Cadmium-Binding Capacity of a Soybean Protein Fraction

(Received April 25, 1985)

Seisaku YOSHIDA, Ryoichi TANAKA and Takashi KASHIMOTO^{*1}

(Osaka Prefectural Institute of Public Health: 3-69, Nakamichi,
1-chome, Higashinari-ku, Osaka, Japan)

The cadmium-binding capacity of the soybean protein fraction was determined by equilibrium dialysis. The aqueous supernatant was extracted from the soybeans with 0.01 *M* Tris-hydrochloric acid buffer (pH 7.4) by ultracentrifugation. The protein fraction (F-I, >10,000 in molecular weight) was separated from the extract by Sephadex G-50 chromatography. Binding measurement was carried out in 0.01 *M* Tris-hydrochloric acid buffer (pH 7.4). The binding data were analyzed according to the Scatchard equation. A Scatchard plot of the binding data showed several phases, indicating heterogeneity of the binding sites. The maximum amount of cadmium bound by F-I was calculated to be 43,000 $\mu\text{g/g}$. When cadmium was added to the aqueous supernatant, five cadmium-binding fractions (at molecular weights of >100,000, 75,000, 38,000, and two low-molecular species) were detected by chromatography on Sephadex G-100. The major cadmium-binding fraction in F-I was the macromolecular species of >100,000 in molecular weight.

Key words: soybeans; soybean proteins; cadmium; cadmium binding capacity; equilibrium dialysis; Scatchard equation; gel filtration

Introduction

Soybeans are a very important foodstuff since they provide both protein and minerals. Soybeans contain large amounts of heavy metals. We obtained high-level-cadmium (Cd)-containing soybeans from a Cd-polluted field. The molecular distributions of Cd, copper (Cu) and zinc (Zn) in the aqueous supernatant of these soybeans were reported previously^{1)~5)}. As regards the binding of heavy metals by soybean protein, we have reported the Cu-binding capacity of the unfractionated protein from these soybeans⁶⁾. The maximum amount of Cu bound by the unfractionated soybean protein was evaluated as 6,000 $\mu\text{g/g}$ by equilibrium dialysis. Soybean protein can also bind larger amounts of Cd and Zn. The amounts of Cd bound by glycinin were found to be larger than those by ovalbumin, gelatin and β -lactoglobulin⁶⁾. Binding of Zn (II)

by 11S and 7S fractions was reported to occur possibly through the imidazole groups of histidine residues^{7),8)}. We report here on the estimation of the Cd-binding capacity of the soybean protein fraction by equilibrium dialysis. A very high Cd-binding capacity of the protein fraction was found by analysis of the binding data according to the Scatchard equation.

Materials and Methods

Plant material

The soybeans were obtained from Toyama prefecture, Japan. The heavy metals constituents in the soybeans were as follows: Cd, 1.4 $\mu\text{g/g}$; Cu, 14.9 $\mu\text{g/g}$; Zn, 103 $\mu\text{g/g}$. The Cd level was ten times the background level⁹⁾, and the Zn content was nearly twice the background. Cu content was the same as the background.

Reagents

Tris(hydroxymethyl)aminomethane, cadmium chloride and the standard solutions for heavy metal analysis were obtained from Wako Pure Chemical Industries. Visking tubing (8/32) was

^{*1} Present address; Department of Pharmacology, Setsunan University: 45-1, Nagaotoge-cho, Hirakata, Osaka, Japan

used for dialysis. Sephadex from Pharmacia Fine Chemicals was used for the gel filtration. Standard proteins for use as molecular weight standards (bovine serum albumin, mol. wt. 67,000; ovalbumin, mol. wt. 43,000; chymotrypsinogen A, mol. wt. 25,000; ribonuclease A, mol. wt. 13,700; bacitracin, mol. wt. 1,400) were from Sigma and Pharmacia. All other chemicals were of reagent grade or better.

Extraction and gel filtration

The aqueous supernatant from the soybeans was prepared according to the previous paper⁴⁾. The soybeans were homogenized with a high-speed blender after being soaked overnight in ten volumes of 0.01 M Tris-hydrochloric acid buffer (pH 7.4). The supernatant was separated from the lipid layer and the residues by ultracentrifugation at 105,000 g for 60 min using a Hitachi Model 55P-2. The heavy metals contents in the extract were as follows: Cd, 2.9 $\mu\text{g/g}$; Cu 30.3 $\mu\text{g/g}$; Zn, 119 $\mu\text{g/g}$. The protein fraction (F-I) was separated from the extract by gel filtration on Sephadex G-50 using a 2.6×35 cm column equilibrated with 0.01 M Tris-hydrochloric acid buffer (pH 7.4). F-I was pooled, desalted on a Sephadex G-25 column and lyophilized. The heavy metals constituents of F-I were as follows: Cd, 4.5 $\mu\text{g/g}$; Cu, 17.3 $\mu\text{g/g}$; Zn, 132 $\mu\text{g/g}$.

After the addition of Cd, the aqueous supernatant was chromatographed on a 2×92 cm Sephadex G-100 column with the buffer at a flow rate of 15 ml/hr. The fraction size was 3.75 ml. The column had previously been calibrated with the molecular weight markers. The absorbance at 280 nm of the effluent was measured in a flow cell.

Equilibrium dialysis

F-I was dissolved in the buffer to give a concentration of 0.1 mg/ml or 1.0 mg/ml. The protein solutions (1 ml) were packed into dialysis tubing and then dialyzed for 6 hours against 100 ml of the buffer containing various concentrations of Cd (in the form of cadmium chloride). Then the Cd concentrations of the inner protein solutions and the outer medium were measured. The amounts of Cd bound by the proteins were calculated from the difference in the Cd concentrations.

Fig. 1. Cd-binding equilibrium curves of F-I

F-I (●: 1.0 mg/ml, ○: 0.1 mg/ml) was dissolved in 0.01 M tris-hydrochloric acid buffer (pH 7.4). The protein solutions (1 ml) were dialyzed for 6 hr against 100 ml of the same buffer containing various concentrations of Cd.

Fig. 2. Scatchard plot of the binding of Cd by F-I

Dotted lines are least-squares lines

Metals analysis

Metals were measured by atomic absorption spectrometry using a Nippon Jarrell-Ash Model AA 781. The eluate from the gel filtration column was measured directly, whereas plant materials were first ashed in a low temperature asher (International Plasma Corporation, Model 1101B)¹⁰⁾.

Fig. 3. Sephadex G-100 elution profile of Cd exogenously added to the aqueous soybean supernatant

The column (2×92 cm) was equilibrated with 0.01 M tris-hydrochloric acid buffer (pH 7.4) and eluted with the same buffer at a flow rate of 15 ml/hr. The fraction size was 3.75 ml. 200 µg of Cd was added to 200 mg of the aqueous soybean supernatant. The elution positions of standard proteins are indicated as follows: a, BSA; b, ovalbumin; c, chymotrypsinogen; d, ribonuclease. They are plotted *versus* the log of molecular weight.

Results

Cd-binding equilibrium curves of F-I measured by equilibrium dialysis are shown in Fig. 1; r , the number of Cd ions bound by F-I, is plotted *versus* the free Cd concentration, A . The curve of F-I at 1.0 mg/ml showed a rapid increase in the amount of bound Cd even at a low concentration of Cd in the outer medium. A further increase was observed at higher free Cd concentration (over 10 µM). The Cd-binding equilibrium curve of F-I at 0.1 mg/ml showed a linear increase in the amount of Cd bound by F-I at a low free Cd concentration of about <5 µM, but the rate of increase in the amount of bound Cd decreased with increase in the Cd concentration of the outer medium. The curve became nearly flat at 20 µM to 30 µM free Cd concentration.

The binding data were analyzed according to the Scatchard equation to evaluate the dissociation constant, K_d , and the maximum binding number of Cd by F-I, n . The equation is $r/A =$

$(n-r)/K_d$. The Scatchard plot of the Cd-binding equilibrium curve of F-I (0.1 mg/ml) showed two or more phases, indicating heterogeneity of the binding sites (Fig. 2). Two least-squares lines are drawn in Fig. 2. The initial linear portion gave K_d 3.1×10^{-5} M and the second linear portion gave K_d 3.3×10^{-8} M. The maximum binding number of Cd (the intercept of the straight line on the abscissa) was 39 corresponding to 43,000 µg Cd per 1 g F-I.

Cd was added exogenously to the aqueous supernatant and the mixture was chromatographed on a Sephadex G-100 column (Fig. 3). Five regions of Cd-content increase were observed. Three regions of Cd-content increase were found in the protein fractions and their molecular weights were calculated as >100,000, 75,000 and 38,000, respectively. The other two Cd-binding components were low-molecular compounds. The largest proportion of the bound Cd was present in the fraction of >100,000 mol wt (38% of the total Cd bound; 72% of the Cd bound by proteins). The lowest-molecular bound Cd was eluted with

Cu and Zn in the fraction of $<1,000$ in mol wt^{b)}.

Discussion

Soybean proteins were shown to have a very high Cd-binding capacity by equilibrium dialysis. The maximum amount of Cd bound by F-I was calculated to be $43,000 \mu\text{g/g}$ (4.3%) by analysis of the binding data according to the Scatchard equation. This was 30,000 times the initial Cd content of the sample soybeans ($1.4 \mu\text{g/g}$) and 10,000 times the Cd content of F-I ($4.5 \mu\text{g/g}$). The maximum binding amount of Cu by F-I was $6,000 \mu\text{g/g}$, as reported previously⁸⁾. The maximum amount of Cd bound by F-I was one order higher than that of Cu.

Appu Rao and Narasinga Rao^{7),8)} reported that the maximum binding number of Zn (II) to 100,000 g of 11S fraction was 17–18, and that to 100,000 g of 7S fraction was 7. From our results, the maximum binding number of Cd to 100,000 g of F-I was 39, i.e., more than twice that of Zn to 11S or 7S protein. If the molecular weight of F-I is assumed to be 200,000, the maximum binding of Cd by 1 mole of F-I is calculated to be 78 mole. Appu Rao and Narasinga Rao suggested that the binding of Zn, Ca and Mg by 11S and 7S protein of soybeans may occur through the imidazole groups of histidine residues on the basis of the total number of binding sites estimated from a Scatchard plot of the equilibrium dialysis data. The probable binding sites of Cd could not be identified on the basis of the results of this experiment. Some other binding sites for Cd besides the histidine residues may present in F-I.

When Cd ($200 \mu\text{g}$) was added exogenously to the aqueous supernatant (200 mg), almost all the Cd added was bound by the extract. Half the bound Cd was found in the protein fractions and half was associated with low-molecular species. Most of the Cd bound by proteins was associated with macromolecules of $>100,000$ in mol wt, including 7S and 11S globulins and larger molecules. When Cd ($100 \mu\text{g}$) was added similarly

to F-I (100 mg), almost all the added Cd was bound; 24% of the bound Cd was associated with the molecules of $>100,000$ in mol wt and 40% was eluted in the region of fraction numbers 80 to 85, as shown in Fig. 3. These fractions contained many phosphorus-containing materials, and they were considered to be phytate^{*1}. The lowest-molecular bound Cd was eluted together with Cu and Zn. The ligand of Cd might possibly be the same as that of Cu and Zn. However, for determination of the ligand for Cd further gel filtration on Sephadex G-25 or G-10, or an ion exchange column, seems necessary.

A Cd-binding protein of about 10,000 mol wt has been isolated from the soybean protein fraction of $>100,000$ mol wt by treatment with a sulfhydryl reductant^{*2}. The characterization of this component, including the Cd-binding capacity, is in progress. If both Cd and the soybean water-soluble fraction were taken simultaneously, soybean proteins and other components would be expected to bind Cd nonspecifically. It would be interesting to know what kind of chemical forms those Cd-complexes take in the gastrointestinal tract, and to know how the toxicity of Cd is affected.

References

- 1) Tanaka, R., Yoshida, S., Kashimoto, T.: J. Food Hyg. Soc. Japan **21**, 243~246 (1980).
- 2) Yoshida, S., Tanaka, R., Kashimoto, T.: *ibid.* **21**, 247~251 (1980).
- 3) Yoshida, S., Tanaka, R., Kashimoto, T.: *ibid.* **23**, 53~58 (1982).
- 4) Yoshida, S., Tanaka, R., Kashimoto, T.: *ibid.* **23**, 319~324 (1982).
- 5) Yoshida, S., Tanaka, R., Kashimoto, T.: *ibid.* **24**, 550~557 (1983).
- 6) Kojima, S., Kiyozumi, M., Kamiya, M., Ishimatsu, Y.: Eisei Kagaku, **25**, 245~250 (1979).
- 7) Appu Rao, A. G., Narasinga Rao, M. S.: J. Agric. Food Chem. **24**, 487~490 (1976).
- 8) Appu Rao, A. G., Narasinga Rao, M. S.: *ibid.* **24**, 490~494 (1976).
- 9) Tanaka, R., Ikebe, K., Tanaka, Y., Kunita, N.: J. Food Hyg. Soc. Japan **24**, 488~499 (1983).
- 10) Tanaka, R., Ikebe, K., Kashimoto, T.: *ibid.* **24**, 136~141 (1983).

*1 Yoshida, S., Tanaka, R., Kashimoto, T.: proceedings of the 47th meeting of the Food Hyg. Soc. Japan in 1984, in Tokyo, p. 13.

*2 submitted for publication