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Note

Effect of Squid Protein Hydrolysate on Freeze-Induced Denaturation of Lizardfish (*Saurida wanieso*) Myofibrillar Protein

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In order to utilize low-cost squid effectively as a functional material for food processing and preservation, squid protein hydrolysate (SPH) was prepared from 4 species of squid by protease treatment. Peptides are the major components (84–88%) of SPH. The protective effect of 5% SPH (dry weight/wet weight) on the freeze-induced denaturation of lizardfish *Saurida wanieso* myofibrillar protein (Mf) was evaluated on the basis of Mf Ca-ATPase activity and the amount of unfrozen water in Mf, which was determined by differential scanning calorimetry (DSC); the effect was compared with that of sodium glutamate. Mf with SPH showed markedly higher Ca-ATPase activity than did Mf without SPH (control) during frozen storage. Mf with SPH had a higher amount of unfrozen water than the control. These findings suggest that SPH stabilized water molecules in the hydration sphere of Mf and thus, suppressed freeze-induced denaturation of Mf. The effect by SPH was less than that by sodium glutamate.

Keywords: squid protein hydrolysate, fish myofibrilllar protein, unfrozen water, Ca-ATPase, frozen storage

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. Protein sources with appropriate amino acid profiles and a method of modification are the most important factors in the preparation of functional foods (Hall & Ahmad, 1995). Enzymatic modifications have several advantages; they minimize nutrient loss and produce a decrease in peptide size, which improves the protein functionality (Sugiyama *et al*., 1991).

Myofibrillar proteins of fish are generally considered to be unstable because of microbial proliferation that occurs at ambient temperature due to the high moisture and nutrient contents. Frozen storage is used as the long-term preservation method, which creates an environment deleterious to the spoilage mechanisms of fish and fishery products. Nevertheless, the mechanisms are inevitably associated with some deterioration particularly in terms of water retention, protein solubility (Matsuda, 1973), viscosity and structural changes of actomyosin (Ohnishi *et al*., 1978), exposure of hydrophobic residues (Niwa *et al*., 1986) and ATPase inactivation (Arai *et al*., 1970). The folding and the structural stability of protein during drying and frozen storage are extensively controlled by addition of a variety of anti-denaturants such as sugars, amino acids, organic acids, phosphates, and polyalcohols (Matsumoto *et al*., 1985; Park & Lanier, 1987; Nozaki *et al.,* 1991). Currently, enzymatically degraded materials from various ingredients, such as fishery by-products and residues from the seafood processing industries, are considered as potential natural suppressors for freeze-induced denaturation and stabilizers of protein molecular structure (Darmanto *et al*., 1997; Zhang *et al*., 2002). However, functional protein from squid by protease treatment has not been reported yet for the purpose of food preservation and product development although squid contributes an important global source of protein, and its chemical constituents and nutritive value are worthy of mention.

Therefore, the present study was designed to prepare proteolytic functional food from squid and to investigate its effect on the state of water and freeze-induced denaturation of lizardfish myofibrillar protein. Lizardfish meat was used in this study because of its high utility for the production of surimi products in the fish processing industries in Nagasaki Prefecture, Japan.

Materials and Methods

Material Four species of squid, viz., Japanese flying squid, *Todarodes pacificus* (weight 330.7±78.0 g), bigfin reef squid, *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 the Nagasaki Fish Market, Japan and transported to our laboratory in a frozen condition.

Preparation of squid protein hydrolysate (SPH) SPH was prepared according to the previously reported method (Iwamoto *et al*., 1991) with a slight modification. Five kilograms of squid of each species was chopped, ground into minced and reconstituted in 2 volumes of distilled water of the materials. The mixture was heated at 90°C for 30 min to terminate the native enzymatic activity, then homogenized and adjusted to pH 8.0

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with 0.1 M NaOH. 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). The enzymatic activity was again terminated by increasing the temperature to 90°C for 30 min. The pH of the mixture was adjusted to 6.0 by adding malic acid and hydrolyzed further by exo-type protease derived from *Aspergillus oryzae* (Shin-Nihon Chemical Industries, Inc.), and the reaction was then terminated by the same method. The product was centrifuged at $3800\times g$ for 15 min and filtered. The filtrate was heated at 80°C for 10 min, and the lipid layer was removed from the surface and subjected to ultrafiltration (Millipore, PK 30,000 NMWL, Minitan, Pellicon XL, Billerica, USA) for collection of materials having a molecular weight of <30,000. The sample was passed through a desalting panel (Model G3, Asahi Kasei, Inc., Kawasaki, Kanagawa) and spray-dried (Model GA32, Yamato Scientific, Inc., Tokyo). Finally, SPH was obtained in powder form.

Components, molecular weight distribution and amino acid composition of SPH Moisture, crude proteins, crude lipids, crude ash and sugar contents were measured by the method of heat drying (105°C), Kjeldahl (%N×6.25), Soxhlet, heating (550°C) and phenol-sulfuric acid, respectively. Salt content was measured using a salt analyzer (Model SAT-2A, Toa Denpa, Inc., Shinjuku, Tokyo). Molecular weight distribution of SPH was determined by gel filtration chromatography (Sephadex G-25 column, inner diameter 2.2 cm, length 60 cm). Absorbance of each fraction was determined at 220 nm. The molecular weight of SPH was calculated based on that of the standard compounds (Fig. 1). Amino acid analysis grade HCl (Nacalai Tesque, Inc., Kyoto) was used for hydrolysis reaction. The reaction proceeded at 110°C for 20 h. The product was then analyzed by an automatic amino acid analyzer (ALC 1000, Shimadzu Seisakusho Co., Kyoto).

Preparation of fish myofibrillar protein (Mf) Mf was prepared according to the method of Katoh *et al*. (1977) with slight modification (Nozaki *et al*., 1991). SPH or sodium glutamate (Na-glu) was added to Mf at a 5% level (dry weight of SPH or Na-glu/wet weight of pelleted Mf) and mechanically dispersed for 20 min at 5°C, and 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 micro-tube (inner diameter 8 mm, length 45 mm) and stored at −25°C. For the control, neither SPH nor Na-glu was added to Mf.

Measurement of Mf Ca-ATPase activity Mf Ca-ATPase activity was measured according to the method of Arai *et al*. (1970) and Zhang *et al*. (2002) in order to determine the protective effect of SPH and Na-glu against freeze-induced denaturation of Mf. The Ca-ATPase activity of the frozen Mf was expressed as the ratio of the specific activity before freezing (relative %).

Measurement of unfrozen water by differential scanning thermal analysis The amount of unfrozen water in Mf was measured according to the method of Wakamatu and Sato (1979) and Zhang *et al*. (2002) using a differential scanning calorimeter (DSC) (model SSC-5200, Seiko Electronic Industry, Inc., Tokyo), to assess changes in the state of water in Mf after addition of SPH or Na-glu and the stability of Mf during long-term frozen storage. The results were compared by means of one-way analysis of variance using Excel Statistical Analysis 2000 (Shakai-Joho Service Co., Tokyo).

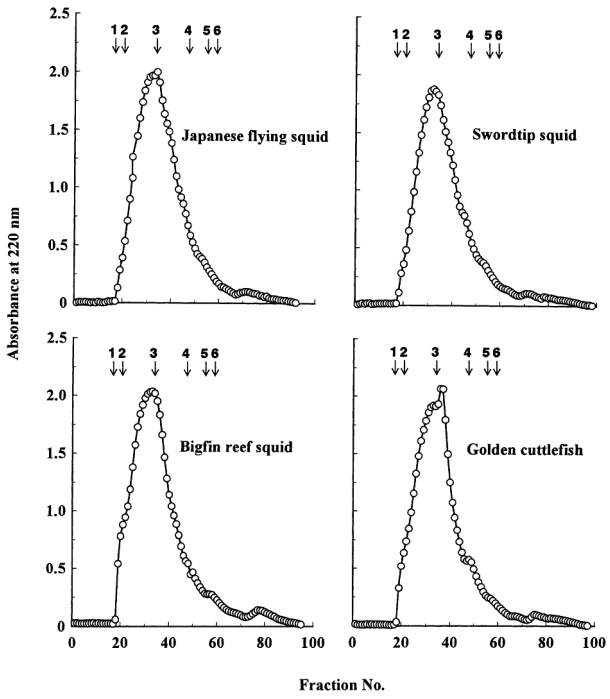

Fig. 1. Gel chromatograms of squid protein hydrolysate on Sephadex G-25 column. Elution: 30 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl. Flow rate: 30 ml/h. Fraction volume: 5 ml/tube. The numbers denote the elution position of the following standard compounds: (1) cytochrome C (M.W. 12,500); (2) aprotinin (M.W. 6511); (3) bacitracin (M.W. 1411); (4) glutathione, reduced (M.W. 307); (5) glycyl-L-phenylalanine (M.W. 222); (6) L-phenylalanine (M.W. 165).

Results

Properties of SPH The major components of SPH were peptides (84–88% of dry matter). The amino acid composition of SPH is presented in Table 1. Glx (glutamic acid+glutamine) was the most abundant in SPH, which accounted for 11.17, 10.41, 10.23, and 9.99 (g/100 g of dry matter) in SPH of Japanese fly-

Table 1. Amino acid composition and protein content of squid protein hydrolysate. (g/100 g of dry 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	1.90	2.16	1.89	1.84
Cys	0.07	0.20	0.15	0.13
Protein	87.67	87.72	87.92	84.28

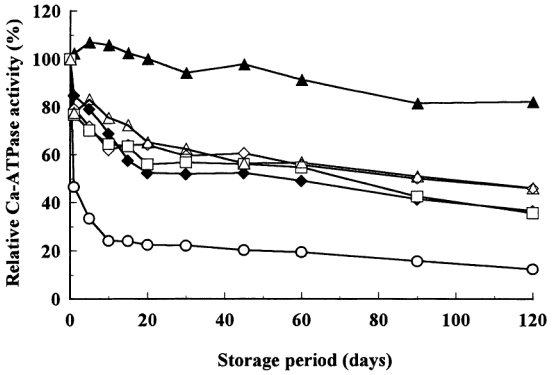

Fig. 2. Changes in Ca-ATPase activity of lizardfish myofibrillar protein in presence of 5% (dry matter) of squid protein hydrolysate or sodium glutamate during frozen storage at −25°C. Myofibrillar protein without SPH or sodium glutamate is considered as the control. (○), Control; (▲), Sodium glutamate; (◆), Swordtip squid; (◇), Japanese flying squid; (□), Bigfin reef squid; (△), Golden cuttlefish.

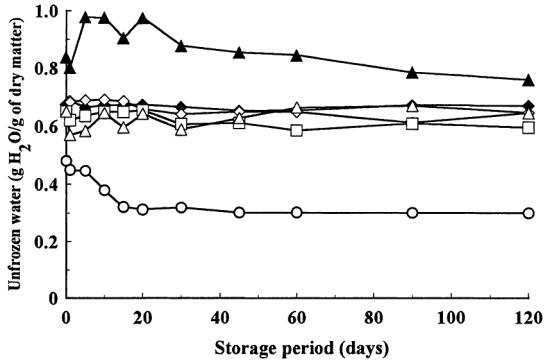

Fig. 3. Amount of unfrozen water (g H_2O/g dry matter) in lizardfish myofibrillar protein with 5% (dry matter) of squid protein hydrolysate or sodium glutamate during frozen storage. Symbols are the same as in Fig. 2.

ing squid, bigfin reef squid, swordtip squid and golden cuttlefish, respectively. Asx (aspartic acid+asparagine) found in the SPH accounted for 6–7 g/100 g of dry matter. Arginine and alanine and serine were also found to be abundant.

Figure 1 shows the elution pattern of Sephadex G-25 gel filtration of SPH. The large peaks corresponded to fractions ranging from 1400 to 300 daltons, suggesting that peptides were the major components of SPH.

Mf Ca-ATPase activity Figure 2 shows the changes in Mf Ca-ATPase activity during 120 days of frozen storage. In the control, the residual activity dropped quickly to about 24% of the initial value in the first 10 days of storage then decreased gradually up to 120 days, indicating that the denaturation process proceeded in two steps. In contrast, the activity remained higher at 72–64% in SPH added Mf up to 15 days of storage, and decreased gradually, showing a two-step denaturation pattern as observed in the control. The activity of Mf with Na-glu was almost unchanged up to 20 days of storage, then decreased very slowly, indicating a one-step denaturation pattern. The findings revealed that SPH suppressed the freeze-induced denaturation of Mf, and SPH from golden cuttlefish exhibited the highest suppression effect followed in order by SPH of swordtip squid, Japanese flying squid, and bigfin reef squid. The suppression effect of SPH was less than that of Na-glu.

Unfrozen water The amount of unfrozen water in Mf increased significantly ($p<0.01$) compared to the control after the addition of Na-glu and SPH, irrespective of species differences (Fig. 3). The amount of unfrozen water in the control decreased to a level of 67% from the initial value (0.482 g H_2O/g of dry matter) within 15 days of frozen storage, and then gradually decreased (0.312–0.301 g H_2O/g of dry matter) up to 120 days. Mf in presence of SPH of swordtip squid was contained the unfrozen water accounted for 0.686–0.675 g H_2O/g of dry matter followed in order by that of Japanese flying squid (0.683–0.651 g H_2O/g of dry matter), bigfin reef squid (0.651–0.600 g H_2O/g of dry matter), and golden cuttlefish (0.650–0.590 g H_2O/g of dry matter). No significant differences were observed among the unfrozen water in Mf with SPH of 4 squid species throughout the frozen storage period. Mf with Na-glu contained a significantly ($p<0.01$) higher amount of unfrozen water that did Mf with SPH, accounting for 0.978–0.752 g H_2O/g of dry matter. Although the effect of SPH on the stabilization of the amount of unfrozen water in Mf was lower than Na-glu, the findings revealed that SPH constructed bound water in the Mf structure and stabilized the Mf molecular structure during the frozen storage.

Discussion

In the present study, we attempted to elucidate the effect of squid protein hydrolysate (SPH), as a natural suppressor, on the freeze-induced denaturation and on the state of water of the lizardfish myofibrillar protein by determining Mf Ca-ATPase inactivation and unfrozen water. As shown in Fig. 2, the Mf Ca-ATPase activity of the control decreased markedly on the second day of frozen storage and then gradually afterward for 120 days, indicating a time-dependent biphasic denaturation pattern of Mf, which was closely similar to the freeze-denaturation pattern of carp (Matsumoto *et al.,* 1985), sardine and chub mackerel (Fukuda *et al.,* 1984). Although Mf with SPH showed a biphasic time-dependent denaturation pattern, it proceeded very slowly, which suggested the suppressive effect of SPH against the freeze-induced denaturation of Mf. This finding corresponds well with the reports of Noguchi *et al*. (1975) and Zhang *et al*. (2002) in which these authors postulated that peptides and protein hydrolysates might have a preventive effect against freeze-induced denaturation of fish muscle protein. The Mf with Na-glu showed a one-step denaturation pattern, revealing that Na-glu strongly suppressed the freeze-induced denaturation of Mf. This finding is in agreement with the report of Nozaki (1987), where he stated that sodium asparaginate and Na-glu caused a monophasic denaturation of Mf during 150 days of frozen storage. The reasons for the lower protective effect of SPH than Na-glu might be due to the complex interactions of the various peptides (hydrophobic and hydrophilic) and other components of SPH, which to some extent hindered the anti-denaturation effect of SPH on Mf during frozen storage.

Despite the numerous reasons responsible for the denaturation of fish proteins during frozen storage, hydrophobic interactions proceeded by the formation of ice-crystals and the destruction of hydrate layers surrounding polar residues are believed to be prime causes for freeze-induced denaturation of fish protein

(Hanafusa, 1973; Nemethy & Scheraga, 1962). The stabilization of protein structure and many protein's reactions are maintained to a large extent by covalent disulfide bonds and by the non-covalent interactions of side-chain groups such as hydrogen bonds, electrostatic interactions, hydrophobic bonds between non-polar residues, and hydration of polar residues (Kauzman, 1959; Nemethy & Scheraga, 1962). The suppressive effect of SPH demonstrated in this study was probably because of interaction between various reactive side chains of peptides and hydrated water molecules produced by non-polar residues in Mf, which made a structural alteration of protein molecules, and this structural rearrangement prevented ice-crystal formation around the polar residues of protein. Thus, in SPH-added Mf, the formation of amorphous hydrate water layer by hydrophobic residues was suppressed by the polar side-chain of peptides in particular, and a certain amount of bound water was retained surrounding the protein, resulting in the suppression of protein denaturation. Niwa *et al*. (1986) 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 intensity of denaturation was suppressed by the addition of Na-glu.

After addition of SPH, the amount of unfrozen water increased in Mf to a greater extent (Fig. 3), showing strong evidence of the stabilization of water molecules by the hydrophilic peptides that occurred in the hydration sphere of Mf. Consequently, the interactions between the reactive side-chain of peptides and functional groups of the polypeptide chain of Mf probably exerted a concerted effect on the bound water construction in Mf molecular structure. These findings were supported by the previously reported results (Noguchi & Matsumoto, 1971; Nozaki, 1987; Nozaki *et al*., 1991), where hydrophilicity of amino acids is thought to be involved in the structural stabilization mechanism of protein molecules. However, the water-binding properties of SPH itself in bulk water were not measured by DSC in the present study, which limits to some extent the clarification of the functional mechanism of SPH in the structural stabilization of Mf. The role of SPH behind the state of water and structural stabilization of protein, however, has not been adequately elucidated in the present report, and remains to be addressed in future.

Thus, in light of the previous discussion it can be concluded that the peptides in SPH have potential to stabilize hydrated water molecules surrounding the Mf structure, so that structural alteration of protein molecules has the capacity to suppress freeze-induced denaturation. Our future attention will be devoted to purifying the individual peptides of SPH having a high protective effect on protein stabilization.

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