Effects of Sodium Dodecyl Sulfate, 2-Mercaptoethanol and EDTA on the Chemical Forms of Copper and Zinc in a Soybean Protein Fraction

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The effects of sodium dodecyl sulfate, 2-mercaptoethanol and EDTA on the distribution and binding status of copper and zinc in the soybean protein fraction were examined by gel filtration on Sephadex G-100. Copper and zinc in the protein fraction were both associated primarily with the major components of $>100,000$ in mol wt. The molecular distribution of zinc in the protein fraction of $>100,000$ mol wt differed slightly from that of copper. Gel filtration of the protein fraction on Sephadex G-100 in the presence of sodium dodecyl sulfate resulted in conversion of both copper- and zinc-binding components to lower molecular weights (30,000 to 100,000) accompanied by denaturation of the soybean proteins. Separation of the major protein fraction of $>100,000$ mol wt in the presence of 2-mercaptoethanol did not cause any significant denaturation of the large proteins but caused a shift of copper to the fraction of about 10,000 mol wt. Only a small amount of zinc was shifted by 2-mercaptoethanol. Zinc in the protein fraction was removed by EDTA in the absence and presence of sodium dodecyl sulfate and/or 2-mercaptoethanol. Copper in the fraction of about 10,000 mol wt in the presence of 2-mercaptoethanol was not affected by EDTA. The stabilization of copper binding by 2-mercaptoethanol was also observed in the presence of sodium dodecyl sulfate.

Key words: soybean; copper; zinc; sodium dodecyl sulfate; 2-mercaptoethanol; EDTA; gel filtration; chemical form

As part of a series of studies to clarify the chemical forms of metals in food, we have been investigating the binding status of cadmium (Cd), copper (Cu) and zinc (Zn) in soybeans. The molecular distributions of Cd, Cu and Zn in the water-soluble fraction of soybeans have been examined by ultrafiltration or by gel filtration^{1)~3)}. Cu and Zn in the water-soluble fraction were distributed over a wide molecular weight (mol wt) range, whereas nearly all the Cd was present in the protein fraction⁴⁾. Cu and Zn in the low-molecular-weight fractions may share the same binding components³⁾. Cd and Cu in the protein fraction were associated mainly with macromolecules of $>100,000$ in mol wt^{2)~4)}. The Cd distribution in the protein fraction was greatly changed by the action of sodium dodecyl sulfate and/or 2-mercaptoethanol.

The Cd-binding components in the fraction of $>100,000$ mol wt were transformed to the fraction of about 10,000 mol wt in the presence of 2-mercaptoethanol.

In this paper, the effects of denaturing agents and a chelating agent, i.e., sodium dodecyl sulfate, 2-mercaptoethanol and ethylenediaminetetraacetate, on the binding of Cu and Zn in the soybean protein fraction were examined.

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 con-

tent was ten times the background level⁶), and the Zn content was nearly twice the background. The Cu content was the same as the background.

Reagents

Tris(hydroxymethyl)aminomethane, disodium ethylenediaminetetraacetate (EDTA), sodium dodecyl sulfate (SDS), 2-mercaptoethanol (2-ME) and the standard solutions for heavy metal analysis were obtained from Wako Pure Chemical Industries Ltd. Sephadex from Pharmacia Fine Chemicals was used for the gel filtration. Standard proteins for use as molecular weight markers (bovine serum albumin, mol wt 67,000; ovalbumin, mol wt 43,000; chymotrypsinogen A, mol wt 25,000; cytochrome c, mol wt 12,300; bacitracin, mol wt 1,400) were from Sigma and Pharmacia Fine Chemicals. All other chemicals were of reagent grade or better.

Extraction and protein isolation

The aqueous supernatant from the soybeans was prepared according to the previous paper². The soybeans were homogenized in a high-speed blender after being soaked overnight in ten volumes (w/w) of 0.01 M Tris-hydrochloric acid buffer (pH 7.4). The buffer-soluble fraction was separated from the lipid layer and the residues by ultracentrifugation at 105,000 *g* for 60 min using a Hitachi 55P-2 centrifuge. The heavy metal contents in the extract were as follows: Cd, 2.9 $\mu\text{g/g}$; Cu, 30.0 $\mu\text{g/g}$; Zn, 119 $\mu\text{g/g}$.

The protein fraction (F-I) was separated from the extract by gel filtration on Sephadex G-50 (or G-25) using a 2.6 $\times$ 35 cm column equilibrated with 0.01 M Tris-hydrochloric acid buffer (pH 7.4). F-I was pooled, desalted on Sephadex G-25 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 $\mu\text{g/g}$. F-I was chromatographed on a 2 $\times$ 75 cm Sephadex G-100 column with the buffer at a flow rate of 15 ml/hr. The fraction size was 3 ml. The column had previously been calibrated with the molecular weight markers. The absorbances at 280 nm and 254 nm of the effluent were measured in a flow cell.

Chemical treatment

F-I (200 mg) was dissolved in 2 ml of the buffer followed by the addition of EDTA to a

Fig. 1. Sephadex G-100 gel filtration chromatogram of the soybean protein fraction (F-I)

The column (2 $\times$ 75 cm) was equilibrated and eluted with 0.01 M Tris-hydrochloric acid buffer (pH 7.4) at a flow rate of 15 ml/hr. The fraction size was 3 ml.

final concentration of 0.01 M. After being stirred for 30 min, the mixture was applied to a Sephadex G-100 column. A part of this fraction was also dissolved in the buffer containing SDS and/or 2-ME and chromatographed on Sephadex G-100 in the presence of SDS and/or 2-ME. The concentrations of SDS and 2-ME were 0.2% and 0.01 M in the buffer, respectively.

Metals analysis

Metals were measured by atomic absorption spectrometry using a Nippon Jarrell-Ash AA781 instrument. The eluate from gel chromatography was measured directly, whereas plant materials were first ashed in a low temperature asher (International Plasma Corporation, model 1101B).

Results

The elution profiles of Cu and Zn in F-I obtained from the Sephadex G-100 column are shown in Figure 1. Five peaks of absorption at 280 nm were detected. F-IA was the major component, containing the major protein, conglycinin, and glycinin or larger molecules. F-ID and F-IE had trypsin-inhibitory activity. Both Cu and Zn were found in all the fractions of F-I and both were distributed mainly in the fraction of >100,000 mol wt. The Zn distribu-

Fig. 2. Elution profile of F-I on Sephadex G-100 in the presence of SDS

F-I was dissolved in the elution buffer containing 0.2% SDS, and the solution was stirred for a few minutes then applied to the column. The elution conditions were as described in the legend to Fig. 1.

tion in the fractions from 100,000 to 10,000 in mol wt was slightly different from that of Cu.

F-I was chromatographed on Sephadex G-100 in the presence of 0.2% SDS (Fig. 2). The pattern of absorption at 280 nm became very different from that in the absence of SDS. Soybean globulins were found to dissociate into small molecules or subunits. Both Cu and Zn in F-I appeared over a broad range from 30,000 to 100,000 in mol wt on treatment with SDS. Dissociation of Cu and Zn from the binding components occurred only to a very small extent.

Figure 3 shows the gel filtration profiles of Cu and Zn in F-I on Sephadex G-100 in the presence of 2-ME. The absorption at 280 nm was almost the same as that in the absence of 2-ME, but the Cu-elution profile changed greatly. Almost all the Cu in F-I was shifted to the fraction of about 10,000 mol wt. The change of the Zn-elution profile was less marked. Half of the Zn in F-I was transferred to lower-molecular-weight fraction and the other half was unaffected by 2-ME.

The elution pattern of F-I on Sephadex G-100

Fig. 3. Elution profile of F-I on Sephadex G-100 in the presence of 2-ME

F-I was dissolved in and eluted with the buffer containing 0.01 M 2-ME.

Fig. 4. Elution profile of F-I on Sephadex G-100 in the presence of both SDS and 2-ME

The concentrations of SDS and 2-ME in the buffer were 0.2% and 0.01 M, respectively.

in the presence of both SDS and 2-ME is shown in Figure 4. The shift of Cu and Zn to the fraction of about 10,000 mol wt was again observed. About half of the Zn was not shifted to the fraction of about 10,000 mol wt, but appeared in the range of 30,000 to 100,000 in mol wt.

The effects of EDTA on the binding status of Cu and Zn in F-I were determined. F-I was chromatographed after treatment with EDTA in the absence and presence of SDS and/or 2-ME (Fig. 5). In all cases, nearly all the Zn in F-I was removed from F-I by EDTA. As regards

Fig. 5. Effects of EDTA on the binding status of Cu and Zn in F-I

F-I was dissolved in the buffer with and without SDS and/or 2-ME. EDTA was added to the sample solution to a final concentration of 0.01 *M*. The mixture was stirred for 30 min and then applied to the Sephadex G-100 column.

Fig. 6. Sephadex G-100 elution profile of F-IA in the presence of 2-ME

F-IA from the Sephadex G-100 chromatography of F-I under the initial conditions was chromatographed in the buffer containing 0.01 *M* 2-ME. The other elution conditions were as described in the legend to Fig. 1.

the Cu, about half of the Cu in F-I remained bound to the protein in the absence of 2-ME and in the presence of SDS. In the presence of 2-ME, almost all the Cu was bound to the fraction of about 10,000 mol wt. Stabilization of the Cu-binding by 2-ME against treatment with EDTA was also observed in the presence of SDS.

The fraction >100,000 in mol wt, F-IA, was gel-filtered on Sephadex G-100 in the presence of 2-ME (Fig. 6). Almost all the Cu in F-IA shifted to the fraction of about 10,000 mol wt, whereas only a small amount of the Zn in F-IA shifted. Almost all the Zn in F-IA remained bound to large protein molecules. No significant change in the absorption at 280 nm was observed. The Cu-containing eluates showed a slight absorbance at 280 nm.

Discussion

Cu and Zn in the soybean protein fraction were associated primarily with macromolecules of >100,000 mol wt, as found for Cd in an earlier study⁴. All the Cu and Zn in large molecules were transferred to lower-molecular-weight fractions on treatment with SDS, with no change in the binding capacity. This result is similar to that in the case of Cd. The ionic detergent SDS presumably destroyed ionic bonds making up the soybean protein aggregates, without affecting the binding affinity for Cu, Zn and Cd.⁴

The effects of 2-ME on Cu and Zn binding were found to differ from those of SDS. In the presence of 2-ME, almost all the Cu and half of the Zn shifted to the fraction of about 10,000 mol wt. 2-ME did not cause any significant denaturing of large protein structures, but did affect the binding of Cu and Zn. The effect of 2-ME on Cu binding was greater than that on Zn binding. A small amount of Zn in F-IA shifted to the fraction of about 10,000 mol wt on treatment with 2-ME. The Zn binding by soybean protein may differ somewhat in character from Cu and Cd binding.

Zn was removed from F-I by EDTA in the absence and presence of SDS and/or 2-ME. Cu in the fraction of about 10,000 mol wt in the presence of 2-ME was not affected by EDTA in

the absence or presence of SDS. From the results of treatments with SDS, 2-ME and EDTA found in this work and previously⁴⁾, the order of binding affinity of the soybean protein fraction is considered to be Cu>Cd>Zn.

The binding sites of Cu, Zn and Cd in soybean globulin have so far not been clarified. Soybean globulins do not contain very many cysteine residues, but sulfhydryl functional groups are likely to be important for metals binding. The shift of metals to the fraction of about 10,000 mol wt indicates the disaggregation of metal-binding components into subunits or the release of the binding components from large carrier molecules in F-IA. The sulfhydryl groups may participate in the association of Cu-, Zn- and Cd-binding components to form large molecules.

The amounts of the metal-binding compo-

nents having a molecular weight of about 10,000 should be very small, but the binding capacity is considered to be very high. Characterization of the metal-binding components of about 10,000 mol wt obtained by treatment with 2-ME is in progress. The details will be reported soon.

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