JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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# Effective Removal of Cadmium from Fish Sauce Using Tannin

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**ABSTRACT:** Fish sauce prepared from squid organs contains cadmium (Cd), which may be present at hazardous concentrations. In this study, we report a new, inexpensive, and acceptable method for removing Cd from fish sauce using tannin, which is an approved food additive in Japan. Decreases in Cd concentrations of 13-fold were observed (0.39−0.03 mg/100 mL) by incorporating the soluble Cd into a precipitate generated by tannin treatment. The total nitrogen content, free amino acid content, 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity, and angiotensin I-converting enzyme inhibitory activity of the treated fish sauce were the same as those of the untreated fish sauce.

**KEYWORDS:** *Fish sauce, heavy metal, cadmium, food additive, tannin*

## INTRODUCTION

Fish sauce is a traditional seasoning produced by fermentation of fish under a high concentration of sodium chloride for 1−2 years. It contains large quantities of free amino acids and peptides[1,2] and has been shown to have beneficial antioxidative and angiotensin I-converting enzyme (ACE) inhibitory activities.[3,4] Recently, for the purpose of market expansion of fish sauce, several technologies that improve the quality of fish sauces have been reported. These include reduction in histamine content with bentonite[5] and improvement in flavor and color using halo-tolerant starter microorganisms.[6]

Fish sauce products contain certain amounts of heavy metals, including cadmium (Cd), arsenic (As), lead (Pb), and mercury (Hg).[7] In particular, fish sauce prepared from squid organs contains Cd, which may be present at hazardous concentrations, because squid organs, such as liver, accumulate Cd from seawater.[8,9] Studies on the removal of heavy metals from fish sauces are scarce; however, there have been many reports on the removal of heavy metals from fish organs.[10−13] In most of these studies, strong acids, such as sulfuric acid, are used to remove heavy metals, but these acids are not acceptable for food processing. Some industrially acceptable methods, which avoid the use of strong acids, use citric acid or proteases to extract Cd from squid or scallop organs. However, removal of Cd with cation-chelating resins or microbial cultivation is time-consuming. This limits the utility of these methods.[14,15]

The purpose of this study was to develop a new, inexpensive, and acceptable method for removing Cd from fish sauce. We assumed that Cd molecules are present in protein-bound forms in fish sauce, as well as in squid organs. We therefore surveyed food additives that can remove metal-bound proteins from fish sauce by selective precipitation, without influencing other components and biological functions. This method may be applicable to other fish sauces.

## MATERIALS AND METHODS

**Removal of Cd from Fish Sauce.** A commercial fish sauce, which is prepared from squid liver, was purchased from the market in Japan. Tannin, glucono delta-lactone (GDL), and bentonite, which are all approved as food additives in Japan, were tested as clarification agents. Tannin, which was prepared from sumac, was purchased from Fuji Chemical Industry Co., Ltd. (Wakayama, Japan). GDL was purchased from Fuso Chemical Co., Ltd. (Osaka, Japan), and bentonite was purchased from Shinwa Foods Chemical Co., Ltd. (Tokyo, Japan).

Aqueous tannin (1 mL, 1%), aqueous GDL (1 mL, 1%), or bentonite (10 mg) solutions were added to the fish sauce (10 mL), and after shaking for a few seconds, the mixtures were centrifuged at 8000*g* for 10 min to separate supernatants from precipitates.

The concentration dependency of tannins was estimated. Concentrations of aqueous tannin solutions were adjusted to 0.01−5 g/100 mL, and the solutions were used to remove Cd, as described above.

**Measurement of Cd Concentrations in Fish Sauce.** Fish sauce (1 mL) was diluted in 3 N nitric acid (20 mL) and heated at 200 °C for 8 h using a hot plate. The sample was then diluted to 100 mL with distilled water and was analyzed using inductively coupled plasma−atomic emission spectrometry (ICP−AES, IRIS Advantage/SSEA, Jarrell-Ash Co., Ltd., Tokyo, Japan). The wavelength for detection of Cd was 214.4 nm. Results are described as the mean of three independent measurements.

**Measurement of Cd Concentrations in Precipitate.** The precipitates generated by tannin treatment were collected and dried at 120 °C for 24 h. The weight was measured using an electronic balance (CP224S, Sartorius Mechatronics Japan, Tokyo, Japan). The Cd concentration in the precipitate was measured as described above.

**Analyses of Chemical Components and Functional Properties of Fish Sauce.** The total nitrogen content of fish sauce was determined using a nitrogen/protein analyzer (KJEL-Auto DTD-4, Mitamura Riken Kogyo Co., Ltd., Tokyo, Japan) by the Kjeldahl method. The free amino acid content was analyzed using an amino

Received: October 27, 2012
Revised: January 4, 2013
Accepted: January 8, 2013
Published: January 8, 2013

acid autoanalyzer (L-8900, Hitachi High-Technologies Corporation, Tokyo, Japan). The sodium chloride content was calculated from the sodium concentration, which was analyzed using ICP–AES. The lactic acid content was analyzed using ion chromatography (ICS-1500, Dionex Corporation, Tokyo, Japan) equipped with a Dionex IonPac ICE-AS6 ion-exchange column (250 × 9 mm inner diameter). The column was equilibrated and eluted with 0.4 mM perfluorobutyric acid in aqueous solution at a flow rate of 1 mL/min.

The antioxidant activity of the fish sauce was evaluated using the radical scavenger 1,1-diphenyl-2-picrylhydrazyl (DPPH).[16] In brief, 0–150 μL of membrane-filtered fish sauce was diluted to 1.8 mL with distilled water and mixed with 600 μL of 0.2 mM DPPH in ethanol. The mixture was shaken and left to stand for 2 min. The absorbance of this solution was measured at 520 nm using a spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan). Antioxidant activities were calculated from the linear decrease in DPPH absorbance and were expressed as values equivalent to concentrations (μmol/mL) of the standard gallic acid solution. Results are presented as the mean of three independent measurements.

The concentration of test agent required to inhibit ACE (Sigma Chemical, St. Louis, MO) activity to 50% ($IC_{50}$) was assayed using hippuryl-L-histidyl-L-leucine (Sigma Chemical) as a substrate according to the methods by Horiuchi et al.[17] and Ohta et al.,[18] with some modifications. In brief, 50 μL of a solution containing 2.35 mM hippuryl-L-histidyl-L-leucine, 600 mM NaCl, and 480 mM phosphate buffer (pH 8.5) was added to 100 μL of serially diluted samples. Subsequently, 200 μL (2.5 mU) of aqueous ACE in distilled water was mixed with the above substrate solution and incubated in a shaking water bath at 37 ± 1 °C for 60 min. Finally, 100 μL of 3% sodium metaphosphate was added to terminate the reaction. Hippuric acid formed during the reaction was measured using a high-performance liquid chromatography (HPLC) system (LC-10AVvp, Shimadzu Corporation) equipped with a C18 column (250 × 4.6 mm, 5 μm particle size, Shinwa Chemical, Tokyo, Japan). Iodide ions were detected by absorbance at 226 nm. The column was equilibrated and eluted with 20% acetonitrile in 0.01 M aqueous potassium phosphate solution at a flow rate of 1 mL/min. The substrate with distilled water was used as a blank, and the reaction mixture with distilled water instead of the sample was used as a control. The inhibitory ratio (%) of ACE was calculated as $[1-(A-B)/(C-B)] \times 100\%$, where $A$ is the absorbance of the testing sample, $B$ is the absorbance of the blank, and $C$ is the absorbance of the control. Samples were tested in triplicate.

**Quantification of the Tannin Concentration in Fish Sauce.** The tannin content of fish sauce was analyzed using the same HPLC system described for ACE inhibition experiments, except that the column was equilibrated with a 0.1% aqueous phosphoric acid solution and was eluted with a methanol gradient of 20–100% in 0.1% phosphoric acid.[19] Elution was monitored by absorbance at 280 nm.

**Gel Filtration Chromatography of Fish Sauce.** To evaluate Cd forms in fish sauce, gel filtration chromatography was performed using the method by Takeuchi et al.,[20] with some modifications. Untreated fish sauce (2 mL) was applied to a Sephadex G-15 gel filtration column (50 × 1.5 cm interior diameter), which was equilibrated and eluted at 1 mL/min with 0.1 M aqueous sodium chloride solution. Fractions were collected every 4 min. Standard solution was prepared by dissolving 100 mg of human albumin (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), 100 mg of alanine (Wako Pure Chemical Industries, Ltd.), and 100 μL of 1000 ppm Cd standard solution (Kanto Chemical Co., Inc., Tokyo, Japan) in 10 mL of distilled water. This standard solution (2 mL) was then applied to a gel filtration column in the same manner as the fish sauce. The Cd concentration and absorbance (280 nm) of the fractions were analyzed by ICP–AES and spectrophotometry (UV-1800, Shimadzu Corporation), respectively. For analysis of amino acid and peptide, a mixture of 80 μL of each fraction, 80 μL of 1% ninhydrin solution, and 1.84 mL of distilled water was heated in boiling water for 15 min and the absorbance at 570 nm was measured by spectrophotometry.

## RESULTS AND DISCUSSION

**Removal of Cd from Fish Sauce Using Tannin.** Initially, we evaluated the efficiency of three food additives, tannin, GDL, and bentonite, in removing Cd from fish sauce (Figure 1). Prior to the use of these additives, we found that simple

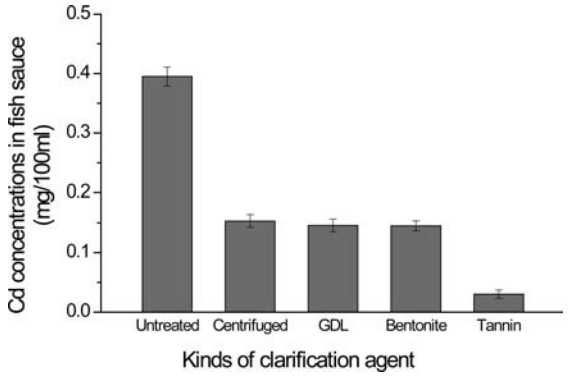


**Figure 1.** Cadmium concentrations in fish sauces treated with various food additive experiments were performed on three independent samples, and data are shown as the mean ± standard error.

centrifugation effectively decreased the Cd concentration to some extent. Cd concentrations in the untreated and centrifuged fish sauce were 0.39 ± 0.02 and 0.15 ± 0.01 mg/100 mL, respectively, indicating that $^2/_5$ of total Cd in fish sauce is bound to substances that are readily precipitated by centrifugation. Next, we attempted to remove Cd using GDL or bentonite. After this treatment, Cd concentrations were equal to those in the centrifuged fish sauce (0.15 ± 0.01 mg/100 mL). On the other hand, the Cd concentration in the fish sauce was reduced to 0.03 ± 0.004 mg/100 mL by tannin, which is $^1/_5$ of that in the centrifuged fish sauce. These results indicate that GDL and bentonite treatments were ineffective, whereas tannin treatment was effective in reducing the Cd concentration in the fish sauce.

The high Cd-removing efficiency of tannin was further confirmed by weights of the precipitates generated by the additives, as shown in Figure 2. GDL treatment generated a small increase in the mass of the precipitate compared to centrifugation (from 11 ± 3 to 14 ± 2 mg), confirming that GDL is ineffective in removing Cd-containing substances. GDL is generally used as a pH-lowering agent because of gluconic

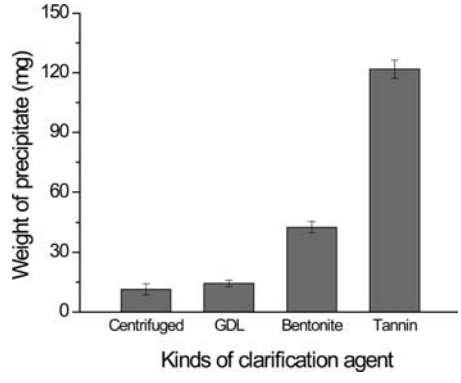


**Figure 2.** Weights of precipitates generated by each clarification treatment experiment were performed on three independent samples, and data are shown as the mean ± standard error.


dx.doi.org/10.1021/jf304520v | J. Agric. Food Chem. 2013, 61, 1184–1188

acid generated by chemical equilibrium. Therefore, GDL is used to coagulate soymilk proteins while manufacturing tofu.[21] In this study, the proteins of fish sauce may not have been coagulated by this mechanism. The weight of the precipitate generated by bentonite treatment was 43 ± 3 mg. However, this precipitate contained 10 mg of added bentonite, because bentonite is insoluble, and approximately 30 mg of the fish sauce precipitate. The weight of this precipitate was 3 times higher than that of the precipitate generated by centrifugation. Proteins are adsorbed onto bentonite primarily because of a cation-exchange action[22,23] that removes positively charged histamine from fish sauce.[7] Hence, our results indicate that bentonite adsorbs positively charged protein molecules. However, these proteins were not necessarily bound to Cd. On the other hand, the weight of the precipitate generated by tannin treatment was 122 ± 5 mg, much higher than that generated by the other two additive treatments with additives. Although this precipitate contained added tannin (10 mg), its weight was 10 times higher than the weight of the precipitate generated by centrifugation. Tannin binds to proteins (presumably proline residues) in beverages, such as beer and apple juice, and the protein−tannin network produces large colloidal particles and maximum light scattering.[24−26] Accordingly, tannin exhibited high clarification efficacy and generated colloidal particles in fish sauce that were effective in removing Cd. The amount of Cd in the precipitate was almost the same as that removed from the fish sauce (data not shown), indicating that Cd is co-precipitated with proteins after tannin treatment.

**Dependency of Cd Removal on the Tannin Concentration.** Figure 3 shows the effect of increasing the tannin concentration from 0.01 to 0.5% (w/v) on Cd remaining in fish sauces. The Cd concentration gradually decreased with increasing amounts of added tannin. Using 0.1% tannin, almost maximum removal of Cd (from 0.39 ± 0.02 to 0.03 ± 0.004 mg/100 mL) was achieved. The suitable amount of tannin for removal Cd is 0.1% (w/v). On the other hand, it is necessary to further evaluate the effect of tannin addition on components and biological functions of fish sauce, and these results were described below.

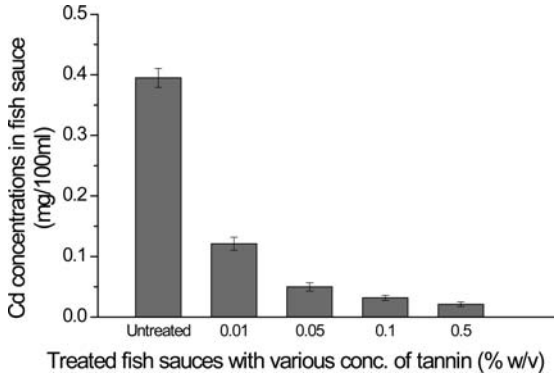


**Figure 3.** Effects of various concentrations of tannin on Cd removal from fish sauces. Each bar represents the mean ± standard error of three experiments.

**Chemical Composition and Functionality of Tannin-Treated Fish Sauces.** Total nitrogen and free amino acid content of tannin-treated fish sauces were determined as summarized in Figures 4 and 5, respectively. The total nitrogen content of treated fish sauces with tannin decreased from 2.4 ± 0.05 to approximately 2.2 g/100 mL, but the reduction was less than 10%. The total free amino acid content of all fish sauce samples was approximately 9000 mg/100 mL. Sodium chloride and lactic acid contents were also stable between all fish sauce samples at approximately 18 and 2.4 g/100 mL, respectively. The ACE $IC_{50}$ and taurine content of all fish sauce samples were approximately 9 μL/mL and 540 mg/100 mL, respectively. ACE inhibitory activity is related to the control of blood pressure, and taurine is related to fatigue recovery. Low-molecular-weight peptides with ACE inhibitory activity are isolated from fish sauce,[4,27] and our results indicate that these peptides, taurine, and amino acids are not precipitated by tannin treatment. Taken together, tannin treatment does not affect the chemical composition and functionality of fish sauce. In subsequent experiments, the DPPH radical scavenging activities in the tannin-treated fish sauces were determined using gallic acid as a standard radical scavenger (Figure 6). The

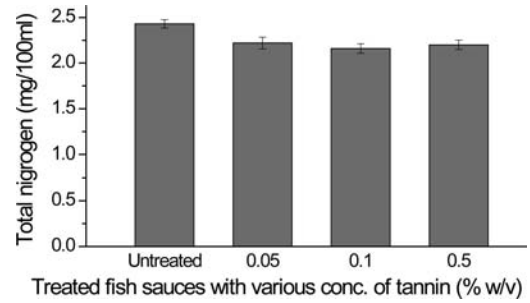


**Figure 4.** Total nitrogen in fish sauces treated with various concentrations of tannin. Each bar represents the mean ± standard error of three experiments.

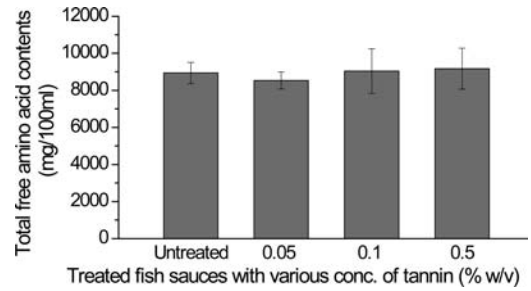


**Figure 5.** Total free amino acid contents of fish sauces treated with various concentrations of tannin. Each bar represents the mean ± standard error of three experiments.

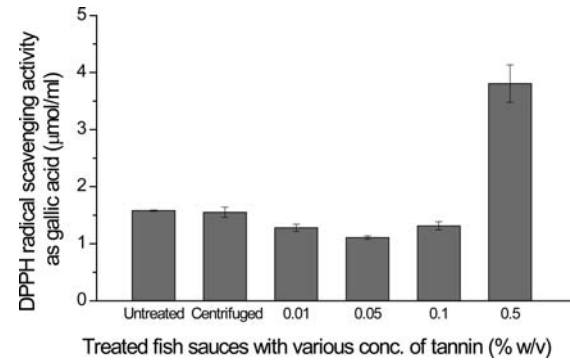


**Figure 6.** DPPH radical scavenging activity in fish sauces treated with various concentrations of tannin. Each bar represents the mean ± standard error of three experiments.


dx.doi.org/10.1021/jf304520v | J. Agric. Food Chem. 2013, 61, 1184−1188

antioxidant activity decreased from 1.58 ± 0.01 to 1.11 ± 0.03 μmol/mL in samples treated with 0.05% (w/v) tannin. Thus, tannin treatment reduced the radical scavenging activity by approximately 30%. Some antioxidant components of fish sauce are high-molecular-weight proteins (molecular weight > 100 000), such as catalase.[28] It is therefore expected that these proteins were precipitated by tannin treatment. However, it unexpectedly increased to 3.81 ± 0.33 μmol/mL after 0.5% (w/v) tannin treatment. To assess the possibility that residual tannin influenced DPPH radical scavenging activities in the fish sauce, the tannin concentration was determined by HPLC. Tannin was only detected in the fish sauce treated with 0.5% (w/v) tannin (33 ± 14 mg/100 mL; the limit of detection was 4.3 mg/100 mL). As calculated from the DPPH radical scavenging activity of tannin (7.1 μmol/g), the radical scavenging activity of residual tannin in the fish sauce treated with 0.5% (w/v) tannin was determined to be 2.3 μmol/mL. This value corresponds well with the difference between DPPH radical scavenging activities of the fish sauce treated with 0.05 and 0.5% (w/v) tannin (2.7 μmol/mL). Thus, residual tannin in fish sauce may have strong antioxidant properties.

**Determination of the Cd Form in Fish Sauce.** To determine whether Cd in fish sauce is bound to proteins, we investigated the Cd form by gel filtration chromatography (Figure 7). In the chromatography of standard solutions, albumin, alanine, and liberated Cd were detected in fractions 9, 13, and 17, respectively. When the fish sauce was analyzed, Cd was detected in fractions 8−15 and the maximum concentration was observed in fraction 11. Ninhydrin-reactive fractions (fractions 11−16) almost corresponded with the main peak of absorbance at 280 nm (fractions 12−16), indicating that these fractions are predominantly composed of amino acids and peptides. The peak of the Cd was eluted between the peaks of albumin (fraction 9) and alanine (fraction 13) in the standard solution and did not correspond to the peak of liberated Cd (fraction 17). This result suggested that Cd is, as expected, present in organic molecule-bound forms in fish sauce and not in liberated forms. Cd in squid liver and pancreas binds to glycoproteins of a structure similar to that of metallothionein;[29−31] thus, we consider that Cd in fish sauce may bind to glycoproteins in the liver and pancreas of squid. Therefore, the technique established in this study to remove Cd-bound organic molecules by tannin treatment could be expanded to the removal of other heavy metals, such as Hg, bound to squid glycoprotein.[29]

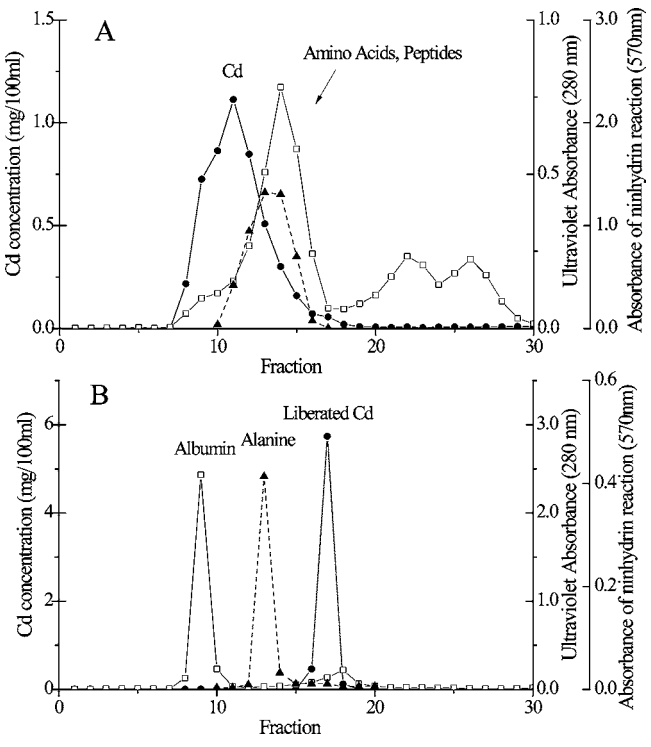


**Figure 7.** Gel filtration chromatography of (A) fish sauce and (B) standard solution. Standard solutions contained albumin, alanine, and liberated Cd. The Cd concentration (●), absorbance at 280 nm (○), and absorbance at 570 nm after ninhydrin reaction (▲) were measured for each fraction.

In conclusion, we developed a simple method to remove Cd from fish sauce prepared from squid organs using tannin that is an approved food additive in Japan. The method involves the addition of 0.1% (w/v) tannin to fish sauce and subsequent removal of the resulting precipitate. The Cd concentration in the starting fish sauce product decreased from 0.39 to 0.03 mg/100 mL (a 13-fold decrease) following this treatment. Chemical components, such as free amino acids, present in fish sauce and the health beneficial effects of the fish sauce were retained after tannin treatment. This method is inexpensive, safe, and effective and has promising applications in fish sauce factories. This method may be applicable to other fish sauces.

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### Notes

The authors declare no competing financial interest.

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