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# Effect of Proteolytic Squid Protein Hydrolysate on the State of Water and Dehydration-Induced Denaturation of Lizard Fish Myofibrillar Protein

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With the goal of preparing low-cost functional food, squid protein hydrolysate (SPH) was extracted from four squid species by protease treatment. Peptides are the major components (~84−88%) of the SPH. The stabilization effects of 5% SPH (dried weight/wet weight) on the state of water and the denaturation of frozen lizard fish *Saurida wanieso* myofibrillar protein (Mf) were evaluated on the basis of desorption isotherm curves with respect to $Ca^{2+}$-ATPase inactivation and the presence of unfrozen water, which was determined using differential scanning calorimetry during dehydration, and the effects were compared with those of sodium glutamate. The Mf with SPH was found to contain higher levels of monolayer and multilayer sorption water, resulting in decreased water activity and $Ca^{2+}$-ATPase inactivation. The amount of unfrozen water in Mf with SPH increased significantly, suggesting that the peptides of SPH stabilized water molecules on the hydration sphere of Mf, which maintained the structural stability of Mf, and therefore suppressed dehydration-induced denaturation. The effect by SPH was less than that by sodium glutamate.

**KEYWORDS: Squid protein hydrolysate; water activity; unfrozen water; $Ca^{2+}$-ATPase; dehydration**

## INTRODUCTION

Functional proteins as food ingredients command high prices because of the textural and stabilizing effects they produce by means of covalent attachment with the amino acids in peptides when incorporated in recipes (*1*, *2*). Protein sources with appropriate amino acid profiles and the method of modification are the most important factors for the preparation of functional food (*2*, *3*). Enzymatic modifications have several advantages; they minimize nutrient loss and produce a decrease in peptide size, which improve the protein functionality (*4*−*6*).

Fishery products are easily putrefied by the microbial proliferation that occurs at ambient temperatures due to the high moisture and nutrient content of these products. In addition, the substance of these products is labile because the content of substrate proteins is relatively low, and the products are prone to physical, chemical, and enzymatic damage. Drying and frozen storage, widely used as long-term preservation methods, create an environment deleterious to the spoilage mechanisms of fish and fishery products. Nevertheless, the mechanisms of the processes are inevitably associated with some deterioration, particularly in terms of water retention and protein solubility (*7*−*9*), because water is closely associated with the maintenance of protein structure and functionality (*10*, *11*). The folding and structural stability of protein during dehydration and frozen storage are extensively controlled by the addition of a variety of anti-denaturants such as sugar, amino acids, organic acids, and phosphate (*12*−*18*). Currently, the potential uses and functionality of enzymatically degraded materials from various ingredients, such as the use of fishery byproducts and residues from seafood processing industries, have drawn the attention of food scientists (2, *5*, *19*−*23*). However, there is no report on the extraction of functional protein from squid by protease treatment for the purpose of food preservation, except a very few studies (*24*, *25*) on squid protein hydrolysate for the characterization of angiotensin converting enzyme inhibitor and microdiets for fish larvae, although squid contributes an important source of global protein and its chemical constituents and nutritive value are worthy of mention (*26*). Therefore, the present study was designed for the preparation of proteolytic functional food from squid and for the investigation of its effect on the state of water and dehydration-induced denaturation of fish myofibrillar protein. The basic purpose of this study is to develop a functional food from squid as a new perspective contemplating the potential use of low-cost squid meat in food processing and preservation.

## MATERIALS AND METHODS

**Materials.** Four squid species, namely, Japanese flying squid *Todarodes pacificus* (weight = 330.7 ± 78.0 g), bigfin reef squid

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*Sepioteuthis lessonania* (weight = 1120.6 ± 273.7 g), swordtip squid *Loligo edulis* (weight = 161.2 ± 23.5 g), and golden cuttlefish *Sepia esculenta* (weight = 190.3 ± 30.1 g), were purchased at Nagasaki Fish Market, Japan, and transported to our laboratory in a frozen condition. Sodium glutamate (Na-glu) was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

**Preparation of Squid Protein Hydrolysate (SPH).** The SPH was prepared according to the method of Iwamoto et al. (*27*) with slight modification. Five kilograms of squid for each species was chopped and ground into mince and reconstituted in 200% distilled water. The mixture was heated at 90 °C for 30 min to inactivate the endogenous enzymatic activity. The mixture was homogenized and adjusted to pH 8.0 with 0.1 N sodium hydroxide at 60 °C. Hydrolysis was carried out for 2 h at 60 °C by adding 0.2% (w/w) endo-type protease derived from *Bacillus subtilis* (Shin-Nihon Chemical Industries, Inc., Anjo, Aichi, Japan). The enzymatic activity was terminated by increasing the temperature to 90 °C for 30 min. The pH was adjusted to 6.0 by adding malic acid, the mixture was hydrolyzed further by exo-type protease derived from *Aspergillus oryzae* (Shin-Nihon Chemical Industries, Inc.), and then the reaction was terminated by the above-described method. The product was centrifuged at 3800*g* for 15 min and then filtered. The filtrate was heated at 80 °C for 10 min, after which the lipid layer was removed from the surface. The hydrolyzed mixture was then subjected to ultrafiltration (Millipore, PK 30000 NMWL, Minitan, Pellicon XL, Millipore Corp., Billerica, MA) for collection of materials having a molecular weight of <30000 Da. The sample was passed through a desalting panel (model G3, Asahi Kasei, Inc., Kawasaki, Kanagawa, Japan) and spray-dried (model GA32, Yamato Scientific, Inc., Tokyo, Japan). Finally, SPH was obtained in powder form.

**Proximate Composition.** Moisture, crude protein, crude lipids, and crude ash contents were measured by the heat drying (105 °C), Kjeldahl (%N × 6.25), Soxhlet, and heating (550 °C) methods, respectively (*28*). Sugar content was determined according to the phenol−sulfuric acid method (*29*). Salt content was measured using a salt analyzer (model SAT-2A, Toa Denpa, Inc., Shinjuku, Tokyo, Japan).

**Molecular Weight (MW) Distribution.** Gel filtration chromatography was carried out on a Sephadex G-25 column (2.2 cm i.d. × 60 cm) in order to determine the MW distribution of the SPH. Samples were prepared at a rate of 15 mg of protein/mL with 30 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl and centrifuged at 7900*g* for 20 min. Then 2 mL of supernatant was loaded on the column and eluted with 30 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl at a flow rate of 30 mL/h. Absorbance for each fraction was determined at 220 nm in order to identify the components of the SPH. The molecular weight of the SPH was calculated on the basis of that of the standard compounds: cytochrome *c* (MW 12500), aprotinin (MW 6511), bacitracin (MW 1411), glutathione, reduced (MW 307), glycyl-L-phenylalanine (MW 222), and l-phenylalanine (MW 165).

**Amino Acid Composition.** Hydrochloric acid (MW 36.46, amino acid analysis grade, Nacalai Tesque Inc., Kyoto, Japan) was used for the hydrolysis reaction of the SPH. The reaction was carried out at 110 °C for 20 h, and the sample was dried using the freeze-dryer. The extracted nitrogen was dispersed in sodium citrate buffer (pH 2.2). The amino acid composition of the SPH was analyzed by an automatic amino acid analyzer (ALC 1000, Shimadzu Seisakusho Co., Kyoto, Japan).

**Preparation of Fish Myofibrillar Protein (Mf).** The Mf was prepared according to the method of Katoh et al. (*30*) and that of Nozaki et al. (*15*). Fresh muscles of lizard fish were cut into thin sections and washed three times with 5 volumes of 0.1 M KCl−20 mM Tris-maleate buffer (pH 7.0). The meat was diluted with 3 volumes of the buffer and homogenized at 10000 rpm for 90 s by a foam preventive-type blender (model JM-H131, Mitsubishi, Tokyo, Japan). The minced meat was then passed through a nylon net (no. 16) to remove the connective tissues. Subsequently, 20% Triton X-100 solution was added to obtain a final concentration of 1%. The suspension was left to stand for 30 min and then centrifuged at 750*g* for 10 min. The sediment was mixed with 5 volumes of the buffer, stirred, centrifuged (750*g*, 10 min), and washed. This procedure was repeated four times. To reduce buffer action as much as possible, the sediment was mixed with 5 volumes of cold distilled water, washed by stirring, and centrifuged at 3800*g* for 10 min. The sediments were further centrifuged at 27000*g* for 20 min. Finally, the obtained sediment was used as Mf with the following characteristics: moisture content, 87.1%; crude protein, 12.4%; crude lipid, 0.04%; and crude ash, 0.43%. All of the procedures were performed at 5 °C.

**SPH or Na-glu Dispersion in Mf.** The SPH and Na-glu were added to Mf at a 5% level (dried weight of SPH or Na-glu/wet weight of pelleted Mf) and mechanically dispersed for 20 min at 5 °C, and then the pH of the mixture was adjusted to 7.0 using 0.01 M NaOH or 0.01 M HCl. The Mf mixture was sealed in a cellophane bag, which was embedded in silica gel in a desiccator, and dehydrated at 5 °C with periodic replacement of the silica gel. This procedure was continued to 10% moisture content of the Mf. A vacuum desiccator was used for further dehydration under reduced pressure.

**Analysis of Desorption Isotherm Curves.** The moisture content and concomitant $A_w$ of the chronologically dehydrated samples were measured by the atmospheric heat drying method at 105 °C and the indirect equilibrium vapor pressure method (*13*), respectively, to analyze the desorption isotherm curves of the Mf. The desorption isotherm curves (**Figure 2**) were established by plotting the values of moisture content against those of $A_w$. The values for the inflection point ($M_1$) on the desorption isotherm curves were obtained by Brunauer, Emmet, and Teller (BET) analysis (*31*). The moisture content at $M_1$ is considered to be the amount of monolayer-sorbed water. The inflection point of $A_w$ on the desorption isotherm curves corresponding to the minimal point on the $M/A_w - A_w$ curves is considered as $M_2$ (*32*). The moisture content at this point is regarded as the amount of multilayer-sorbed water. The sorption area ($S$) of Mf was calculated using the equation

$$S = M_1 \times S_w \times N/(Mw \times 10^3) \quad (1)$$

where $S$ = the sorption surface area per mg of sorbed water (m$^2$/mg), $M_1$ = the amount of monolayer-sorbed water (g/g of dried matter), $S_w$ = the cross-sectional area of water molecules (10.8 Å$^2$), $N$ = Avogadro's number ($6.02 \times 10^{23}$/mol), and Mw = the molecular weight of water (18 g/mol).

**Determination of Unfrozen Water.** The amount of unfrozen water in the Mf was determined according to the method of Wakamatsu and Sato (*33*) using differential scanning calorimetry (DSC) (model SSC-5200, Seiko Electronic Industry Inc.). The dehydrated Mf and $Al_2O_3$, each ∼20 mg, were packed into separate calorimetric aluminum cells. The $Al_2O_3$-containing cell was used as a reference in order to balance the heat capacity of the cell containing Mf. The cells were subjected to DSC analysis in which the heat of fusion was measured by elevating the temperature from −40 to 25 °C at a rate of 1 °C/min. The measured endothermic peak area reflected the heat of fusion necessary to melt the ice, which corresponded to the free water in Mf. The peak melting temperature was initially observed at −1.91 °C, and the peak points were shifted gradually (up to −6.14 °C) with the reduction of the moisture content in the sample. The total water content in the Mf was measured by drying at 105 °C for 24 h. The unfrozen water in dehydrated Mf calculated by the linear relationship between transition heat $\Delta H_f$ (J/g of dried matter) and water content ($x$) per g of dried sample, which was expressed by

$$f(x) = A_x - B \; (A, B > 0) \quad (2)$$

where $f(x) = 0$, $x$ = unfrozen water content per g of dried sample (g of $H_2O$/g of dried matter).

**Mf $Ca^{2+}$-ATPase Activity.** The dehydrated Mf was suspended in 30 volumes of 0.1 M KCl−20 mM Tris-maleate buffer (pH 7.0), left to stand overnight at 5 °C for water restoration, and then homogenized (Nichion-irika Kikai Seisakusho Histocolon NS-560) at 1000 rpm for 1 min. The homogenate was centrifuged at 750g for 10 min. The Mf precipitate was suspended in the same buffer. The protein concentration of the Mf suspension was determined using the biuret method (*34*) employing bovine serum albumin (fraction V) as a standard and corrected by the previously mentioned Kjeldahl method. The Mf $Ca^{2+}$-ATPase activity was measured in a reaction medium composed of 100 mM KCl, 5 mM $CaCl_2$, 25 mM Tris-maleate (pH 7.0), 1 mM adenosine

**Table 1.** Proximate Composition of Squid Protein Hydrolysate

| | g/100 g of dried matter | | | |
|---|---|---|---|---|
| composition | swordtip squid | Japanese flying squid | bigfin reef squid | golden cuttlefish |
| crude protein | 87.67 | 87.72 | 87.92 | 84.28 |
| ± SD[a] | 0.50 | 1.19 | 0.40 | 0.52 |
| crude lipid | 0.07 | 0.26 | 0.21 | 0.12 |
| ± SD | 0.04 | 0.01 | 0.02 | 0.01 |
| crude ash | 6.99 | 6.07 | 6.13 | 6.91 |
| ± SD | 0.04 | 0.03 | 0.16 | 0.03 |
| sugar | 3.32 | 3.40 | 3.40 | 3.55 |
| ± SD | 0.02 | 0.14 | 0.11 | 0.29 |
| NaCl | <0.01 | <0.01 | <0.01 | <0.01 |

[a] Standard deviation.

**Table 2.** Amino Acid Composition of Squid Protein Hydrolysate

| | g/100 g of dried matter | | | |
|---|---|---|---|---|
| amino acid | swordtip squid | Japanese flying squid | bigfin reef squid | golden cuttlefish |
| Asx | 6.46 | 7.44 | 6.67 | 6.52 |
| Glx | 10.23 | 11.17 | 10.41 | 9.99 |
| Arg | 6.20 | 7.01 | 7.46 | 6.09 |
| Lys | 2.31 | 1.55 | 1.18 | 1.11 |
| His | 1.67 | 2.22 | 1.98 | 2.04 |
| Gly | 0.19 | 0.22 | 0.19 | 0.19 |
| Ala | 4.51 | 4.27 | 4.15 | 3.52 |
| Ser | 7.28 | 7.59 | 6.99 | 6.48 |
| Thr | 2.68 | 3.03 | 2.69 | 2.65 |
| Val | 3.00 | 3.59 | 2.58 | 2.43 |
| Leu | 4.76 | 5.12 | 4.98 | 4.90 |
| Ile | 1.76 | 2.11 | 1.76 | 1.81 |
| Phe | 1.56 | 1.53 | 1.38 | 1.46 |
| Tyr | 1.48 | 1.67 | 1.51 | 1.60 |
| Pro | 4.84 | 3.95 | 5.15 | 2.48 |
| Met[a] | 1.90 | 2.16 | 1.89 | 1.84 |
| Cys[a] | 0.07 | 0.20 | 0.15 | 0.13 |

[a] Values may not be accurate as no special treatment was considered for Met and Cys.

triphosphate (ATP), and 0.2−0.4 mg/mL Mf at 25 °C. The reaction was terminated after 5 min by adding 30% trichloroacetic acid solution at a final concentration of 5%, and free inorganic phosphate was measured by colorimetry (*30*).

## RESULTS

**Chemical Composition of SPH.** The proximate composition of the SPH is summarized in **Table 1**. Protein was the major component, ranging in content from 84 to 88%, whereas ash and sugar contents accounted for about 6−7 and 3%, respectively. Crude lipids and NaCl were found as trace components of the SPH. The amino acid composition of the SPH was categorized as acidic, basic, and neutral (**Table 2**). The SPHs of Japanese flying squid, bigfin reef squid, swordtip squid, and golden cuttlefish were found to contain amino acids accounting for 64.8, 61.1, 60.9, and 55.2% (grams per 100 g of dried matter of SPH), respectively. Hydrophilic amino acids accounted for about 62.1, 60.9, 61.1, and 63.5% (grams per 100 g of amino acid) in the SPHs of Japanese flying squid, swordtip squid, bigfin reef squid, and golden cuttlefish, respectively.

**Molecular Weight Distribution. Figure 1** shows the MW distribution of the SPH corresponding to a fraction of <6500 Da. The large sharp peak indicates the average MW of the SPH, ranging from 1400 to 300 Da, suggesting that peptides are the major components of the SPH.

**Desorption Isotherm Curves of Myofibrils.** As shown in **Figure 2**, the desorption isotherm curves elucidate the correlation between $A_w$ and moisture content of the Mf. The curves for the Mf containing SPH and Na-glu and for the control were sigmoid with two inflection points of $A_w$, at 0.05−0.17 and 0.5−0.7. The $A_w$ values of the Mf containing SPH of the four squid species were similar but markedly lower than that of the control at all moisture levels. The Mf containing Na-glu showed a lower $A_w$ value than that of the Mf containing SPH at the same moisture content.

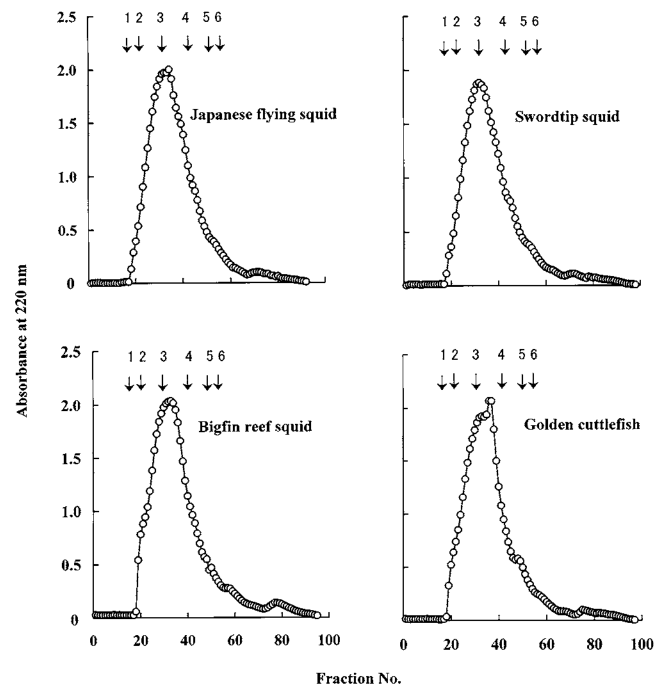


**Figure 1.** Gel chromatograms of squid protein hydrolysate on a Sephadex G-25 column: elution, 30 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl; flow rate, 30 mL/h; fraction volume, 5 mL/tube. Numbers denote elution position of the following standard compounds: 1, cytochrome *c* (MW 12500); 2, aprotinin (MW 6511); 3, bacitracin (MW 1411); 4, glutathione, reduced (MW 307); 5, glycyl-L-phenylalanine (MW 222); 6, L-phenylalanine (MW 165).

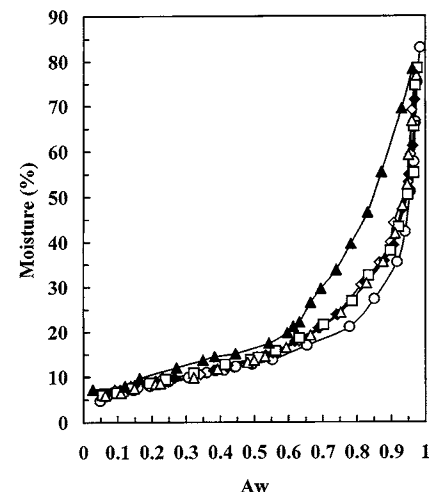


**Figure 2.** Effect of squid protein hydrolysate and sodium glutamate on the desorption isotherms of lizard fish myofibrillar protein at 20 °C during dehydration process: (○) control; (▲) sodium glutamate; (◆) swordtip squid; (◇) Japanese flying squid; (□) bigfin reef squid; (△) golden cuttlefish. Hydrolysate or sodium glutamate at 5% (dried matter) is added to 100 g of lizard fish myofibrillar protein. Myofibrillar protein without additives was considered as the control.

The $M_1$, $M_2$, and $S$ values calculated from the desorption

**Table 3.** Amount of Monolayer and Multilayer Water, Sorption Surface Area, and Remaining $Ca^{2+}$-ATPase Activity at the Inflection Points of the Desorption Isotherm of Lizard Fish Myofibrillar Protein (Mf) at 20 °C[a]

| system | monolayer water[b] $M_1$[c] | $A_{w1}$[d] | ATPase[e] | multilayer water[f] $M_2$[c] | $A_{w2}$[g] | ATPase[h] | $M_2/M_1$ | $S$[i] |
|---|---|---|---|---|---|---|---|---|
| control | 7.58 | 0.148 | 1.50 | 13.90 | 0.560 | 1.97 | 1.83 | 0.29 |
| sodium glutamate | 9.09 | 0.149 | 7.03 | 17.10 | 0.531 | 35.10 | 1.88 | 0.36 |
| swordtip squid | 8.28 | 0.159 | 9.27 | 15.00 | 0.542 | 21.13 | 1.81 | 0.32 |
| Japanese flying squid | 8.19 | 0.140 | 13.54 | 15.84 | 0.581 | 27.12 | 1.93 | 0.32 |
| bigfin reef squid | 7.58 | 0.145 | 7.74 | 15.32 | 0.552 | 22.62 | 2.02 | 0.29 |
| golden cuttlefish | 7.67 | 0.164 | 6.09 | 14.60 | 0.530 | 21.77 | 1.90 | 0.30 |

[a] Squid protein hydrolysate or sodium glutamate at 5% (dried matter) is added to 100 g of Mf (wet material). Mf without additives is considered as control. [b] Estimated by BET analysis (*31*). [c] Moisture content (g/100 g of the Mf). [d] Water activity of the Mf at $M_1$ point. [e] Remaining relative $Ca^{2+}$-ATPase activity (%) of the Mf at $M_1$ point. [f] Estimated by Bull's analysis (*32*). [g] Water activity of the Mf at $M_2$ point. [h] Remaining relative $Ca^{2+}$-ATPase activity (%) of the Mf at $M_2$ point. [i] Sorption surface area ($m^2$/mg) of the Mf.

isotherm curves of the Mf and the $Ca^{2+}$-ATPsae activity together with water activity ($A_{w1}$ and $A_{w2}$) at the inflection points are summarized in **Table 3**. The Mf with Na-glu showed a higher $M_1$ value (9.09 g of $H_2O$/100 g of sample) followed by 8.28, 8.19, 7.67, and 7.58 g of $H_2O$/100 g for the Mf containing the SPHs of swordtip squid, Japanese flying squid, bigfin reef squid, and golden cuttlefish, respectively, whereas the Mf with Na-glu showed a higher $M_2$ value, accounting for 17.10 g of $H_2O$/100 g of Mf, followed by 15.84, 15.32, 15.00, and 14.60 g of $H_2O$/100 g for Mf containing the SPHs of the Japanese flying squid, bigfin reef squid, swordtip squid, and golden cuttlefish, respectively. The $M_1$ and $M_2$ values of the Mf with the SPHs of Japanese flying squid, swordtip squid, and golden cuttlefish were higher than that of the control (7.58 and 13.90 g of $H_2O$/100 g of Mf). Although the $M_1$ value of the Mf with the SPH of bigfin reef squid was similar to that of the control, it exhibited a higher $M_2$ value than the control. The Mf with Na-glu and SPHs of the Japanese flying squid, swordtip squid, and golden cuttlefish showed higher $S$ values (0.30−0.36 $m^2$/mg) than the control (0.29 $m^2$/mg). The Mf with the SPH of bigfin reef squid showed an $S$ value similar to that of the control. The findings suggest that the Na-glu and the SPH stabilized monolayer and multilayer-sorbed water in the Mf structure, whereas the Na-glu had a higher effect followed by the SPHs of the Japanese flying squid, swordtip squid, and golden cuttlefish. The SPH of bigfin reef squid exhibited a slight effect on the stabilization of water molecules in the Mf structure.

**Determination of Unfrozen Water.** DSC analysis showed that the Mf with SPH or Na-glu exhibits lower enthalpy changes than the control at different levels of moisture content (data not shown). A graphical representation of the amount of unfrozen water in Mf, calculated from the linear relationship ($r = 0.99$, $n = 6$, $P < 0.001$) between transition heat (J/g of dried matter) and moisture content (g of $H_2O$/g of dried matter), is depicted in **Figure 3**. The amount of unfrozen water in the Mf containing SPH or Na-glu was significantly higher ($P < 0.05$) than in the control. The Mf with SPHs of the swordtip squid and Japanese flying squid exhibited significantly higher ($P < 0.05$) amounts of unfrozen water accounting for 0.449 and 0.426 g of $H_2O$/g of dried matter, respectively, than that in the Mf containing SPHs of the bigfin reef squid (0.390 g of $H_2O$/g of dried matter) and golden cuttlefish (0.385 g of $H_2O$/g of dried matter). The Mf containing Na-glu showed unfrozen water accounting for 0.429 g of $H_2O$/g of dried matter. These findings suggest that the SPH and Na-glu stabilized water molecules in the Mf molecular structure. The SPH of swordtip squid showed the highest effect, followed by Na-glu and then the SPH of Japanese flying squid.

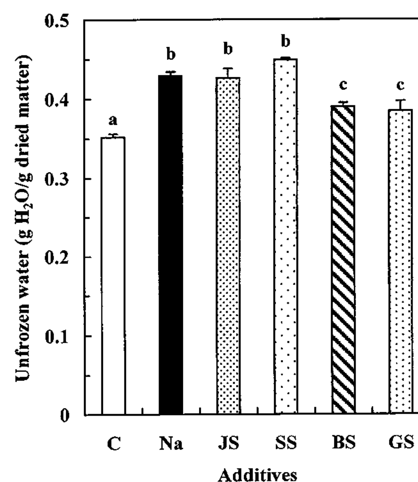


**Figure 3.** Amount of unfrozen water (grams of $H_2O$/gram of dried matter) in lizard fish myofibrillar protein with squid protein hydrolysate or sodium glutamate during the dehydration process: C, control; Na, sodium glutamate; JS, Japanese flying squid; SS, swordtip squid; BS, bigfin reef squid; GS, golden cuttlefish. Values with different letters are significantly different at $P < 0.05$.

**Mf $Ca^{2+}$-ATPase Activity.** The $Ca^{2+}$-ATPase activity was measured to confirm the cryoprotective effect of the SPH against the dehydration-induced denaturation of the Mf compared with that by Na-glu and that in the control. The correlation between $Ca^{2+}$-ATPase activity and the $A_w$ of the Mf is depicted in **Figure 4**. The Mf $Ca^{2+}$-ATPase of the control was inactivated over 85% at the $A_w$ level of 0.9, indicating the capillary condensation domain, and the remaining activity gradually reduced between the capillary condensation domain and the multilayer domain. Zhang et al. (*22*) reported that >90% of the $Ca^{2+}$-ATPase activity of lizard fish myofibrillar protein reduced at an $A_w$ level of 0.7. In contrast, the $Ca^{2+}$-ATPase activity of the Mf with SPH and that with Na-glu diminished very slowly with decreasing moisture content, and the relative values were higher than those of the control at all $A_w$ levels. Although the $Ca^{2+}$-ATPase inactivation of the Mf with SPH was more or less similar among the four species, the SPH of Japanese flying squid exhibits a higher cryoprotective effective against dehydration-induced denaturation of the Mf followed in order by that of golden cuttlefish, swordtip squid, and bigfin reef squid. The Mf with Na-glu showed a better result than did Mf with SPH.

## DISCUSSION

The results presented in this study aim to contribute to an understanding of the phenomena that occur upon the stabilization

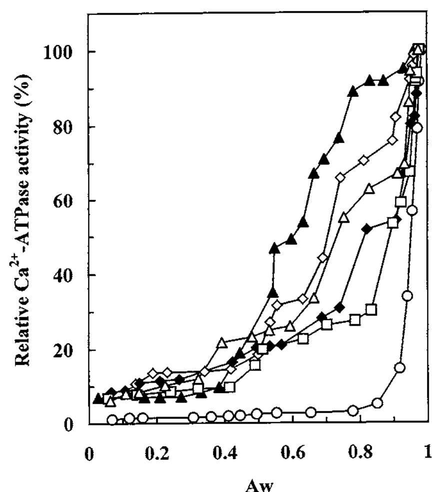


**Figure 4.** Correlation between relative $Ca^{2+}$-ATPase activity (percent) and water activity ($A_w$) of lizard fish myofibrillar protein during the dehydration process: (○) control; (▲) sodium glutamate; (◆) swordtip squid; (◇) Japanese flying squid; (□) bigfin reef squid; (△) golden cuttlefish.

of Mf structure by SPH constituents during silica gel-based dehydration. Analyses of the state of water and the mode of $Ca^{2+}$-ATPase inactivation during dehydration are useful for obtaining a good understanding of the structural alteration and denaturation of the protein. The water sorption isotherm is an extremely important parameter to predict changes in food stability.

In our study, the desorption isotherm curves for the control and for the Mf containing SPH were more or less similar to those of atka mackerel meat (*13*) and white croaker myofibrils (*15*). The $M_2/M_1$ ratio of the Mf ranged from 1.81 to 2.02, which corresponds well to that of 1.5−2.0 for the fibrous protein such as collagen and gelatin (*32*). Comparison among $M_1$, $M_2$, and $S$ values from the desorption isotherm of the Mf containing Na-glu and those containing SPH show superiority over those of the control. Similarly, in comparison with the control, the DSC analysis showed a lower enthalpy change and a higher amount of unfrozen water in the Mf with SPH and that containing Na-glu, suggesting that the SPH constituents and the Na-glu provided a stabilization effect on the Mf molecular structure by increasing the amount of bound water. This result is in accordance with the findings of Park (*35*), who stated in a DSC study that protein additives, which reduce the enthalpy of endothermic peaks, appear to delay denaturation of fish protein. The stabilization mechanism of protein structures and many of their reactions are maintained to a large extent by covalent disulfide bonds and by the non-covalent interactions of the side-chain groups such as hydrogen bonds, electrostatic interactions, hydrophobic bonds between nonpolar residues, and hydration of polar residues (*10*, *11*). Furthermore, hydrophilic amino acids play important roles in the protein stabilization because these amino acids are very polar and are known to have large water-constraining denaturation-inhibiting effects (*15*, *36*, *37*). In the present study, the analysis of the amino acid composition of the peptides indicated that the hydrophilic amino acids (Glx, Asx, Arg, and Ser) are the major components in SPH, suggesting that the interaction between the hydrated water molecules of the Mf and the functional groups of the hydrophilic peptides produced bound water in the Mf that presumably made a structural alteration to the Mf. Nemethy and Scheraga (*11*) contributed to this area of research when they reported that the polar groups of amino acids strongly interact with water and interfere with the stabilization of water molecules. Recently, Zhang et al. (*22*) reported that Antarctic krill protein hydrolysate consisted of largely hydrophilic amino acids that exhibited a stabilization effect on Mf structure during the dehydration process. Conversely, the nonpolar groups can neither accept nor donate hydrogen bonds, so the water molecules at the surface of the cavity occupied by the nonpolar groups cannot make hydrogen bonds with other molecules in their usual fashion. The water molecules tend to straddle the nonpolar residues such that two or three of their tetrahedral directions are tangential to its surface, which permit them to form hydrogen bonds (*36*). However, the nonpolar amino acids of the SPH may exert a slight effect on the stabilization mechanism of the Mf structure. Thus, it may be concluded that the SPH constituents, particularly their polar groups of peptides, produced bound water through stabilization of the hydrated water in Mf and that water, in turn, maintained the structural stability of the Mf during dehydration.

Dehydration induces the destruction of hydrogen bonds or hydrophobic bonds and disturbs the intramolecular hydrogen bonds, which causes structural rearrangement and destruction of protein morphology (*12*, *38*). In the present study, the inhibitory effect of the SPH against the dehydration-induced destruction of the Mf was evaluated by determining $A_w$ with respect to $Ca^{2+}$-ATPase activity at different levels of moisture content and comparing the values with those from Na-glu and from the control. The overall features of $Ca^{2+}$-ATPase activity and $A_w$ of the Mf suggest that the SPH suppresses the dehydration-induced denaturation of the Mf at all moisture levels. This suppression is an indication that the peptides of the SPH inhibited the hydrophobic forces in Mf and stabilized the Mf molecular structure by preventing any changes of hydration and of hydrogen bonds surrounding the Mf. Niwa et al. (*39*) reported that the protein of white muscle fishes easily deteriorates due to the exposure of hydrophobic amino acid residues of myosin molecules and that the increment of the fluorometric intensity was suppressed by the addition of Na-glu. We previously reported (*21*, *22*) that protein hydrolysates consisting largely of hydrophilic residues inhibit the dehydration-induced denaturation of Mf by preventing any changes in hydration and hydrogen bonds through their interaction with water molecules in the hydration sphere of Mf. This kind of effect would result in structural rearrangement of water molecules in the solution, which inhibit dehydration-induced denaturation of protein molecular structure. Hanafusa (*12*) reported that the interaction between water molecules and protein is responsible for dehydration-induced denaturation. Furthermore, Castonuovo et al. (*40*) reported that hydrophilic structure makers promote structuring of solvent conducive to increased solute−solvent hydrogen bonding and that the hydrophilic structure breakers act as denaturing agents of proteins. The main components of the SPH used in the present study were peptides; therefore, the mechanism underlying the hydration and stabilization of bound water in the Mf structure might be due to the hydrophilic side chain of the peptides, and hydration and stabilization led to suppression of the dehydration-induced denaturation of Mf.

The SPH exhibited smaller differences in the species-specific stabilization effect on the Mf structure. We speculated that the conformation of the peptides, their chain lengths, and the number of active groups exposed in the SPH might provide a varying qualitative effect on the stabilization mechanism of the Mf. However, to clarify the concentration-dependent protective effect of the SPH against dehydration and freeze-induced denaturation of Mf, studies in this area are now in progress, and the results will soon be published elsewhere.


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