in metal ion binding might be weakened by increasing temperature. However, because electrostatic interactions are exothermic, one would expect maximum Pb^{2+} binding at 5°C, not at 25°C. This might be because of slow relaxation of protein backbone in the gel matrix at 5°C. The electrostatic neutralization of initially exposed (swelling-induced) COO^- groups by Pb^{2+} also may have caused the gel to collapse on the periphery before swelling and complete exposure of all COO^- groups in the core of the gel particles. This may have limited diffusion of ions into the gel. The results suggest that a combination of optimum extent of swelling and electrostatic interactions facilitated the maximum extent of Pb^{2+} binding at 25°C.

Effect of pH on Metal Binding

In most equilibria between metal ions and proteins, the metal ions compete with protons for the

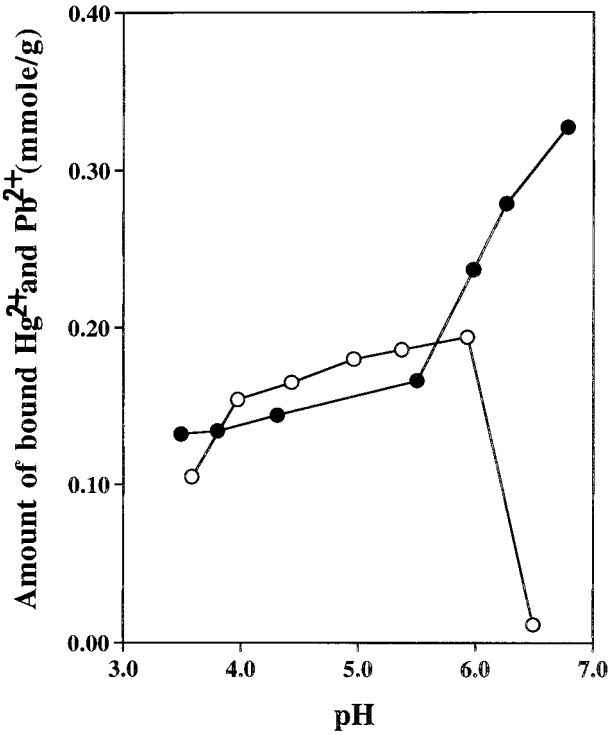

Figure 6 Effect of pH on the Pb^{2+} and Hg^{2+} binding capacity of EDTA-SPI hydrogel at 25°C. (○) Pb^{2+}; (●) Hg^{2+}.

binding sites on proteins so that, as in almost all aqueous equilibria including acid dissociations, pH will be of dominant importance. The distribution of metal cations between free and bound states will depend on pH, and much less metal ion should be bound under acidic conditions than at basic pH. Also, the extent of ionization of functional groups in protein is pH-dependent, i.e., the carboxyl, hydroxyl, and sulfhydryl groups on protein side chains are protonated at low pH values. Conversely, at higher pH the deprotonated groups (e.g., negatively charged thiols or carboxylate) will be more nucleophilic than the protonated species, and therefore can form ion pairs or complex with the metal ions as shown in Figure 6. A rapid increase in binding of lead occurred between pH 3.5 and 4.0 and increased further as the pH was increased from 4 to 6. Since the pK of carboxyl groups in proteins is about 4.6, at pH greater than 4.6 the ionizing carboxyl groups of EDTA moieties and those of endogenous glutamic and aspartic acid residues contributed to increased Pb^{2+} binding in the range of pH 4 to 6. At pH 6.5, the Pb^{2+} binding dramatically dropped to 0.015 mmol/g gel. This may be due to the low solubility of $PbCl_2$ above pH 6.5.

The effect of pH on the mercury-binding of this EDTAD-SPI gel is also shown in Figure 6. The gel exhibited a low affinity for Hg^{2+} in acidic condition (pH < 4), a somewhat higher affinity between pH 4 and 5.5, and a sharp increase of binding above pH 6.0. Since the pK_1, pK_2, and pK_3 values of carboxyl groups of EDTA are 2.0, 2.6, and 6.2, respectively, all three of these carboxyl groups would ionize at pH above 6.2 and the pK_2 and pK_3 carboxyl groups of EDTA would bind metal ions more tightly than the other carboxyl groups (as shown in Fig. 1); this may be the reason for the rapid increase of Hg^{2+}-binding of this gel. It has been reported that the imidazole group becomes a very good site for Hg^{2+}-binding above its pK value (pH 6.0) when it loses its proton.[3]

Competitive Adsorption

Given that the EDTAD-SPI hydrogel has differing affinities for different metals, it is reasonable to

Table I Competitive Adsorption of Metal Ion Pairs to EDTAD-SPI Hydrogel

	Initial Concentration of Each Ion in the Mixture (m*M*)					Decrease of Equilibrium Binding Capacity[a] (%)
Metal Pair	0.13	0.25	0.50	1.00	1.25	
Ca/Pb	—	0.218/0.059[a]	0.282/0.202	0.170/0.347	0.084/0.449	−58/−36[b]
Ca/Zn	—	0.174/0.051	0.337/0.102	0.378/0.180	0.384/0.130	−42/−74
Ca/Hg	0.064/0.016	—	0.189/0.350	0.180/0.340	0.279/0.370	−55/−59
Hg/Zn	0.055/0.006	—	0.210/0.044	0.300/0.270	—	−59/−37
Hg/Pb	0.030/0.009	—	0.105/0.107	—	0.201/0.457	−59/−31

[a] Amount of bound metal (mmol/g dry gel).
[b] Relative difference of equilibrium binding capacity:

$$\frac{\text{Equilibrium binding capacity of metal (mixed)} - \text{Equilibrium binding capacity of metal (alone)}}{\text{Equilibrium binding capacity of metal (alone)}} \times 100\%$$

expect competition among metal ions in a solution for the binding sites on the hydrogel. In the present study the selectivity of this gel for metal ions was investigated. Table I shows the results of studies on competitive adsorption between several ion pairs. As mentioned in the Experimental section, this study was performed by incubating the gel in equimolar concentrations of ion pairs, and the metal salts we used here were assumed to completely dissociate into ions at pH 6.0 under such low concentrations, i.e., 0.1 to 1.25 m*M*. It can be seen (Table I) that the equilibrium binding capacity of each metal ion was reduced by 30 to 70% in the presence of an equimolar concentration of another metal ion; in other words, these metal ions might share the same binding sites in the gel or inhibit the metal binding of adjacent sites for other metal ions. In the case of Ca^{2+}/Pb^{2+} and Ca^{2+}/Hg^{2+} systems, calcium was found to bind preferentially to the gel at low concentrations (<0.25 m*M*), and the gel adsorbed more Pb^{2+} and Hg^{2+} than Ca^{2+} at higher metal concentrations. However, for the Ca^{2+}/Zn^{2+} pair, the Zn^{2+} binding by the gel was always lower than Ca^{2+} binding. This indicates that the affinity of the gel for Zn^{2+} was lower than for Ca^{2+}. The preferential binding of Ca^{2+} ion by the gel at low Ca^{2+} concentrations implies that the gel might contain higher-affinity binding sites for Ca^{2+} than for Hg^{2+}, Pb^{2+}, and Zn^{2+} ions. On the contrary, Hg^{2+} and Pb^{2+} with large ionic radii (1.10 and 1.20 Å, respectively) might bind predominantly to low affinity sites on the gel at high concentrations and thus decrease the binding capacity of Ca^{2+} (ionic radius of 0.99 Å). In the case of the Hg^{2+}/Zn^{2+} pair, the gel binds Hg^{2+} more than Zn^{2+} throughout the concentration range studied. In the case of the Hg^{2+}/Pb^{2+} pair, the gel showed a higher relative affinity for Hg^{2+} than for Pb^{2+} at 0.13 mM concentration, but the opposite was observed at higher concentration (1.25 m*M*). The results indicate that the binding of metal ions by EDTAD-SPI gel was ionic size-dependent at high concentrations of metal ions. The larger the ionic radius, the better the binding capacity.

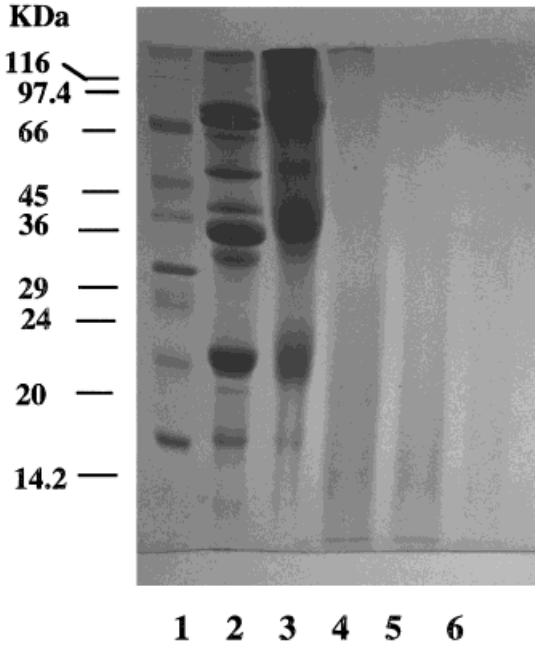

Figure 8 SDS-PAGE of enzyme hydrolysates of EDTAD-SPI hydrogel. Sample 1, molecular marker; sample 2, native SPI; sample 3, 65% EDTAD-SPI; sample 4, α-chymotrypsin hydrolysate; sample 5, bacterial protease hydrolysate; sample 6, trypsin hydrolysate.

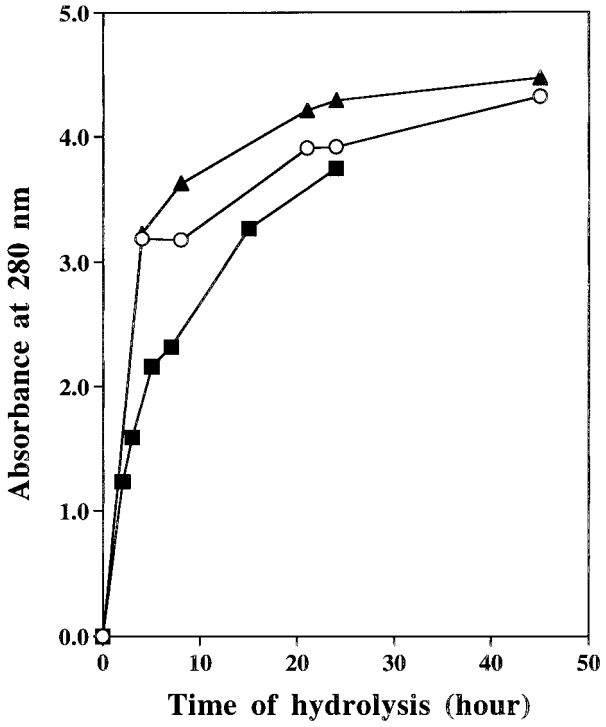

Figure 7 Time course of proteolytic degradation of EDTA-SPI hydrogels by three different enzymes at 1 : 100 (wt/wt) enzyme/substrate ratio. (○) α-Chymotrypsin; (▲) bacterial protease; (■) trypsin.

Enzyme Hydrolysis

Since proteins are readily degraded by many proteolytic enzymes, e.g., trypsin, chymotrypsin, protease, pepsin, etc., the EDTAD-modified protein-based hydrogels should be biodegradable. When

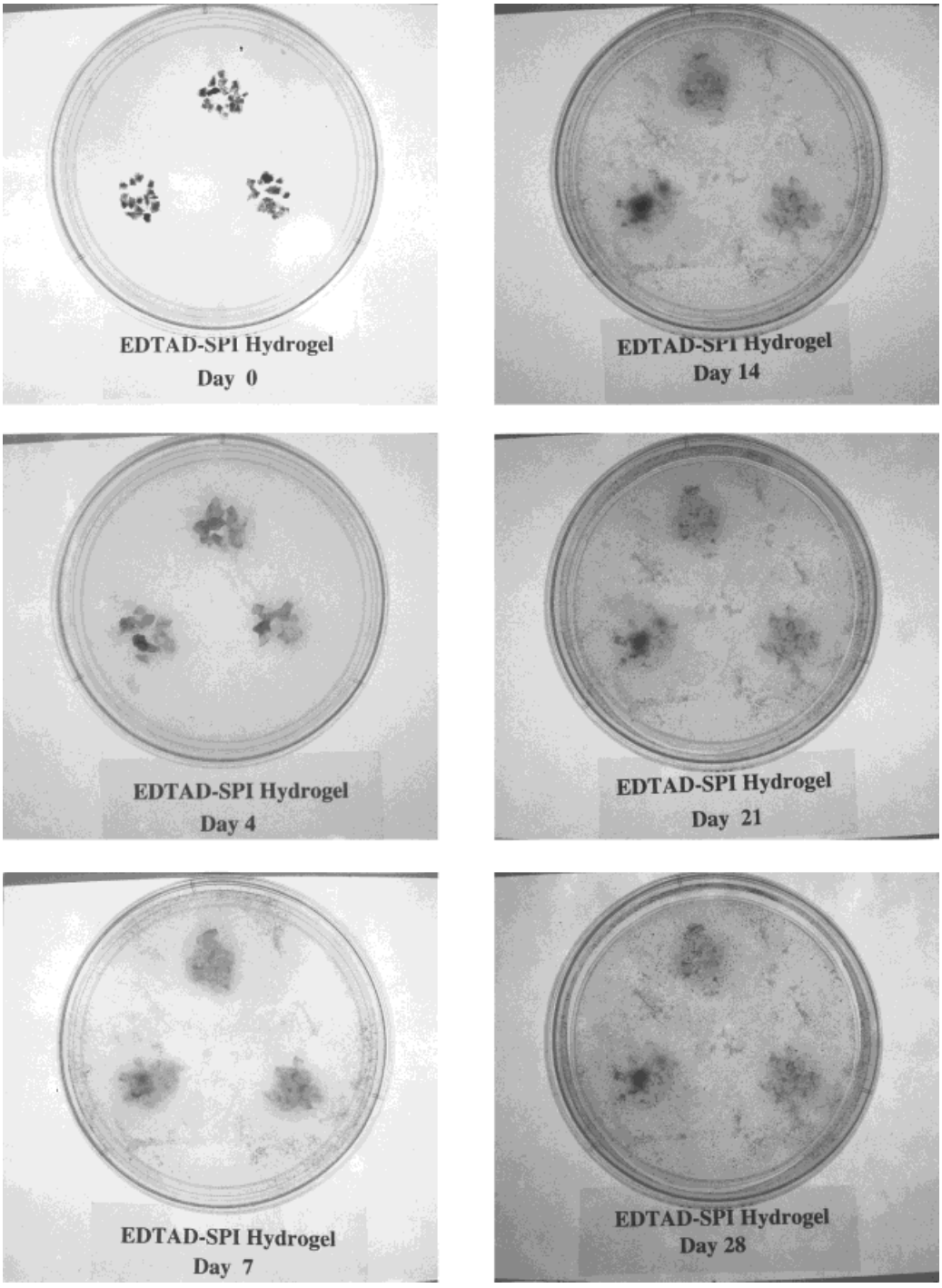

Figure 9 Fungal overgrowth test of EDAD-SPI hydrogels.

the protein-based hydrogel was hydrolyzed by enzymes, amino acids and peptides of various sizes should be released from the gel. Thus, in the present study, the absorbance at 280 nm of the gel-enzyme mixture was used to monitor the proteolysis of the protein-based hydrogel.

Figure 7 shows the time course of hydrolysis of EDTAD-SPI hydrogel with chymotrypsin, bacterial protease, and trypsin. In all cases, the proteolysis proceeded at a rapid rate during the initial 5 h and then slowed thereafter. Among the enzymes tested, protease was the most effective, followed by chymotrypsin and trypsin. These results were not surprising because protease, being a nonspe-

cific enzyme, could act on several sites in SPI. Trypsin was the least effective; this is perhaps because of the blockage of lysine sites by EDTAD and glutaraldehyde.

After a 24-h hydrolysis, no visible gel solid was observed in any of the reaction samples, indicating that all protein gels were broken down into small polypeptides or amino acids. This finding was further supported by SDS-PAGE analysis. Figure 8 shows the SDS-PAGE analysis of each hydrolysate taken from the supernatant of the gel-enzyme mixture after 24-h digestion. Samples 4 to 6 contain the hydrolysate from chymotrypsin, protease, and trypsin digestions, respectively. It is evident that all protein bands of SPI subunits, 7S and 11S, had disapeared in these hydrolyzed samples.

The results presented in Figures 7 and 8 clearly indicate that the 65% EDTAD-SPI hydrogel was readily hydrolysed by trypsin, chymotrypsin, and protease as studied, and can be considered as enzymatically degradable.

Fungal Overgrowth Test

Biodegradable polymers can be consumed by microorganisms and are reduced to simple compounds such as carbon dioxide, water, and ammonia.[19] To examine whether EDTAD-SPI hydrogel can fit in this category, the ASTM G21 (1993) fungal overgrowth test specification was adopted here. Figure 9 shows the biodegradability test of EDTAD-SPI hydrogel.

Because the gels used in this study were in dry form, they began to swell as soon as they were placed on nutrient-salt agar plates, resulting in increased gel volume as evident on day 4 of Figure 9. The first sign of fungal growth was observed around the gel specimen on day 3 (not shown); rapid fungal growth was continual thereafter. On day 7 the swollen gel specimens began to collapse and some of fungal mycelia appeared. Fungal growth slowed but was constant until EDTAD-SPI gel specimens were almost completely degraded on day 28. From day 14 on, fungal mycelia covered the gel samples; dark spots seen in photographs are mycelia, not gel.

The four stages for describing the polymer degradation suggested by Kumar[20] can be employed to explain our observation here, and they are summarized as follows:

1. Hydration, which is translated into disruption of van der Waals' forces and hydrogen bonds. This was observed between day 0 and day 4.
2. Gel strength loss linked to initial cleavage of backbone covalent bonds (biodegradation). This stage began on approximately day 3, resulting from the enzymatic cleavage of protein peptide bonds followed by collapse of the gel structure (day 7).
3. Loss of mass integrity, which is related to further cleavage of covalent bonds, leading to even lower molecular weights.
4. Dissolution of low-molecular-weight species followed by their assimilation.

Stages 3 and 4 may have occurred throughout the period of 7 to 28 days.

CONCLUSION

EDTAD-modified soy protein hydrogel exhibited promising metal-chelating properties and biodegradability. The gel absorbed 0.65, 0.7, and 0.95 mmole of Zn^{2+}, Pb^{2+}, and Hg^{2+} ions per gram of dry gel, respectively. The results of enzyme hydrolysis and the fungal overgrowth test indicated that the EDTAD-SPI hydrogels were biodegradable and that they can be broken down readily by fungi and used as carbon and nitrogen sources for continued growth of the fungi. The gel may be used in wastewater treatment to scavenge heavy metals.

Financial support from the USDA-NRICGP program (Grant # 93-37500-9242) is gratefully acknowledged.

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