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

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To utilize fishery waste products as functional food material, the fish protein hydrolysate (FPH) was prepared from fish scraps of three marine species by protease treatment. The added-concentration dependent effects of FPH (2.5–12.5%, dry weight/wet weight) on the state of water and denaturation of lizard fish myofibrils were evaluated by desorption isotherm curves, myofibrillar Ca-ATPase activity, and unfrozen water which was determined by differential scanning calorimetry during dehydration. The water activity (A_w) in myofibrils was distinctly decreased with the increasing concentration of FPH from 2.5% up to 10.0%, although the A_w added with 12.5% of FPH was almost equal to that with 10.0% of FPH. The amount of unfrozen water in myofibrils during dehydration was increased by the addition of FPH, and the greatest increase was found at 5.0–10.0%. The FPH suppressed the inactivation of myofibrillar Ca-ATPase activity during dehydration, and a larger effect of suppression was observed at 7.5% addition of FPH. These findings suggest that peptides produced in FPH functioned to stabilize the hydrate water surrounding the myofibrils and suppressed their dehydration-induced denaturation.

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

Generally, fish and fishery products are easily spoiled by the growth of microbes due to their high moisture content and nutritional quality. Therefore, drying is used in the food processing industry as a method of preserving fish and fishery products. The quality of fish and fishery products is changed by decreasing the water holding capacity and solubility of protein during dehydration (Migita *et. al.*, 1956; Suzuki, 1971), since water in meat is closely associated with the maintenance of the structure and functional properties of proteins (Kavanau, 1965). To inhibit such unwanted changes, sugars, amino acids, organic acids, phosphates and protein hydrolysates are added as denaturation-inhibiting substances (Hanafusa, 1973; Matsuda, 1973; Nakano & Yasui, 1976; Nozaki *et al.*, 1993; Darmanto *et al.*, 1997). In recent years, the potential uses and functionality of enzymatically-degraded materials from various food ingredients, such as fishery products (Suetsuna *et al.*, 1988; Sugiyama *et al.*, 1991), residues from seafood processing (Miyake, 1982; Murata *et al.*, 1991; Darmanto *et al.*, 1997; Zhang *et al.*, 2002a), have drawn attention. However, fish protein hydrolysates (FPH) are the subjects of an expanding demand as natural protein sources with good functional properties for the enrichment of food products (Whitaker, 1986). Therefore, we described earlier the preparation of FPH derived from five marine species: white croaker (white meat), horse mackerel and flying fish (medium meat), chub mackerel and sardine (red meat) for color function, and their suppressive effects have been compared with lizard fish myofibrils at the concentration of 5.0% FPH during dehydration (Khan *et al.*, 2003a). But the effects of the five FPH on the myofibrils were more or less similar, and their proximate components and amino acid components were almost the same among the five species. In the present study, we examined the added-concentration dependent effects of FPH (2.5–12.5%, dry weight) of three species on the state of water and Ca-ATPase activity of lizard fish myofibrils during dehydration. Lizard fish meat was used in this study, because it is in high demand as a suitable raw material for the preparation of high-quality fish jelly products in food industries in Japan.

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Materials and Methods

Materials Fish scraps (head, viscera, scale, skin, caudal fin, and bone) of three marine fish species viz., horse mackerel (*Trachurus japonicus*, mean body weight, 263.0±12.8 g), chub mackerel (*Scomber japonicus*, mean body weight, 561.0± 51.5 g), and white croaker (*Argyrosomus argentatus*, mean body weight, 163.2±13.5 g) were obtained from the Fish Processing Factory, Faculty of Fisheries, Nagasaki University, Japan.

Preparation of FPH FPH was prepared according to the previously reported method of Iwamoto *et al.* (1991) with a slight modification, which is described in our previous paper (Khan *et al.*, 2003a).

Proximate composition, molecular weight distribution, and amino acid analysis of the FPH Moisture, protein, lipid, ash, and sugar substances were determined by the methods of heat drying (105°C), Kjeldahl, Soxhlet, ashing in furnace (550°C), and phenol-sulfuric acid, respectively. The salt (NaCl) was measured using a salt analyzer (model SAT-2A, Toa Denpa Inc., Shinjuku, Tokyo, Japan).

The molecular weight distribution of the FPH was analyzed using gel filtration chromatography (column, Sephadex G-25 column 2.2 cm in diameter×60 cm length; elution solvent, 30 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl; applied sample volume, 2 ml; flow rate, 30 ml/h; and fraction volume, 5 ml/tube). The absorbance at 220 nm was measured for each fraction, and the average molecular weight was calculated by comparison with standard materials.

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

Preparation of myofibrils The lizard fish myofibrils were prepared according to the method of Katoh *et al.* (1977) with a slight modification that was described in our previous paper (Zhang *et al.*, 2002b; Khan *et al.*, 2003a). The general components of myofibril specimens were moisture 87.1%, protein 12.4%, lipid 0.04%, and ash 0.43%.

Addition of FPH and dehydration The myofibrils were placed in a mortar, and FPH (2.5–12.5 g dry matter) was added to100 g of myofibrils and mixed thoroughly for 20 min at 5°C after adjusting the pH 7.0 with 0.01 N NaOH or 0.01 N HCl. The sample was sealed in a cellophane bag, embedded in silica gel in a desiccator, and dehydrated at 5°C with occasional replacement of the silica gel. When the moisture content reached approximately 10%, further dehydration was performed in the desiccator under reduced pressure by a vacuum pump. Myofibrils without FPH were used as a control.

Measurement of water activity, moisture, and analysis of desorption isotherm curves The water activity (A_w) of the dehydrated myofibrils was measured by the indirect equilibrium vapor pressure method (Akiba *et al.*, 1974) using an oil manometer at 20°C. The moisture of the specimens was also measured by the atmospheric heating method at 105°C for 24 h. Desorption isotherm curves were produced by plotting moisture 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 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/A_w-A_w curves, was defined as M_2 according to a report of Bull (1944), and the moisture 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:

$$S=M_1\times S_w\times N/(M_w\times 10^3),$$

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 dry mater), S_w is the cross-sectional area of water molecules (10.8 $Å^2$), N is Avogadro's number (6.02×10^{23}/mol), and M_w is the molecular weight of water (18 g/mol).

Measurement of unfrozen water The amount of unfrozen water was determined by the colorimetric method (Wakamatsu & Sato, 1979) using a differential scanning calorimeter (model SSC-5200, Seiko Electronics Inc.). About 20 mg of the samples was placed in a tightly sealed aluminum container and weighed accurately. Twenty mg of Al_2O_3 was sealed in another aluminum container, which was used as a reference. The heat of melting (mJ/mg) was determined using a starting temperature of −40°C rising to 25°C at a rate of 1.0°C/min. The total amount of water in the sample was measured using the heating method (105°C).

Measurement of myofibrillar Ca-ATPase activity The dehydrated myofibrils sample was added with 30 volumes of 0.1 M KCl-20 mM Tris-maleate buffer (pH 7.0) and left to stand overnight at 5°C for water restoration. The sample was homogenized (Nichionirika Kikai Seisakusho Histocolon NS-560) at 1,000 rpm for 1 min, 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. The obtained sediment 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 corrected using the Kjeldahl method.

Results and Discussion

Properties of FPH Details of the proximate composition, amino acid analysis, and molecular weight distribution of the FPH of horse mackerel, chub mackerel, and white croaker were described in our previous report (Khan *et al.*, 2003a), and the properties of the three FPH are described briefly here. The major components of the FPH were peptides (85.11–85.8%). According to the gel filtration chromatography of FPH, many peaks corresponding to the molecular weight of less than 12,000 were observed. In particular, the large sharp peaks with the molecular weight between 1,400–300 revealed that FPH was a mixture of various peptides. The average molecular weight of the peptides in the FPH were 1,250 for horse mackerel, 1,000 for chub mackerel, and 1,250 for white croaker, as described in our previous report (Khan *et al.*, 2003b). The analyses of the amino acid composition of the peptides showed a large amount (about 16%) of the glutamic acid+glutamine (acidic amino acid) in the peptides. Arginine, lysine (basic amino acid), and serine, alanine, and leucine (neutral amino acid), were also found in abundance (6–13%) in the peptides. Although the free amino acid contents in the FPH were not measured in the present study, according to the gel filtration chromatography it was presumed that these contents were very low (Khan *et al.*, 2003a).

Desorption isotherm curves of myofibrils Figure 1 shows the effect of FPH on desorption isotherm curves of the myofibrils during dehydration. Desorption curves of myofibrils with

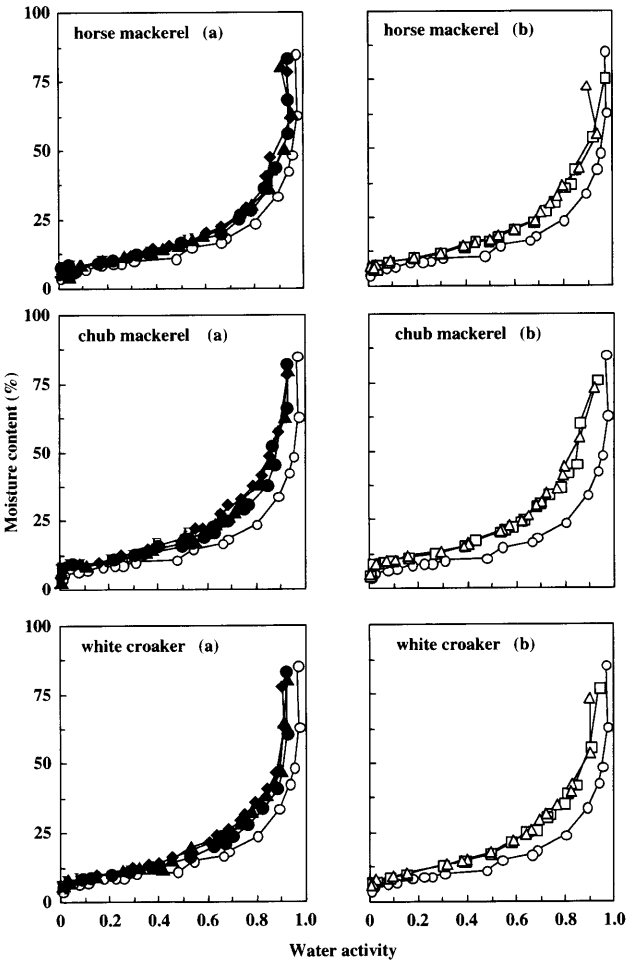

Fig. 1. Effect of fish protein hydrolysate (FPH) on the desorption isotherms of lizard fish myofibrils at 20°C during the dehydration process. FPH was added at the rate of 2.5–12. 5% (dry matter) to 100 g of myofibrils. The desorption isotherm corresponding to 2.5–7.5% FPH is shown in (a), and that corresponding to 10.0 and 12.5% FPH does in (b). Symbols: ○, control; ●, 2.5%; ▲, 5.0%; ◆, 7.5%; □, 10.0%; △, 12.5%.

FPH (2.5–12.5%) were sigmoid with 2 inflection points of A_w at 0.05-0.15 and 0.5–0.7. The Aw of the myofibrils with FPH were markedly lower than that of control (0.4–0.98) at the same moisture content. The A_w activity was remarkably decreased by addition of increasing the concentration from 2.5% to 10.0% of FPH to myofibrils, although the decreased A_w level with 12.5% of FPH was almost equal to that with 10.0% of FPH. Only a slight difference of A_w during the dehydration was found among the three FPH of horse mackerel, chub mackerel, and white croaker. Furthermore, the A_w of myofibrils with the FPH was almost the same as that of the control in the range below 0.4.

The monolayer (M_1) and multilayer (M_2) water, and sorption surface area (S) of the myofibrils are presented in Table 1. The myofibrils with FPH showed larger M_1 and M_2 values than those of the control. The M_1 and M_2 values (g H_2O/g dry matter) of myofibrils with added 2.5–12.5% of FPH were 0.097–0.131 and 0.183–0.252 in chub mackerel, 0.096–0.121 and 0.159–0.222 in white croaker, and 0.101–0.112 and 0.189–0.269 in horse mackerel, respectively. The sorption surface area (S) values of the myofibrils with FPH ranged from 0.34–0.46 (m^2/mg), and S values tended to be similar to the M_1 and M_2 values. These results indicate that the FPH stabilized the monolayer and multilayer sorbed water and increased the sorption surface area in myofibrils during dehydration.

Table 1. Amount of monolayer and multilayer water, sorption surface area of lizard fish myofibrils with 2.5–12.5% fish protein hydrolysate (FPH) on the basis of desorption isotherm at 20°C.

System	Concentration	Monolayer water (M_1)		Multilayer water (M_2)		M_2/M_1	$S^{a)}$
Control		8.44[b)]	0.092[c)]	13.6[b)]	0.157[c)]	1.61	0.32
Horse mackerel	2.5%	9.18	0.101	15.9	0.189	1.73	0.35
	5.0%	9.72	0.108	21.2	0.269	2.18	0.38
	7.5%	10.1	0.112	17.2	0.208	1.70	0.39
	10.0%	9.51	0.105	18.6	0.229	1.96	0.37
	12.5%	9.32	0.103	17.7	0.215	1.90	0.36
Chub mackerel	2.5%	9.73	0.103	15.5	0.183	1.59	0.38
	5.0%	10.01	0.111	16.3	0.195	1.63	0.39
	7.5%	8.88	0.097	18.7	0.230	2.11	0.34
	10.0%	10.44	0.117	19.6	0.242	1.88	0.41
	12.5%	11.58	0.131	20.1	0.252	1.74	0.46
White croaker	2.5%	8.78	0.096	13.7	0.159	1.56	0.34
	5.0%	10.00	0.111	18.2	0.222	1.82	0.39
	7.5%	9.86	0.109	15.9	0.189	1.61	0.38
	10.0%	10.07	0.112	16.2	0.193	1.61	0.39
	12.5%	10.83	0.121	16.2	0.193	1.50	0.42

[a)] Sorption surface area of sample (m^2/mg). [b)] Moisture content (g/100g of sample). [c)] Moisture content (g/g of dry matter).

As we described earlier (Khan *et al.*, 2003a) an attempt was made to prepare FPH from fish scrap of three marine species and to compare the protective effect of dehydration-induced denaturation on fish myofibrils. We intended in this work to classify the concentration dependent protective effect of FPH on dehydration-induced denaturation of myofibrils. The shapes of the desorption isothermal curves obtained from lizard fish myofibirils with and without (control) FPH were similar to those of Atka mackerel meat (Akiba, 1961), carp actomyosin (Nakano, 1979), water-bleached and crushed carp meat (Akiba, 1973), and white croaker myofibrils (Nozaki *et al.*, 1991). That is, they belonged to type B food (protein rich food, e.g., pork meat, bonito meat, and egg) in the various sorption isotherms classified by Shibasaki *et al.* (1967). The M_1 and M_2 ratios for the various kinds of proteins and those for the myofibrils with 5.0% of FPH from different species were reported to range from 1.5 to 2.0 (Bull, 1944), and from 1.68 to 1.89 (Khan *et al.*, 2003a), respectively. These reports well correspond with our present results (1.5–2.18). The fact that the level of monolayer and multilayer water (M_1+M_2) was increased in myofibrils with the addition of FPH, suggested that the FPH increased bound water molecules in myofibrils and the hydrophobic functional groups of peptides. Such interaction presumably structurally altered the hydration structure that maintained the structural integrity of myofibrils during the dehydration process. The reflections of these results were observed in the DSC study, where unfrozen water regarded as bound water increased in the myofibrils.

Unfrozen waterThe heat of melting of ice water 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 melting ice obtained from pure water was 333.27 mJ/mg, which was in agreement with the known value of 333.05 mJ/mg (Wakamatsu & Sato, 1979). A comparative feature of the amount of unfrozen water in myofibrils is shown in Fig. 2. The myofibrils with FPH (2.5–12.5%) had a larger amount of unfrozen water (0.345–0.417 g H_2O/g dry matter) than that in the control (0.341 g H_2O/g dry

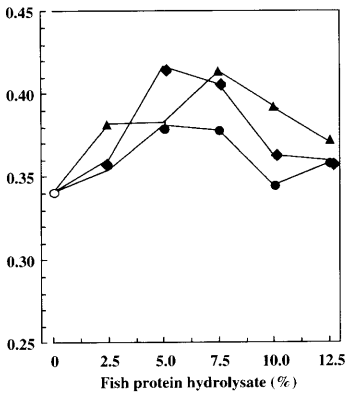

Fig. 2. Amount of unfrozen water (g H_2O/g dry matter) in lizard fish myofibrils with various concentrations of fish protein hydrolysate (FPH) during dehydration process. FPH was added at the rate of 2.5–12.5% (dry matter) to 100 g of myofibrils. Symbols: ○, control; ●, horse mackerel; ▲, chub mackerel; ◆, white croaker.

matter). The myofibrils with 5% FPH of horse mackerel showed a larger amount of unfrozen water (0.381 g H_2O/g dry matter) followed by 7.5% FPH (0.378 g H_2O/g dry matter); 7.5% FPH of chub mackerel (0.414 g H_2O/g dry matter), 10.0% (0.392 g H_2O/g dry matter); and 5.0% FPH of white croaker (0.417 g H_2O/g dry matter), 7.5% FPH (0.406 g H_2O/g dry matter), respectively. These results suggest that the FPH stabilized the hydrate water in the myofibrils through incorporating water molecules in myofibrils by the interaction of peptides in the FPH. Further, the amino acid groups derived from, for example, glutamic acid+glutamine, aspartic acid+asparagine, arginine, and lysine in the FPH are known to have large water-constraining and denaturation-inhibiting effects (Kanna *et al.*, 1972; Nozaki *et al.*, 1991) in myofibrils during dehydration. Thus, FPH captured water molecules surrounding the myofibrils, which were excluded by their hydrophobic residues. Peak increment of unfrozen water in myofibrils with FPH of white croaker was observed at a specific concentration of 5.0%, while that of unfrozen water was observed at 7.5% of FPH of chub mackerel, suggesting a concentration dependent effect of FPH on the myofibril stability. Although the concentration dependent effect of FPH of horse mackerel was not clear, the trend of the increment of unfrozen water suggests that the concentration dependent effect occurred between 5.0–7.5% of FPH.

The mechanism of concentration dependent effect of FPH on the stabilization of water molecules in the myofibril structure could be explained by the hydration and stabilization being dependent on a threshold FPH concentration, which corresponds to the capacity of myofibrils in excluding the FPH from its surface. Above that concentration, the free FPH molecules surrounding the myofibrils take up the hydrate water, resulting in a change in hydration structure. Such structural rearrangements reduced the unfrozen water contents in myofibrils and caused the denaturation to some extent (Fig. 3). This speculation is in accordance with the results of Gekko (1981) and Gekko & Koga (1983).

Myofibrillar Ca-ATPase activity The concentration dependent effect of FPH against dehydration-induced denaturation of myofibrils was assessed determining the myofibrillar Ca-ATPase activity during dehydration. The relative Ca-ATPase activity of the myofibrils with FPH is shown in Fig. 3 and is seen to be higher than that of the control. The myofibrillar Ca-ATPase activity of the control was decreased rapidly, while a markedly slow decreasing tendency was observed in the myofibrils with FPH. The FPH suppressed the inactivation of myofibrillar Ca-ATPase activity during dehydration and higher suppression effect was observed at the concentration of 7.5% FPH in horse mackerel, chub mackerel, and white croaker species, respectively. These findings suggest that FPH suppress the dehydration-induced denaturation of fish myofibrillar protein during dehydration.

The inhibitory effect of FPH on the dehydration-induced denaturation of myofibrils was compared at the different levels of moisture content. The mechanisms behind the suppression of dehydration-induced denaturation of protein of various cryoprotectants have been investigated in terms of structural changes of water surrounding the myofibrillar protein surface. The addition of intermolecular hydrogen bonds, hydrophobic interactions between non-polar residues, and the hydration of polar residues play important roles in the maintenance of the protein molecular structure and development of its function

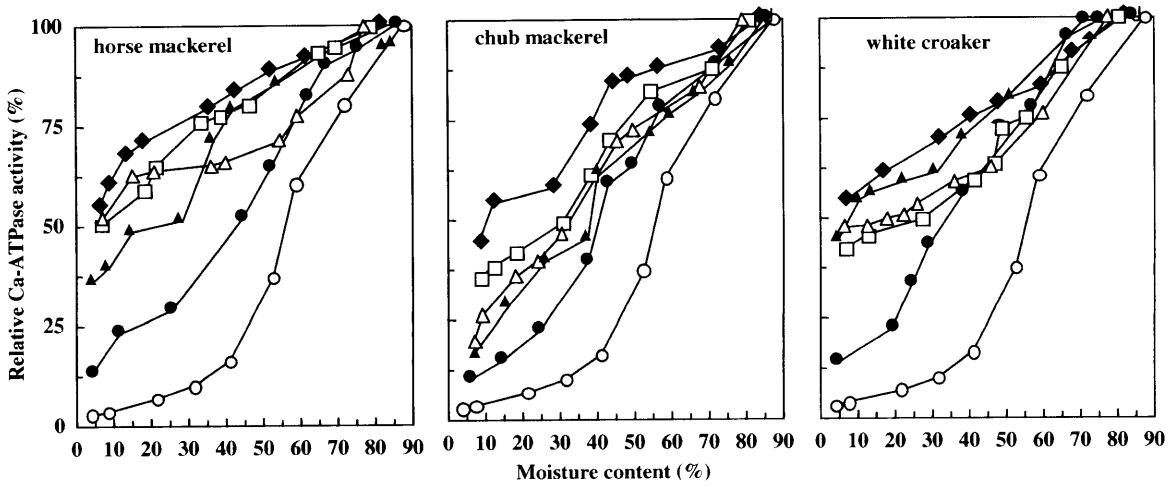

Fig. 3. Changes in the relative Ca-ATPase activity (%) of lizard fish myofibrils with various concentrations of fish protein hydrolysate (FPH) during the dehydration process. FPH was added at the rate of 2.5–12.5% (dry matter) to 100 g of myofibrils. Symbols are the same as those in Fig. 1.

(Kauzman, 1959; Nemethy & Scheraga, 1962). Dehydration induces not only destruction of hydrogen bond or hydrophobic bonds, and but also interferes with the intermolecular hydrogen bonds in myofibrils, which results in destruction of protein morphology and rearrangement of the protein structure (Hanafusa, 1973). The hydrophilic amino acids are the major components of the peptide in the FPH, which might be responsible for inhibition of the dehydration-induced denaturation of myofibrillar protein. As shown in Fig. 3, the trend of Ca-ATPase activity of myofibrils with and without FPH revealed that the FPH inhibited the dehydration-induced denaturation of myofibrils regardless of species differences. But, the inhibitory effects of FPH were concentration dependent and the maximal suppressive effects were observed up to certain concentrations of FPH (5.0–7.5%). Thus, the suppressive effect of FPH correlates with the results of DSC analysis where concentration dependent stabilization effects of FPH were observed on the increment of unfrozen water content in myofibrils. Therefore, the above findings provide clear evidence that FPH acts as a myofibril stabilizer through bound water construction up to a certain level. These findings are similar to the results of Darmanto *et al.* (1997) and Zhang *et al.* (2002b), where protein hydrolysates exhibited concentration dependent protective effects on myofibrils during dehydration. Thus, on the basis of previous discussion, it may be concluded that FPH have a denaturation inhibiting effect on the myofibrillar protein during dehydration. The FPH could therefore be used as a functional food material against myofibrillar protein denaturation during the dehydration process.

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