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Effect of Enzymatic Fish Protein Hydrolysate from Fish Scrap on the State of Water and Denaturation of Lizard Fish (*Saurida wanieso*) Myofibrils during Dehydration

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Fish protein hydrolysate (FPH) was prepared from scraps of 5 marine species, in order to utilize by-products effectively by protease treatment. Protein was the major component of the FPH ranging 82–86%. The effects of 5% FPH (dried weight/wet weight) on the state of water and the denaturation of lizard fish *Saurida wanieso* myofibrils were evaluated by desorption isotherm curves, Ca-ATPase activity, and differential scanning calorimetry (DSC) during dehydration. The myofibrils with FPH showed a decreased water activity (Aw), while they exhibited significantly higher Ca-ATPase activity compared to myofibrils without FPH (control). The myofibrils with FPH had higher amount of unfrozen water than the control. These results suggest that the FPH suppressed dehydration-induced denaturation, which seems to be attributable to the stabilization of the hydrated water surrounding the myofibrils.

Keywords: fish protein hydrolysate, Ca-ATPase activity, water activity, unfrozen water, lizard fish, myofibrils, dehydration

Fishery wastes have been converted by proteolytic hydrolysis into a more marketable and functional form, which is called fish protein hydrolysate, FPH (Hall & Ahmad, 1992; Hoyle & Merritt, 1994; Barzana & Garcia-Garbaly, 1994; Liceaga-Gesualdo & Li-Chan, 1999). Recently, the functional properties of enzymatic hydrolysate from various foods, such as fishery products (Suetsuna *et al*., 1988; Iwamoto *et al*., 1991; Sugiyama *et al*., 1991; Yokoyama *et al*., 1992; Liceaga-Gesualdo & Li-Chan, 1999; Hall & Ahmad, 1992) and residue from seafood processing (Miyake, 1982; Murata *et al*., 1991), have been studied. Further, FPH has the potential to meet the expanding demand for natural protein sources, and it has already been used to enrich food products as a food ingredient, and also as a nutritional supplement for human consumption (Whitaker, 1986; Rutman & Heimlich, 1974; Ballester *et al*., 1977).

Dehydration is widely used as a method for fish and fishery products preservation. The quality of a fishery product generally deteriorates due to the decrease of water holding capacity and solubility of proteins during dehydration (Migita *et al*., 1956; Suzuki, 1971), because water is closely associated with maintenance of the structure and function of proteins (Kauzman, 1959; Nemethy & Scheraga, 1962; Kavanau, 1965). To prevent such undesirable changes, the addition of sugars, amino acids, organic acids, phosphates and functional proteins has been recommended by many food scientists (Hall & Ahmad, 1992, Hanafusa, 1973; Akiba, 1973; Matsuda, 1973; Nakano & Yasui, 1976; Nozaki *et al*., 1993).

In this study, we evaluated the effects of fish protein hydrolysate prepared from scraps of 5 marine species on the state of water and denaturation of lizard fish myofibrils during dehydration. Lizard fish meat has been used as a material to prepare high quality fish jelly products in fish processing industries in Nagasaki. Therefore, we used this species in our study.

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Materials and Methods

Materials Scraps of 5 marine species, viz., horse mackerel (*Trachurus japonicus*, body weight, 263.0±12.8 g), chub mackerel (*Scomber japonicus*, body weight, 561.0±51.5 g), white croaker (*Argyrosomus argentatus*, body weight, 163.2±13.5 g), sardine (*Sardinops melanostictus*, body weight, 78.2±13.1 g), and flying fish (*Cypselurus heterurus*, body weight, 117.8±9.2 g), were obtained from the Fish Processing Factory, Faculty of Fisheries, Nagasaki University, Japan.

Preparation of fish protein hydrolysate (FPH) Fish protein hydrolysate was prepared according to the method of Iwamoto *et al*. (1991) with a slight modification. A double volume of distilled water was added to the fish scraps, and the mixture was heated at 90°C and thoroughly homogenized (700 rpm, MAZELA-Z, EYELA, Tokyo) for 30 min. After homogenization, pH was adjusted to 8.0 by adding 1 N NaOH. The endo-type protease derived from *Bacillus subtilis* (Shin-Nihon Chemical Industries, Inc., Anjo, Aichi) was added at a level of 0.1% (w/w) to the sample. Enzymatic hydrolysis was performed at 60°C for 2 h, then reaction was terminated by heating at 90°C for 30 min. The pH was adjusted to 6.0 by adding malic acid as a powder form. Exo-type protease derived from *Aspergillus oryzae* (Shin-Nihon Chemical Industries, Inc) was added at a level of 0.1% (w/w) to the sample. Hydrolysis was performed at 60°C for 2 h, and the reaction was terminated by heating at 90°C for 30 min. The samples were then centrifuged at 3800×*g* for 15 min. The supernatant was filtered to remove buoyant particles; and the filtrate was heated at 80°C for 10 min; then the lipid layer was removed from the surface. Again pH was adjusted to 7.0 with 1 N NaOH. The obtained sample was ultrafiltrated through a

Millipore membrane (PK 30,000 NMWL, Minitan, Pellicon XL) to collect materials with a molecular weight of less than 30,000 daltons, and the sample was desalted with a desalting panel (Micro Acilyzer, model G3; Asahi Kasei Inc., Kawasaki, Kanagawa) for 24 h. The sample was subjected to spray drying (model IGA 32; Yamato Science Inc., Tokyo). Finally, the FPH was obtained as a powder form.

Proximate composition Moisture, crude protein, crude lipid, crude ash contents, and sugar substances in FPH were determined by heat drying (105°C), Kjeldahl, Soxhlet, ashing in a furnace (550°C), and phenol-sulfuric acid methods, respectively. NaCl content was measured using a salt analyzer (model SAT-2A, Toa Denpa Inc., Tokyo).

Amino acid analysis Amino acid analysis-grade hydrochloric acid (Nacalai Tesque Inc., Kyoto) was used for the hydrolysis of the FPH. The hydrolysis was allowed to proceed at 110°C for 20 h. The amino acid profiles of the FPH were analyzed using an automatic amino analyzer (ALC-1000, Shimadzu Seisakusho Co., Kyoto).

Molecular weight distribution The molecular weight distribution of the FPH was determined using gel filtration chromatography on a Sephadex G-25 column (2.2 cm I.D.×60 cm). The absorbance at 220 nm was measured for each fraction, and the average molecular weight was calculated and compared with that of the standard materials.

Preparation of myofibrils The fish myofibrils were prepared according to the method of Katoh *et al.* (1977) with a slight modification. The dorsal muscles of lizard fish *Saurida wanieso* were chopped up, and then washed by stirring with five volumes of 0.1 M KCl-20 mM Tris-maleate buffer (pH 7.0). After addition of three volumes of the same buffer, the specimens were homogenized in a foam preventive type blender (model JM-H 131; Mitsubishi, Tokyo) at 10,000 rpm for 90 s. The homogenate was filtered through a nylon net (# 16) in order to remove connective tissues. Subsequently, the specimens with 20% Triton X-100 solution (adjusted to a final concentration of 1%) were left to stand for 30 min, and then centrifuged at 750×g for 10 min. The sediment was mixed with five volumes of the same buffer, stirred, centrifuged, and washed. To remove as much as possible the buffer action derived from KCl in the sediment, the sediment was mixed with five volumes of cold distilled water, washed by stirring, and then centrifuged at 3800×g for 10 min. The supernatant was discarded. To remove excess water, centrifugation was performed again at 27,000×g for 20 min, and the obtained

Table 1. Chemical composition of the fish protein hydrolysate. (g/100 g of dried matter)

Composition	Horse mackerel	Chub mackerel	White croaker	Sardine	Flying fish
Crude protein (%)	85.83	82.74	85.11	83.05	82.32
±S.D.*	0.15	0.11	0.05	0.14	0.24
Crude ash (%)	8.70	9.37	7.69	8.67	9.16
±S.D.	0.03	0.04	0.05	0.01	0.03
Crude lipid (%)	0.23	0.17	0.26	0.17	0.18
±S.D.	0.07	0.01	0.01	0.01	0.01
Sugar substance (%)	3.02	3.08	3.2	4.18	3.56
±S.D.	0.01	0.02	0.03	0.01	0.03
NaCl	<0.01	<0.01	<0.01	<0.01	<0.01

*Standard deviation.

Table 2. Amino acid composition of the fish protein hydrolysate. (g/100 g of dried matter)

Amino acid	Horse mackerel	Chub mackerel	White croaker	Sardine	Flying fish
Acidic amino acids					
Aspartic acid+Asparagine	7.18	6.90	7.42	6.98	7.31
Glutamic acid+Glutamine	11.35	10.40	12.31	10.41	11.19
Basic amino acids					
Arginine	4.37	4.45	4.47	3.97	4.16
Lysine	5.75	5.61	6.06	5.95	5.65
Histidine	3.29	4.21	1.79	4.27	3.59
Neutral amino acids					
Glycine	0.21	0.21	0.22	0.21	0.19
Alanine	5.61	5.17	6.08	5.07	5.18
Serine	9.04	8.66	9.56	8.39	8.56
Threonine	2.93	3.08	3.31	3.04	3.03
Valine	1.94	2.24	2.55	2.42	2.14
Leucine	4.95	5.00	5.23	5.21	4.90
Isoleucine	1.48	1.67	1.91	1.79	1.59
Phenylalanine	2.18	2.32	2.35	2.27	2.69
Tyrosine	1.54	1.80	1.65	1.78	1.68
Proline	3.71	3.61	4.26	3.17	3.67
Taurine	1.79	1.64	2.14	1.48	2.07
Methionine	2.12	2.04	2.20	2.09	1.92
Cystine	0.06	0.06	0.09	0.10	0.07

sediment was used as myofibrils. All procedures were conducted at 5°C. The general components of myofibrils were as follows: water 87.1%, crude protein 12.4%, crude lipid 0.04%, and crude ash 0.43%.

The myofibrils were placed in a mortar (Ishikawa type), and FPH (5 g as dried weight/100 g of myofibrils as wet weight) was added and mixed thoroughly for 20 min at 5°C after adjusting the pH to 7.0 with 0.01 N NaOH or 0.01 N HCl. The sample was put in a cellophane bag, embedded in silica gel and placed in a desiccator, and dehydrated at 5°C with occasional replacement of the silica gel. When the moisture content reached about 10%, further dehydration was performed in a desiccator under reduced pressure by a vacuum pump. Myofibrils without FPH were used as a control.

Measurement of water activity, moisture content, and analysis of desorption isotherm curves In the dehydration process, the water activity (Aw) of the hydrated myofibrils was measured by the indirect equilibrium vapor pressure method (Akiba *et al*., 1974) using an oil manometer at 20°C. The moisture content of the specimens was also measured by the atmospheric heating

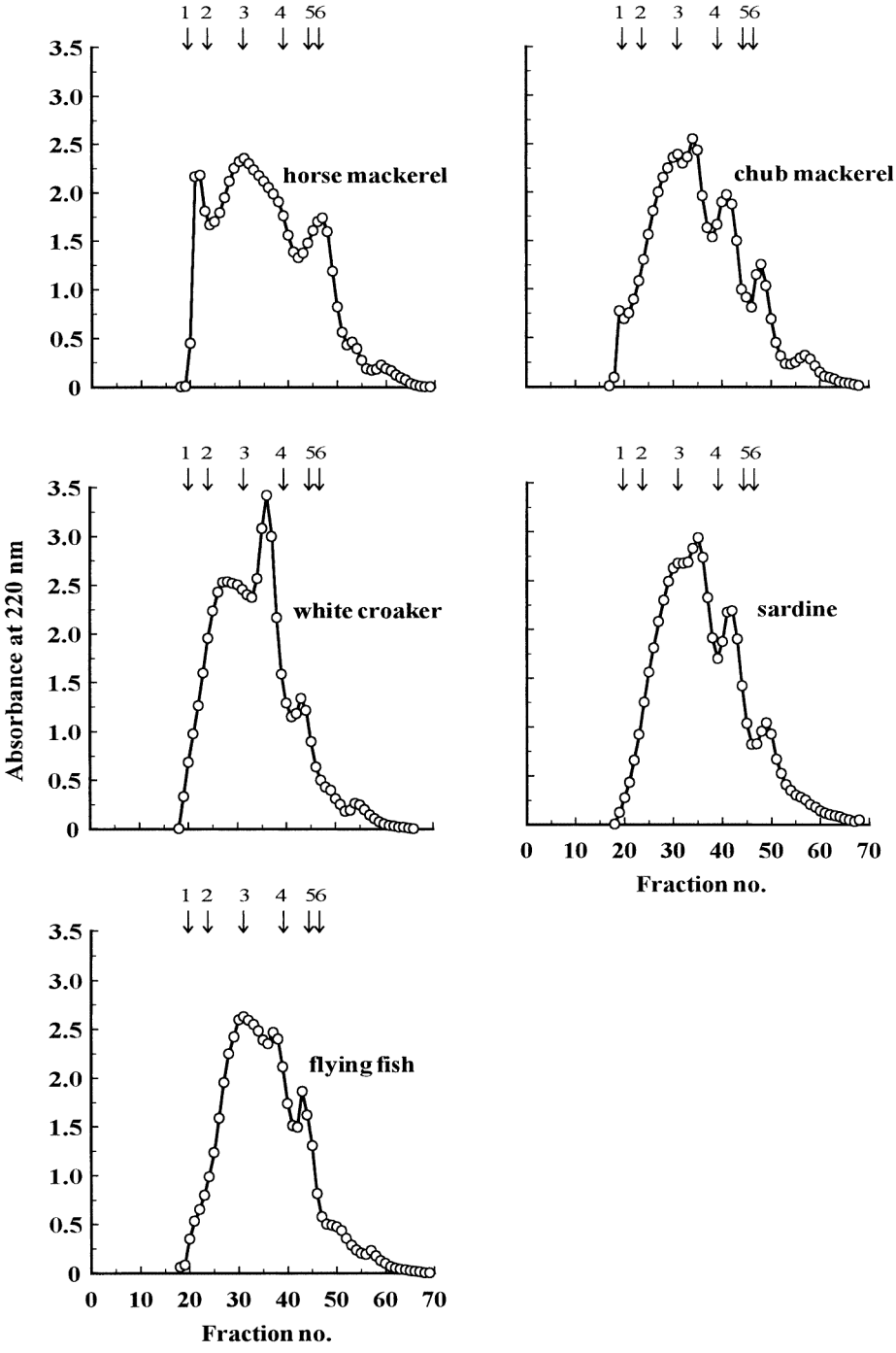

Fig. 1. Gel chromatograms of fish protein hydrolysate (FPH) on Sephadex G-25 column. Elution: 30 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl. Flow rate: 30 ml/h. Fraction volume: 5 ml/tube. The numbers denote elution positions of the following compounds: (1) cytochrome C (M.W. 12,500); (2) aprotinin (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).

method at 105°C for 24 h. Desorption isotherm curves were produced by plotting moisture content against Aw at 20°C. The inflection point (M_1) on the desorption isotherm curve was obtained by Brunauer-Emmet-Teller analysis (Brunauer *et al.*, 1938), and the moisture content at this point was defined as the amount of monolayer sorbed water. The inflection point of Aw on the desorption isotherm curves, corresponding to the minimal point on the moisture content/Aw-Aw curves, was defined as M_2 according to the report of Bull (1944), and the moisture content at this point was regarded as the amount of multilayer sorbed water. The sorption surface area of the myofibril specimens (S) was calculated by the following equation:

$$S=M_1\times Sw\times N/(Mw\times 10^3),$$

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

Measurement of unfrozen water by differential scanning thermal analysis The amount of unfrozen water was determined according to the method of Wakamatsu and Sato (1979) by a differential scanning calorimeter (DSC, model SSC-5200, Seiko Electronics Inc., Tokyo). The heat of melting of ice from distilled water (5–25 mg) was determined initially in order to establish a linear relationship between the amount of pure water and the heat of melting. The heat for ice melting obtained from pure water was 333.271 mJ/mg, which was close to the known value of 333.046 mJ/mg for pure water (Wakamatsu & Sato, 1979). A sample of about 20 mg was placed in a tightly sealed aluminum container and weighed accurately. Twenty milligrams of Al_2O_3 sealed in another aluminum container was used as reference. The heat of melting was measured from −40°C to 25°C with an increasing rate of 1.0°C/min. It was assumed that the heat of melting was due to the free water in the sample. The total amount of water in the sample was determined by the heat drying method (105°C) at atmospheric pressure.

Measurement of myofibrillar Ca-ATPase activity The dehydrated myofibrils were mixed with 30 volumes of 0.1 M KCl-20 mM Tris-maleate buffer (pH 7.0) and left overnight at 5°C for the restoration of water; homogenized (Nichionirika Kikai Seisakusho Histocolon NS-560, Chiba, Japan) at 1000 rpm; and centrifuged at 750×g for 10 min. The sediment was mixed with the same buffer, washed by stirring, and centrifuged at 750×g for 10 min; this step was repeated twice. The obtained sample was suspended in the same buffer. Myofibrillar Ca-ATPase activity was measured in the reaction medium of 100 mM KCl and 5 mM $CaCl_2$, 25 mM Tris-maleate buffer (pH 7.0), 1 mM adenosine triphosphate (ATP), and 0.2–0.4 mg/ml myofibrils at 25°C. The reaction was terminated by adding 30% trichloroacetic acid solution at a final concentration of 5%, and free inorganic phosphate was measured by colorimetry (Katoh *et al.*, 1977). The protein concentration was measured by the biuret method (Gornall *et al.*, 1949) using bovine serum albumin (fraction V) as a standard, and was corrected by the Kjeldahl method.

Results

Chemical properties of FPH The chemical composition of FPH is presented in Table 1. The crude protein was the major component, accounting for 85.83%, 82.74%, 85.11%, 83.05%, and 82.32% in the FPH of horse mackerel, chub mackerel, white croaker, sardine, and flying fish, respectively. The crude lipid, crude ash, and sugar contents ranged 0.17–0.26%, 7.69–9.37%, and 3.02–4.18%, respectively, while trace amount of sodium chloride (NaCl) was also detected.

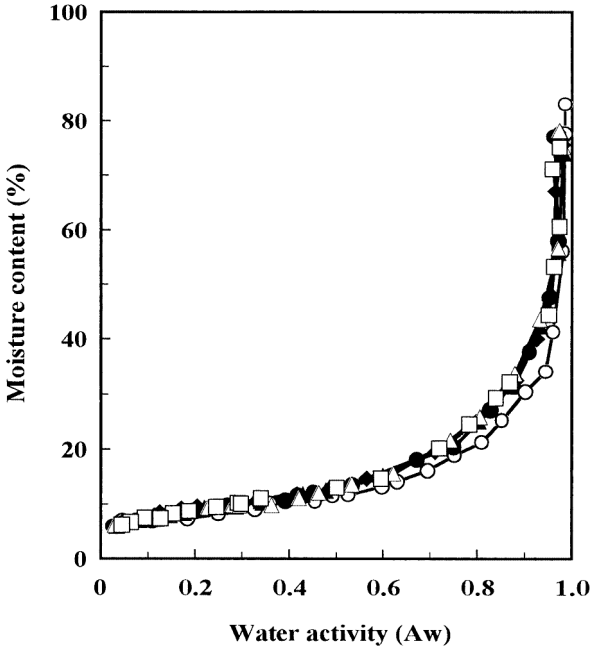

Fig. 2. Effect of fish protein hydrolysate (FPH) on the desorption isotherms of lizard fish myofibrils at 20°C during the dehydration process. FPH added was 5% (dried matter) to 100 g of myofibrils. ○, control; ●, horse mackerel; ▲, chub mackerel; ◆, white croaker; △, sardine; □, flying fish.

The amino acid composition of the FPH is summarized in Table 2. Glutamic acid+glutamine (acidic amino acid) was most abundant, accounting for 11.35%, 10.40%, 12.31%, 10.41%, and 11.19% (g/100 g dried matter) in the FPH of horse mackerel, chub mackerel, white croaker, sardine, and flying fish, respectively. Arginine and lysine (basic amino acid), and serine, alanine, and leucine (neutral amino acid) were also found in abundance.

Figure 1 shows the elution patterns of Sephadex G-25 gel filtration chromatography of the FPH. Many peaks corresponding to molecular weights of less than 12,000 daltons were observed. Large peaks had molecular weight between 1400 and 300 daltons, indicating that FPH was a mixture of various peptides.

Desorption isotherm curves of myofibrils The desorption isotherm curves of myofibrils with and without FPH (the control) are shown in Fig. 2. The Aw (0.4–0.9) of myofibrils with FPH was lower than that of control. The myofibrils with the FPH of flying fish had slightly lower Aw among the FPH. Similar Aw values were observed in the myofibrils with the FPH of sardine, white croaker, horse mackerel and chub mackerel during the dehydration process.

The monolayer (M_1) and multilayer (M_2) water, and sorption surface area (S) of the myofibrils were calculated from the desorption isotherm curves at 20°C (Table 3). The myofibrils with the FPH had higher M_1 and M_2 values than the control; those myofibrils with the FPH had higher sorption surface areas (0.30–0.32) compared to the control (0.27).

Measurement of unfrozen water The amount of unfrozen water in myofibrils with FPH and in the control is shown in Fig. 3; the amount increased in myofibrils with the FPH in comparison with the control (0.349 gH_2O/g dried matter). The myofibrils with the FPH of flying fish had the largest amount of unfrozen water (0.407 g H_2O/ g dried matter) followed in order by white croaker (0.393 g H_2O/g dried matter), chub mackerel (0.383 g H_2O/g dried matter), sardine (0.376 g H_2O/g dried matter), and horse mackerel (0.363 g H_2O/g dried matter). These results indicate that FPH has an suppressing effect on the denaturation of myofibrils, and stabilizes the hydrated water surrounding the myofibrils.

Effect of FPH on the denaturation of myofibrils during dehydration The effect of the FPH was evaluated by determining the changes of myofibillar Ca-ATPase activity during dehydration. The relative Ca-ATPase activity was plotted against water activity as shown in Fig. 4. About 90% of Ca-ATPase activity of the control was reduced at Aw 0.85, which is the domain of the capillary condensation, and gradually fell below the capillary condensation domain. The myofibrils with the FPH showed significantly higher Ca-ATPase activity than the control at the same Aw level. The Ca-ATPase activity gradually decreased with decreasing water activity, and the myofibrils with the FPH of flying fish showed the lowest decreasing tendency among the FPH. On the other hand, myofibrils with the FPH of horse mackerel, sardine, and chub mackerel showed more or less similar results. These results suggest there is a suppressive effect of FPH on the denaturation of myofibrillar protein during the dehydration process.

Discussion

Moisture content, unfrozen water, and water activity of food-

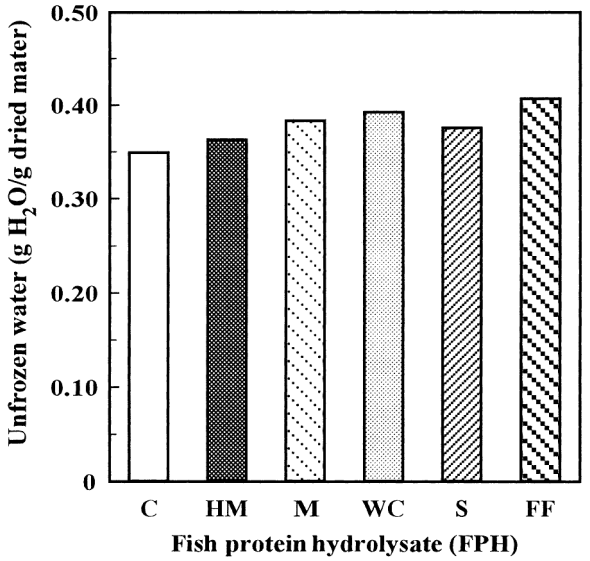

Fig. 3. Amount of unfrozen water (g H_2O/g dried matter) in lizard fish myofibrils with fish protein hydrolysate (FPH) during dehydration. FPH added was 5% (dried matter) to 100 g of myofibrils. C, control; HM, horse mackerel; M, chub mackerel; WC, white croaker; S, sardine; FF, flying fish.

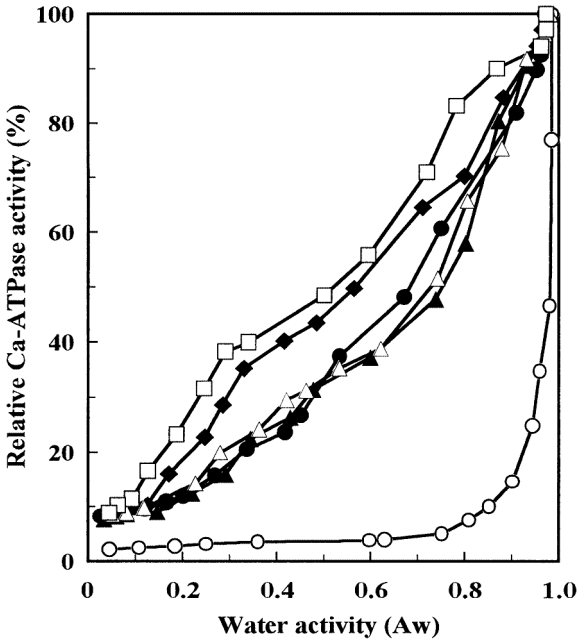

Fig. 4. Correlation between relative Ca-ATPase activity (%) and water activity (Aw) of lizard fish myofibrils during dehydration. Fish protein hydrolysate (FPH) added was 5% (dried matter) to 100 g of myofibrils. Symbols are the same as those in Fig. 2.

Table 3. Amount of monolayer and multilayer water, sorption surface area, and remaining Ca-ATPase activity at the inflection points of myofibrils with 5% fish protein hydrolysate.

System	Monolayer water[a]		Aw_1[b]	Aa_1[c]	Multilayer water[d]		Aw_2[e]	Aa_2[f]	M_2/M_1	S[g]
	M_1[h]	Md_1[i]			M_2[h]	Md_2[i]				
Control	7.27	0.078	0.151	2.83	13.32	0.154	0.613	3.86	1.83	0.27
Horse mackerel	7.80	0.085	0.135	9.67	13.91	0.162	0.541	11.9	1.78	0.30
Chub mackerel	7.96	0.087	0.127	8.62	14.22	0.166	0.561	15.36	1.79	0.30
White croaker	8.05	0.088	0.103	9.79	15.25	0.180	0.595	19.9	1.89	0.31
Sardine	7.80	0.085	0.108	10.24	14.60	0.171	0.579	22.73	1.87	0.30
Flying fish	8.31	0.091	0.188	11.35	13.95	0.162	0.559	13.21	1.68	0.32

[a] Estimated by B.E.T. analysis.
[b] Water activity of the sample at the M_1 point.
[c] Remaining myofibril relative Ca-ATPase activity (%) of the sample at the M_1 point.
[d] Estimated by Bull's analysis.
[e] Water activity of the sample at the M_2 point.
[f] Remaining myofibril relative Ca-ATPase activity (%) of the sample at the M_2 point.
[g] Sorption surface area (m^2/mg) of sample.
[h] Moisture content (g/100g of sample).
[i] Moisture content (g/100g of dried matter).

stuffs have been determined by many scientists in order to improve food quality. In the present study, FPH prepared from the scraps of five marine species were compared and their inhibitory effects determined against denaturation of fish myofibrils during dehydration. The desorption isotherm patterns of myofibrils with FPH and of the control were similar to those of Atka mackerel meat (Akiba, 1961), carp actomyosin (Nakano, 1979), water-washed meat (Akiba, 1973), white croaker myofibrils (Nozaki *et al*., 1991), and belonged to type B in their various protein substances, as classified by Shibasaki *et al*. (1967). In the present study, the M_1 and M_2 values of the myofibrils with the FPH were slightly higher than those of the control. These results correspond well with the findings of Nakano and Yasui (1976). The M_1 and M_2 ratio for the various kinds of proteins ranged from 1.5 to 2.0 (Bull, 1944), which correlates with our results (1.68–1.89).

The amount of unfrozen water was increased to a greater degree in myofibrils containing FPH than that in the control. This is probably due to the stabilization of water molecules in the myofibrils that occurs upon the addition of the FPH, suggesting that the water molecules were incorporated in myofibrils by the interaction of peptides in the FPH. Further, the stabilization of water in myofibrils might be due to the presence of amino acids such as glutamic acid+glutamine, aspartic acid+asparagine, arginine, and lysine in the FPH. These amino acids are known to possess large water-constraining effects and denaturation-inhibiting effects (Nozaki *et al*., 1991; Kanna *et al*., 1972; Matsuda, 1979) in myofibrils during dehydration. Further, the formation of intermolecular hydrogen bonds, the hydrophobic interactions between non-polar residues, and the hydration of polar residues play important roles in the maintenance of protein molecular structure and development of their function (Kauzman, 1959; Nemethy & Scheraga, 1962). Therefore, based on the previous discussion, we speculated that the FPH captured water molecules surrounding the myofibrils, which were excluded by the hydrophobic residues. Thus, the structure of the water molecules was changed through stabilization of the hydrate water on the surface of the protein molecules, which probably contributed to an increase in the amount of unfrozen water in myofibrils. Recently, Zhang *et al*. (2002a) reported that Antarctic krill protein hydrolysates stabilized water molecules in the myofibrils, which increase the amount of unfrozen water during dehydration.

The myofibrillar Ca-ATPase activity has often been used to determine the degree of denaturation of myofibrils. The inhibitory effects of FPH on the dehydration-induced denaturation of fish myofibrils were observed at different levels of Aw compared with that of remaining Ca-ATPase activity. Myofibrils with the FPH had higher Ca-ATPase activity at various Aw levels. Dehydration causes breaking of hydrogen bonds or hydrophobic bonds and intermolecular hydrogen bonds in myofibrils, which results in destruction of the protein morphology and rearrangement of their structure (Hanafusa, 1973). FPH is believed to inhibit the dehydration-induced denaturation of myofibrils by preventing any changes of hydration and of hydrogen bonds through their interaction with water molecules surrounding the myofibrils. Darmanto *et al*. (1997) and Zhang *et al*. (2002b) reported that protein hydrolysates could be used as suppressive additives against the denaturation of myofibrillar proteins during dehydration. Thus, the hydration and denaturation-inhibiting effects on myofibrils are mainly due to the peptides in FPH because the main components of the FPH used in the present study were peptides. Therefore, the FPH could be utilized as a functional food and an inhibitor of the myofibrillar protein denaturation during dehydration. The effects of other components such as sugars and impurities cannot be ignored. Thus, further studies are required for purification of individual components of FPH and to determine their amino acid composition in order to clarify the above facts more precisely in future.

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