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Preparation of Phytate-Removed Deamidated Soybean Globulins by Ion Exchangers and Characterization of Their Calcium-Binding Ability

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Phytate-removed deamidated soybean globulins were prepared using ion-exchange resins to provide them with a functional property to enhance calcium absorption in the body. The phosphorus level was reduced from 45 to 20 μmol/g using 0.05 g/mL of AE-4, an anion-exchange resin with a (2-hydroxyethyl)dimethylammonium group, in 0.2% soybean globulin solution for 1 h at 4 °C, and 90−92% of the phosphorus in defatted soybeans could be removed. As for deamidation, CE-4, a cation-exchange resin of the carboxylate type, showed a much higher deamidation activity than CE-1 and CE-2, cation-exchange resins of the sulfonate type. No peptide bond hydrolysis was observed for any cation-exchange resin treated at 4 °C. There was no significant difference in the amount of acid amide deamidated at temperatures between 4 and 50 °C. The deamidation level was able to increase to 73% using 0.10 g/mL of CE-4 in a 0.2% soybean globulin solution for 6 h at 4 °C. The amount of calcium bound to the soybean globulins decreased with removal of the phytate but increased with deamidation.

KEYWORDS: Soybean globulin; deamidation; phytate removal; ion-exchange resin; calcium-binding property

INTRODUCTION

Calcium is one of the indispensable minerals in our body, but daily intake in Japan has not yet met the recommended value despite the set point for it being set lower than in other countries. To enhance the bioavailability of calcium, it is important to consider not only the amount of calcium ingested but also the substances coexisting with it. Phytate, phosphorus, oxalate, and dietary fibers are known to interfere with calcium bioavailability by binding too strongly with calcium and insolubilizing it. Some compounds such as casein phosphopeptide (CPP) and calcium citrate malate (CCM) are known to enhance calcium bioavailability (*1*−*5*) by weakly binding with calcium and solubilizing it. CPP and CCM have already been included in foods for specified health uses (functional foods) authorized by the Ministry of Health, Labour and Welfare of Japan to enhance calcium absorption.

Soybean proteins are reported to have low calcium bioavailability (*1*, *4*), probably due to phytate that binds too strongly with calcium and insolubilizes it. Therefore, it is better to remove phytate in order to increase calcium's bioavailability. On the other hand, soybean proteins are known to be rich in glutamine and asparagine. Deamidation of these amino acids produces glutamic acid and aspartic acid, which have carboxyl groups that are expected to be weakly bound to calcium and to enhance calcium absorption in the body. We have shown in the preceding paper that the removal of phytate and the deamidation of acid amide by enzymes are effective for improving the calcium-binding properties of soybean globulins (*6*). However, there remain some problems for the practical use of these phytate-removed deamidated soybean globulins: (1) enzymes are too expensive for industrial purposes, and (2) the degree of deamidation by those enzymes is only ∼5% of the total acid amide in the soybean globulins.

The use of a strong acid would be another popular way to deamidate amide groups in a protein, but it would require high temperature and also cause considerable hydrolysis of the peptide bonds. The hydrolysis of proteins may produce bitter-tasting peptides and also reduce the processing property. Shih (*7*) found that deamidation could be made in dilute HCl solution with Dowex 50, a cation-exchange resin of the sulfonate form. The degrees of deamidation were about 15% at 58 °C and about 70% at 85 °C, although considerable peptide bond cleavage

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occurred above 75 °C. A treatment temperature >60 °C is not sometimes desirable because protein denaturation would occur. In addition, there is no report on the deamidation of proteins by ion-exchange resins other than Dowex 50.

In this study, various ion-exchange resins were examined to determine the most effective condition to remove phytate and deamidate soybean globulins below 50 °C, at which there would be little risk of protein denaturation. After optimum conditions had been determined, the calcium-binding property of phytate-removed deamidated soybean globulins was compared with that of untreated soybean globulins.

MATERIALS AND METHODS

Materials. The soybeans were purchased from a local market. The ion-exchange resins used were Amberlite IRA400, IRA458, IRA410, IRA411S, IRC50, IRC718, IR120B, and XT1006 obtained from Organo Co., Tokyo, Japan. The anion-exchange resins, IRA400, IRA458, IRA411S, and IRA410, hereafter will be referred to as AE-1, AE-2, AE-3, and AE-4, respectively. The cation-exchange resins, IR120B, XT1006, IRC718, and IRC50, hereafter will be referred to as CE-1, CE-2, CE-3, and CE-4, respectively. AE-1 and AE-2 were anion-exchange resins with a trimethylammonium group, and AE-3 and AE-4 were those with a (2-hydroxyethyl)dimethylammonium group. CE-1 and CE-2 were cation-exchange resins of sulfonate type, CE-3 was aminodiacetate type, and CE-4 was carboxylate type. All other chemicals used were of reagent grade.

Extraction of Soybean Globulins. Soybean globulins were extracted as described in the preceding paper (*6*) with minor modification. After the soybeans had been dehulled and ground with a mixer, the soybeans were repeatedly defatted with 5 times their weight of hexane and dried under vacuum. The defatted soybean meal was stirred with 20 times its weight of a 0.03 M Tris-HCl buffer at pH 8.0 containing 0.01 M 2-mercaptoethanol for 1 h and then centrifuged at 18000*g* for 20 min at 20 °C. The supernatant was adjusted to pH 6.4 with 2 N HCl and centrifuged at 18000*g* for 20 min at 4 °C. The precipitate was dialyzed against distilled water and freeze-dried.

Removal of Phytate. The ion-exchange resins used to remove the phytate from the soybean globulins were successively washed with 1 N HCl, deionized water, 1 N NaOH, deionized water, 1 N HCl, deionized water, and a 0.05 M Tris-HCl buffer at pH 7.4 prior to use. Each ion-exchange resin and the 0.2% soybean globulins in the 0.05 M Tris-HCl buffer at pH 7.4 were mixed and stirred at 4 °C. After globulins had been filtered through a cotton cloth, the filtrate was dialyzed against distilled water and freeze-dried.

Measurement of Phytate Content. Most of the phosphorus in soybeans is in the phytate form (*8*−*12*), so the amount of phosphorus was taken as an index of the phytate content. Soybean globulins were dissolved in 0.1 N HCl to 0.1%, and the phosphorus content in each sample solution was measured by inductively coupled plasma atomic emission spectroscopy (SPS1700R, Seiko Instruments Inc., Tokyo, Japan).

Deamidation. The ion-exchange resins used to deamidate the soybean globulins were successively washed with 1 N NaOH, deionized water, 1 N HCl, deionized water, 0.1 N NaOH, deionized water, and a 0.05 M Tris-HCl buffer at pH 7.4 prior to use. Each ion-exchange resin and 0.2% soybean globulins in the 0.05 M Tris-HCl buffer at pH 7.4 were mixed and stirred at 4 °C. After the globulins had been filtered through a cotton cloth, the filtrate was dialyzed against distilled water and freeze-dried.

Measurement of the Degree of Deamidation. The degree of deamidation was expressed as the amount of acid amide removed from the soybean globulins with respect to the total acid amide in the soybean globulins before the deamidation treatment by ion-exchange resins. The amount of acid amide removed was obtained from the difference between the amount of acid amide in the soybean globulins before and after the ion-exchange resin treatment because the ammonia released during the treatment would be absorbed by the ion-exchange resin and difficult to measure. The amount of acid amide in the soybean globulins was determined by measuring the amount of ammonia released by the HCl treatment, which completely deamidates a protein. Soybean globulins before and after the deamidation treatment by ion-exchange resins were each dissolved in 2 N HCl, completely deamidated at 100 °C for 3 h, and then neutralized with 1 N NaOH.

The amount of ammonia in a completely deamidated sample was measured according to the procedure in the preceding paper (*6*), which is a combination of Conway's microdiffusion method (*13*) and the indophenol method (*14*). In a sealed vessel, ammonia released from 0.5 mL of the sample solution by 2 mL of a strong alkali (K_2CO_3) was absorbed into 0.5 mL of $^1/_{100}$ N H_2SO_4. After incubation at 37 °C for 2 h, 0.5 mL of the ammonium sulfate solution obtained was taken into a test tube cooled in ice-cold water, and 0.05 mL of 0.003 M $MnSO_4$, 1 mL of an alkaline phenol solution, and 0.5 mL of NaClO were added. The test tube was immediately sealed with a cap, shaken, and placed in boiling water for 5 min. The solution was then diluted with 10 mL of distilled water, and the absorbance of indophenol at 625 nm produced by the reaction was measured with a spectrophotometer (UV-240, Shimadzu Corp., Tokyo, Japan). A calibration curve was obtained by using a known amount of an ammonium sulfate solution.

Measurement of the Degree of Peptide Bond Hydrolysis. The degree of peptide bond hydrolysis is expressed as the proportion of nitrogen contained in the 0.6 M trichloroacetic acid-soluble fraction to the total nitrogen content. The nitrogen content was measured according to the Kjeldahl method (*15*).

Preparation of Calcium-Free Protein Samples. Prior to the evaluation of the calcium-binding property of soybean globulins, calcium was removed from them using cation-exchange resins (Amberlite XT1006, Organo Co., Ltd.), which did not cause deamidation or hydrolysis. The cation-exchange resins were successively washed with 1 N NaOH, deionized water, 1 N HCl, deionized water, 0.1 N NaOH, deionized water, and a 0.05 M Tris-HCl buffer at pH 7.4 prior to use. The cation-exchange resins were mixed with 0.2% soybean globulins in the 0.05 M Tris-HCl buffer at pH 7.4 in the weight ratio of 1:20 and stirred at 4 °C for 10 min. After the globulins had been filtered through a cotton cloth, the filtrate was dialyzed against distilled water and freeze-dried.

Evaluation of the Amount of Calcium Bound to the Soybean Globulins. The amount of calcium bound to the soybean globulins was evaluated according to a previously described procedure (*6*, *16*). The free calcium concentration was measured with an ion meter (IM-40S, TOA Electronics, Ltd., Tokyo, Japan) connected to a calcium-ion-selective electrode (CA-135B, TOA Electronics, Ltd.) and to a double-junction reference electrode (HS-305DS, TOA Electronics, Ltd.). The measurement of the free calcium ion concentration was performed at 25.0 °C. The ion meter was calibrated with $(0-5) \times 10^{-4}$ M calcium chloride solutions. All of the solutions were prepared with 0.2 M Tris-HCl buffer at pH 7.4, the pH of the lower part of the small intestine where most of the calcium is absorbed. The apparent amount of calcium bound to soybean globulins was calculated by subtracting the free calcium concentration from the total calcium concentration.

RESULTS

Figure 1 shows the phosphorus content in defatted soybeans and soybean globulins before and after the anion-exchange resin treatment. During the preparation of the globulins, the levels of phosphorus decreased from ∼200 to ∼45 μmol/g, and the treatment with the anion-exchange resins reduced the levels of phosphorus further to about half and one-third. AE-4 was the most effective species in removing phytate from the soybean globulins, and 92.2% of the phosphorus in defatted soybeans was removed, although there was no significant difference in the phytate removal of the anion-exchange resins. The addition of 0.05 g of AE-4 to 1 mL of protein solution reduced the amount of phosphorus from ∼45 to ∼20 μmol/g, and the further addition of AE-4 did not show a significant decrease in the amount of phosphorus in the soybean globulins (Figure 2). A 1 h treatment time was sufficient to remove the phosphorus from the soybean globulins, reducing the amount of phosphorus from ∼45 to ∼20 μmol/g (Figure 3).

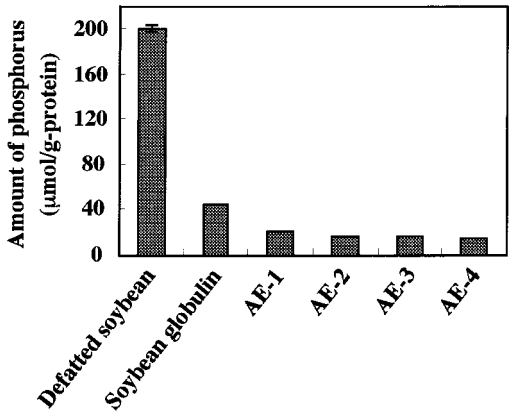

Figure 1. Removal of phytate from soybean globulins during purification and anion-exchange resin treatment. The amount of anion-exchange resins used was 0.05 g/mL–0.2% protein solution, and the time treated with the anion-exchange resins was 3 h.

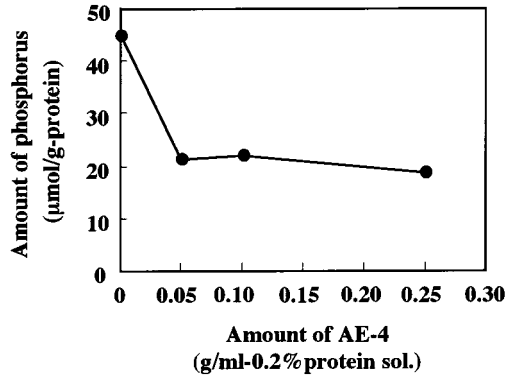

Figure 2. Effect of AE-4 at different amounts on removal of phytate from soybean globulins. The time treated with AE-4 was 1 h.

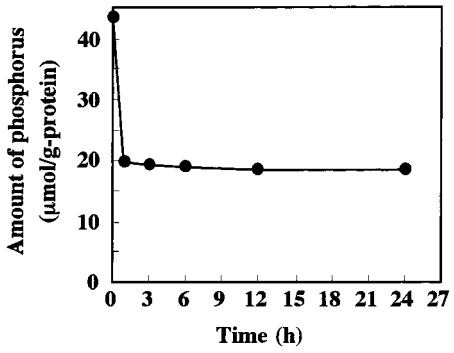

Figure 3. Effect of time treated with AE-4 on removal of phytate from soybean globulins. The amount of AE-4 used was 0.05 g/mL–0.2% protein solution.

Figure 4 shows the amount of acid amide deamidated from the soybean globulins by the cation-exchange resin treatment. Among the tested cation-exchange resins CE-4 was the most effective in deamidating the soybean globulins, deamidating ~1100 μmol/g of protein of acid amide. As the amount of CE-4 mixed with soybean globulins increased, the amount of acid amide deamidated substantially increased to the addition of 0.1 g/mL of protein solution of CE-4 and showed little difference thereafter (Figure 5). As the treatment time increased, the amount of deamidated acid amide increased to ~700 μmol/g of protein using the 0.05 g/mL of protein solution of CE-4 and showed no further significant change after 6 h of treatment time (Figure 6). A treatment temperature between 4 and 50 °C

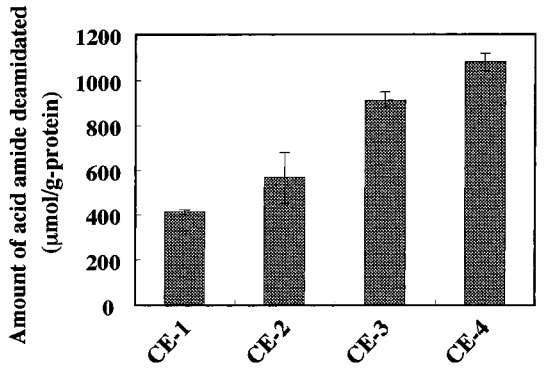

Figure 4. Deamidation of soybean globulins by cation-exchange resins. The amount of cation-exchange resins used was 0.50 g/mL–0.2% protein solution, and the time treated with the cation-exchange resins was 6 h.

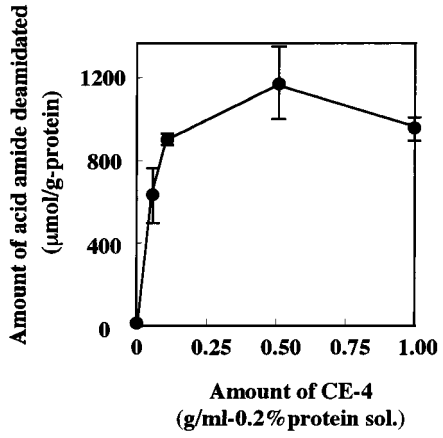

Figure 5. Effect of amount of CE-4 on deamidation of soybean globulins. The time treated with CE-4 was 6 h.

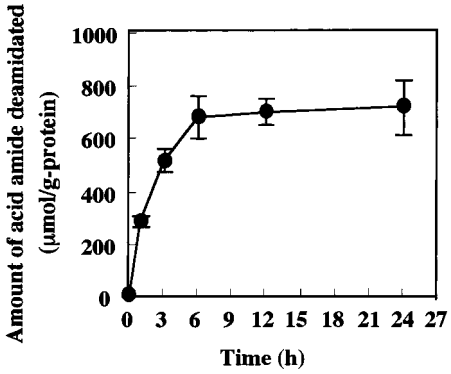

Figure 6. Effect of time treated with CE-4 on deamidation of soybean globulins. The amount of CE-4 used was 0.05 g/mL–0.2% protein solution.

showed no significant difference in the amount of deamidated acid amide (Figure 7). None of the ion-exchange resins hydrolyzed the soybean globulins during treatment at 25 °C for 6 h (data not shown). Figure 8 shows the calcium-binding isotherms of soybean globulins treated with ion-exchange resins. The amount of calcium bound to the soybean globulins decreased with the removal of phytate but increased to levels higher than that found in the untreated globulins after deamidation.

DISCUSSION

Phosphorus in soybeans is principally in the phytate form (*8*−*12*, *17*). Phytate binds to soybean protein and interferes with the dietary mineral absorption in the small intestine by forming

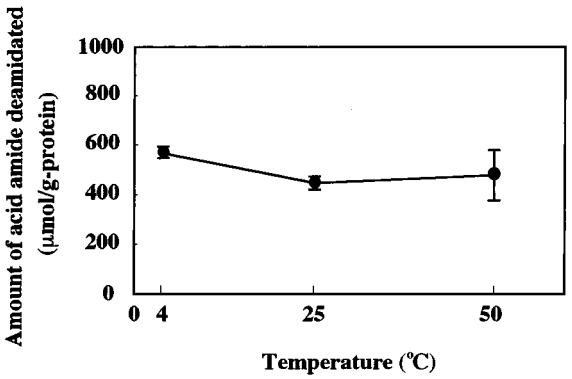

Figure 7. Effect of temperature on deamidation of soybean globulins by CE-4. The amount CE-4 used was 0.05 g/mL–0.2% protein solution, and the time treated with CE-4 was 3 h.

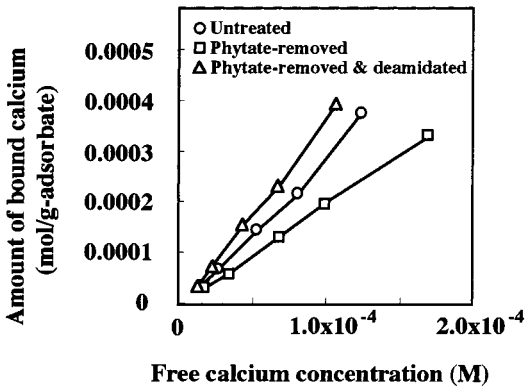

Figure 8. Calcium-binding isotherms of soybean globulins treated with ion-exchange resins.

a complex and insolubilizing it (*18*, *19*). Therefore, many researchers have made efforts to remove phytate from soybean proteins using acidic and alkaline reagents, cations, EDTA, ultrafiltration, and anion-exchange resins (*6*, *9*−*11*, *20*−*25*). We have reported that the anion-exchange resin Amberlite IRA-400, which is referred to as AE-1 in this study, was effective for removing phytate from the soybean protein (*6*), although we have not tried any other anion-exchange resins or examined the optimum conditions for phytate removal. In the present study, four anion-exchange resins with different functional groups or maximum exchange capacities were examined to find better anion-exchange resins. Every anion-exchange resin was effective for removing phytate from the soybean globulins, eliminating 90−92% of the phosphorus in the defatted soybeans. Even if the type of ion-exchange resin, the amount of resin, and the treatment time were varied, however, there always remained ∼8% phosphorus, indicating the existence of phosphorus tightly bound to the protein which cannot be eliminated.

The deamidation of protein has received increasing interest in order to increase protein solubility (*26*−*29*) or to improve its functional properties such as its foaming property (*27*, *30*, *31*), emulsifying property (*27*, *28*, *30*, *32*, *33*), and baking property (*34*). The most popular method for the deamidation of protein is acid treatment (*27*−*33*), but considerable hydrolysis of the peptide bonds is indispensable, which produces bitter-tasting peptides and also reduces the processing property. The enzymatic approach might be another way to deamidate protein. However, the deamidation level is usually quite low (*6*, *35*) without the additional peptide bond hydrolysis (*36*) to unfold the protein structure. Proteolytic enzymes may be used for deamidation (*6*, *37*), but the use of the deamidated products will be limited because small peptides have a low processing property and sometimes have a bitter taste. Motoki et al. (*38*) used transglutaminase to deamidate glutamine in casein, but the chemical modification was necessary to prevent the amine-incorporating reaction and the cross-linking reaction and, also, this enzyme is specific only to the glutaminyl side chain. Moreover, enzymatic approaches are too costly for industrial use. Shih (*7*) used a sulfonate cation-exchange resin (Dowex 50) to deamidate soy extract, suggested by the finding that sodium dodecyl sulfate (SDS) catalyzed the deamidation reaction of cottonseed protein. In his study, the temperature was varied from 58 to 85 °C, with the result that the deamidation level was ∼15% at 58 °C with <1% peptide bond cleavage and ∼70% at 85 °C with 12% peptide bond cleavage. However, he did not examine the results at temperatures <58 °C or cation-exchange resins other than Dowex 50. It is sometimes better to treat proteins at a lower temperature to prevent their denaturation or decay. Therefore, in this study, the cation-exchange resin treatment was conducted at 4 °C and several types of cation-exchange resins were tried in order to examine their effect on the deamidation of soybean globulins. As a result, the cation-exchange resin of the carboxylate type showed a much higher deamidation activity than did that of the sulfonate type. Because the total amount of acid amide in soybean globulins used in this study was ∼1490 μmol/g of protein, the deamidation levels were 28% for CE-1, 38% for CE-2, 62% for CE-3, and 73% for CE-4. We have already confirmed in the preceding paper (*6*) that the untreated soybean globulins were ammonia-free. In addition, no ammonia was released from basic amino acids and tryptophan by the cation-exchange resin treatment. Therefore, the ammonia released by the cation-exchange resin treatment would be simply from the acid amide in glutamine and asparagine. Moreover, no peptide bond hydrolysis was observed for any cation-exchange resin used at 4 °C. This would be the study report that has achieved a high level of deamidation of soybean globulins without any detectable peptide bond hydrolysis.

The calcium-binding isotherms showed that the amount of bound calcium decreased with the removal of phytate but increased with deamidation. As explained before, phytate interferes with calcium absorption in the body by binding too strongly with calcium and insolubilizing it. The binding strength of the carboxyl group to calcium would be weak enough to release calcium in the small intestine, where calcium is absorbed. Therefore, the phytate-removed deamidated soybean globulins obtained in this study are expected to enhance calcium absorption in the body. The in vivo effects of the phytate-removed deamidated soybean globulins are now under investigation.

ABBREVIATIONS USED

CPP, casein phosphopeptide; CCM, calcium citrate malate; AE-1, an anion-exchange resin with the trimethylammonium group (Amberlite IRA400); AE-2, an anion-exchange resin with the trimethylammonium group (Amberlite IRA458); AE-3, an anion-exchange resin with the (2-hydroxyethyl)dimethylammonium group (Amberlite IRA411S); AE-4, an anion-exchange resin with the (2-hydroxyethyl)dimethylammonium group (Amberlite IRA410); CE-1, a cation-exchange resin of the sulfonate type (Amberlite IR120B); CE-2, a cation-exchange resin of the sulfonate type (Amberlite XT1006); CE-3, a cation-exchange resin of the aminodiacetate type (Amberlite IRC718); CE-4, a cation-exchange resin of the carboxylate type (Amberlite IRC50).

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