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Effect of Storage Temperatures on the Formation of Disulfides and Denaturation of Milkfish Myosin (*Chanos chanos*)

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The effects of frozen storage temperatures on the formation of disulfides and the denaturation of myosin, extracted from milkfish (*Chanos chanos*) dorsal muscle, were investigated. The activities of Ca-ATPase and Mg(Ca)-ATPase and solubility in 0.6 M KCl decreased, and the total $NaBH_4$-soluble and -insoluble proteins increased at a much higher rate at −20 °C than at −35 °C. During freezing, the total SHs of samples at −20 °C decreased significantly, but not at −35 °C. The decreasing rate of total SHs at −20 °C was significantly faster than at −35 °C during storage.

The stability of fish proteins during frozen storage is highly influenced by temperatures (Arai et al., 1973; Arai, 1977; Suzuki et al., 1964, 1965; Suzuki, 1967; Hatano, 1968; Tokiwa and Matsumiya, 1969; Seki and Hasegawa, 1978; Fukuda et al., 1981; Fukuda, 1986; Jiang, 1977; Jiang et al., 1988a; Matsumoto, 1980). On the study of the changes in inactivation rate constant of actomyosin (AM) Ca-ATPase (K_D) of frozen mackerel, the K_D value at −15 °C was 5-fold that at −40 °C (Fukuda, 1986). Jiang (1977) and Jiang et al. (1985) found that mullet and amberfish muscle proteins were much more stable when stored at −40 °C than at −20 °C. Although no changes in extractability, sedimentation constant, and intrinsic viscosity of AM were found with fish muscle frozen by liquid nitrogen (Dyer, 1951; Segran, 1956; Suzuki et al., 1964, 1965; Noguchi and Matsumoto, 1970), protein denaturation occurred and further progressed during storage at −8 to −10 °C (Suzuki et al., 1965).

According to previous work (Jiang et al., 1988b), the freeze denaturation of AM was mainly caused by formation of disulfide, hydrogen, and hydrophobic bonds. More disulfides formed on actomyosin frozen at −20 °C than at −35 °C (Jiang et al., 1988a). This study aimed to investigate the effects of storage temperature on the formation of disulfides, solubility, and ATPase activity of freeze-thawed myosin.

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MATERIALS AND METHODS

Preparation of the Myosin. Myosin was extracted from milkfish (*Chanos chanos*) dorsal muscle according to the previous study (Chen et al., 1988). To investigate the effects of storage temperatures on myosin, 30 mL of extracted myosin (5.46 mg/mL) was placed in plastic tubes, and the tubes were stoppered, frozen, and stored at −20 and −35 °C for 8 weeks. At definite time intervals, samples were removed, thawed to 0 °C with running tap water (about 25 °C), and subjected to the following analyses.

Determination of Sulfhydryls (SHs) of Myosin. The total SHs of myosin were determined according to Buttkus (1971). To 1.0 mL of myosin solution (5-10 mg/mL) was added 9 mL of chilled solution [mixture of 50 mM KH_2PO_4-K_2HPO_4, 6 mM ethylenediaminetetraacetic acid (EDTA), 0.6 M KCl, and 8 M urea, pH 8.0] and the resultant mixture stirred for 30 min at 25 °C. To 3 mL of the mixture was added 0.02 mL of 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid) and the resultant mixture incubated at 40 °C for 15 min. The absorbance at 412 nm was measured to calculate the total SHs according to Ellman (1959).

Reactive SHs were determined by incubating the myosin at 5 °C for 1 h in the absence of urea, according to Buttkus (1971). The total and reactive SHs were expressed as moles/5 × 10^5 g of protein.

Solubility. Solubility of myosin was determined according to Hamada et al. (1977). Samples were thawed to 0 °C with running tap water (25 °C) and centrifuged at 15000*g*, 0 °C, for 1 h. The collected supernatant was defined as salt-soluble fraction I, which was considered to be native proteins. To the precipitate was added 5 mL of solution (containing 8 M urea, 6 mM EDTA, and 0.6 M KCl solution) and the resultant mixture stirred for 30

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min at 25 °C. The supernatant obtained after centrifuging this mixture at 15000g, 15 °C, for 1 h was defined as urea-soluble fraction (II), which was considered to be aggregated by formation of hydrogen and hydrophobic bonds. Finally, 5 mL of solution (containing 0.5% $NaBH_4$, 6 mM EDTA, 8 M urea, and 0.6 M KCl) was added to the precipitate and the resultant mixture stirred for 30 min at 25 °C. The supernatant obtained after centrifuging this mixture at 15000g; 15 °C, for 1 h was defined as $NaBH_4$-soluble fraction III, which was aggregated by formation of disulfides, and the final residue as insoluble fraction IV.

Protein concentration was measured by the micro-biuret method (Itzhaki and Gill, 1964). The solubility was expressed as the ratio of the quantity of soluble protein to that of original myosin.

Ca-ATPase Activity. To 1 mL of myosin solution (1–5 mg/mL) were added 0.5 mL of 0.5 M Tris–maleate buffer (pH 7.0), 0.5 mL of 0.1 M $CaCl_2$, 7.5 mL of deionized water, and finally 0.5 mL of 20 mM adenosine 5′-triphosphate (ATP) solution (pH 7.0). After the ATP was added, the rate of release of inorganic phosphate at 25 °C within 3-min reaction was measured. A 5-mL portion of 15% trichloroacetic acid was added to stop the reaction; the quantity of inorganic phosphate released was measured according to the method of Arai (1974). The Ca-ATPase activity was defined as micromoles of inorganic phosphate liberated per milligram of protein within 1 min for the reaction at 25 °C.

Mg(Ca)-ATPase Activity. To 1 mL of myosin solution (1–5 mg/mL) were added 1.0 mL of 0.02 M $MgCl_2$, 1.0 mL of 1 mM $CaCl_2$, 1.0 mL of 0.2 M Tris–maleate buffer (pH 7.0), 5.0 mL of deionized water, and finally 1.0 mL of 20 mM adenosine 5′-triphosphate (ATP) solution (pH 7.0). After the ATP was added, the rate of release of inorganic phosphate at 25 °C within 3-min reaction was measured. A 5-mL portion of 15% trichloroacetic acid was added to stop the reaction; the quantity of inorganic phosphate released was measured according to the method of Arai (1974). The Mg(Ca)-ATPase activity was defined as micromoles of inorganic phosphate liberated per milligram of protein within 1 min for the reaction at 25 °C.

Statistical Analysis. Duncan's multiple-range test was used for statistical analyses.

RESULTS AND DISCUSSION

Effect of Storage Temperatures on the Solubility of Myosin. Immediately after freezing, the percentage ratio of salt-soluble fraction I of myosin stored at −20 and −35 °C decreased to 78.6% and 77.3%, respectively, when compared to the unfrozen sample (Table I). No significant differences in the salt-soluble and 8 M urea soluble proteins were observed between samples frozen at −20 and −35 °C ($P < 0.01$) during the first 2 weeks of storage; similar results were found with the total $NaBH_4$-soluble III and insoluble fraction IV during the first 4 weeks of storage (Table I). The quantity of salt-soluble fraction was significantly lower; 8 M urea soluble and the total of $NaBH_4$-soluble and -insoluble fractions were significantly higher at −20 C than at −35 °C during prolonged storage. According to a previous study (Jiang et al., 1988b), both the $NaBH_4$-soluble (III) and -insoluble (IV) fractions prepared by the method of Hamada et al. (1977) were considered to result from the formation of disulfides. During freezing, the quantity of the total $NaBH_4$-soluble and -insoluble fractions of both samples was much higher than that of urea-soluble proteins. These data indicated that disulfides markedly formed on both samples at −20 and −35 °C during the freezing process.

The decreasing rate of salt-soluble protein (fraction I) was significantly faster at −20 °C than at −35 °C ($P < 0.01$, Table II). The quantity of urea-soluble fraction II of myosin increased during storage at −35 °C; at −20 °C it increased during the first 4 weeks of storage but decreased during prolonged storage (Table I). The increase in the urea-soluble fraction indicated the formation of hydrogen and hydrophobic bonds during storage. After 4 weeks of storage at −20 °C, the decrease in urea-soluble proteins might be due to the formation of disulfides. The total $NaBH_4$-soluble and -insoluble fractions of all samples increased during storage (Table I) at a rate, which at −20 °C was almost 1.4-fold that at −35 °C (Table II). These data suggest that protein denaturation during freezing and subsequent storage was mainly caused by formation of disulfide, hydrogen, and hydrophobic bonds; and more disulfides formed in myosin stored at −20 °C than at −35 °C.

Effect of Storage Temperatures on the SHs of Myosin. After freezing, the total SHs of samples frozen at −20 °C decreased significantly; no significant change was observed at −35 °C ($P < 0.01$) (Table III). During storage, however, the total SHs at −20 °C decreased significantly ($P < 0.01$), while less decrease at −35 °C was observed (Table III). During freezing, the increase in total $NaBH_4$-soluble and -insoluble fractions and the decrease in the total SHs (Tables I and III) indicate the formation of disulfides in both samples at −20 and −35 °C, with more disulfides formed at −20 °C than at −35 °C. During storage, disulfides further increased in both samples (Tables I and III).

Table I. Effect of Storage Temperature on the Solubility[a] of Milkfish Myosin

fraction[b]	treatment	solubility at storage time (weeks) 0	2	4	6	8
I	A[c]	4.29 a (78.6)	4.19 a (76.7)	3.59 b (65.8)	3.44 b (63.0)	3.18 b (58.2)
	B[c]	4.22 a (77.3)	4.23 a (77.5)	3.74 a (68.5)	3.60 a (65.9)	3.45 a (63.2)
II	A	0.05 a (0.9)	0.13 a (2.4)	0.19 a (3.5)	0.14 a (2.6)	0.13 b (2.4)
	B	0.05 a (0.9)	0.11 a (2.0)	0.13 b (2.3)	0.12 b (2.2)	0.16 a (2.9)
III	A	0.05 a (0.9)	0.11 a (2.0)	0.25 a (4.6)	0.31 a (5.7)	1.48 a (8.8)
	B	0.04 a (0.7)	0.07 b (1.3)	0.18 b (3.3)	0.12 b (2.2)	0.04 b (1.0)
IV	A	1.07 b (19.6)	1.03 a (18.9)	1.43 a (26.2)	1.57 a (28.8)	1.67 b (30.6)
	B	1.15 a (21.1)	1.05 a (19.2)	1.36 a (24.9)	1.60 a (29.7)	1.83 a (33.5)
III + IV	A	1.12 a (20.7)	1.14 a (20.9)	1.68 a (30.8)	1.88 a (34.5)	2.15 a (39.4)
	B	1.19 a (21.8)	1.12 a (20.5)	1.54 a (28.2)	1.74 b (31.9)	1.87 b (34.5)

[a] The solubility was expressed as milligrams of soluble protein per milliliter. The concentration of unfrozen protein was 5.46 mg/mL. [b] Key: I, salt soluble; II, urea soluble; III, $NaBH_4$-soluble; IV, insoluble; III + IV, total of fractions III and IV. [c] Key: A, frozen and stored at −20 °C; B, frozen and stored at −35 °C. Values in the same column of each fractions bearing different letters differ significantly ($P < 0.01$). Values in parentheses are the percent ratio relative to the unfrozen sample.

Table II. Effect of Storage Temperature on the Change Rates of Solubility, Total SHs, and Ca-ATPase Activity of Milkfish Myosin

storage temp, °C	change rate (%/week) salt-soluble[a]	III + IV[a,b]	total SH[c] (mmol/ 5×10^5/wk)	Ca-ATPase act.[d] (P_i mmol/ min/mg/wk)
−20	−149 a (−2.7)	140 a (2.6)	−1065 a (−4.1)	−15 a (−6.3)
−35	−109 b (−2.0)	99 b (1.8)	−980 b (−3.7)	−5.4 b (−1.7)

[a] Micrograms per milliliter per week. Values bearing different letters in the column differ significantly ($P < 0.01$). [b] Total $NaBH_4$-soluble and -insoluble fractions. [c] Millimoles/5×10^5 g of protein per week. [d] Nanomoles of inorganic phosphate per minute per milligram per week.

Table III. Effect of Storage Temperature on the Total SH of Milkfish Myosin[a]

storage temp, °C	total SH at storage time (weeks)					
	unfrozen	0	2	4	6	8
-20	26.5 aA[b] (100)[c]	24.5 bB (92.3)	19.4 cB (73.0)	17.5 dB (65.8)	16.9 dB (63.6)	15.1 eB (56.2)
-35	26.5 aA (100)	26.0 aA (98.1)	23.8 bA (89.6)	21.2 cA (79.8)	21.0 cA (79.2)	17.6 dA (66.3)

[a] The total SHs are expressed as moles/5 × 10^5 g of protein. [b] Values in the same row bearing different lowercase letters differ significantly ($P < 0.01$). Values in the same column bearing different uppercase letters differ significantly ($P < 0.01$). [c] Values in parentheses were the percent ratios relative to the unfrozen sample.

Table IV. Effect of Storage Temperature on the Reactive SH of Milkfish Myosin[a]

storage temp, °C	reactive SH at storage time (weeks)					
	unfrozen	0	2	4	6	8
-20	14.0 b[b]	15.1 a	13.1 b	12.5 c	12.5 c	11.0 d
-35	14.0 b	17.0 a	17.0 a	17.0 a	17.3 a	16.2 a

[a] The reactive SHs are expressed as moles/5 × 10^5 g of protein. [b] Values in the same row bearing different letters differ significantly ($P < 0.01$).

Table V. Effect of Storage Temperature on the Ca-ATPase Activity of Milkfish Myosin[a]

storage temp, °C	Ca-ATPase activity at storage time (weeks)					
	unfrozen	0	2	4	6	8
-20	0.32 a[b] (100.0)[d]	0.13 b (39.2)	0.06 c (17.6)	0.03 d (10.2)	c	c
-35	0.32 a (100.0)	0.27 b (83.6)	0.26 b (79.0)	0.25 b (78.1)	0.23 b (70.1)	0.23 b (71.6)

[a] The Ca-ATPase activity was expressed as micromoles of inorganic phosphate released within 1-min reaction at 25 °C per milligram of protein. [b] Values in the same row bearing different letters differ significantly ($P < 0.01$). [c] Undetectable. [d] Values in parentheses were the percent ratios relative to the unfrozen myosin.

The reactive SHs of all samples increased significantly after freezing ($P < 0.01$) and decreased during storage at -20 °C, but not at -35 °C (Table IV). It was considered that the tertiary structure of myosin changed, caused by formation of disulfide, hydrogen, and hydrophobic bonds (Tables I and III); consequently, the reactive SHs masked in myosin molecules were exposed. Based on the changes in total SHs and total $NaBH_4$-soluble and -insoluble fractions of myosin, the oxidation of SHs was significantly faster at -20 °C than at -35 °C (Tables I and II).

Effect of Storage Temperatures on ATPase Activity. The Ca-ATPase activity of myosins at -20 and -35 °C decreased significantly after freezing and during storage at -20 °C, decreasing more slowly at -35 °C (Table V). The decreasing rate of Ca-ATPase activity at -20 °C was almost 3.7-fold that at -35 °C (Table II). According to Tables III and V, the loss of Ca-ATPase activity seemed to parallel the decrease in total SHs. The results suggested that the decrease in Ca-ATPase activity might be partly caused by the oxidation of SHs. This decrease coincided with the studies by Jiang et al. (1988a,b), Buttkus (1971), and Hamada et al. (1977). However, the total SHs only decreased 7.7% and 1.9% after freezing at -20 and -35 °C, respectively (Table III), while the Ca-ATPase activity decreased 60.8% and 16.4%, respectively (Table V). This distinct difference between changes in total SHs and Ca-ATPase activity suggested that not all of the SHs in myosin be related to the ATPase activity.

Considering the change rate of total SHs and Ca-ATPase activity, the decreasing rate of total SHs at -20 °C was 1.1-fold that at -35 °C, while Ca-ATPase at -20 °C was 3.7-fold that at -35 °C. This suggests that factors other than the oxidation of SHs might also affect the ATPase activity of myosin. Suzuki (1967) and Hatano (1968) postulated that, during frozen storage, the loss of enzymatic activity of myosin was due to the tertiary structural changes caused by ice crystallization. However, Buttkus (1971) and Hamada et al. (1977) concluded that the decrease in Ca-ATPase was highly related to the oxidation of SHs. The decrease in the total SHs and increase in total $NaBH_4$-soluble and -insoluble fractions during frozen storage at -20 and -35 °C (Tables I and III) indicated that the loss of Ca-ATPase activity might be due to the oxidation of SHs on the active site of myosin.

Table VI. Effect of Storage Temperature on Mg(Ca)-ATPase Activity of Milkfish Myosin during Frozen Storage

storage temp, °C	Mg(Ca)-ATPase activity at storage time (weeks)					
	unfrozen	0	2	4	6	8
-20	0.16 a[b] (100.0)[d]	0.04 b (25.0)	0.02 c (9.8)	0.02 c (9.8)	c	c
-35	0.16 a (100.0)	0.04 c (25.6)	0.05 c (29.3)	0.06 b (39.0)	0.04 c (23.8)	0.03 d (15.2)

[a] The Mg(Ca)-ATPase activity was expressed as micromoles of inorganic phosphate released within 1-min reaction at 25 °C per milligram of protein. [b] Values in the same row bearing different letters differ significantly ($P < 0.01$). [c] Undetectable. [d] Values in parentheses were the percent ratios relative to the unfrozen myosin.

Because of the inhibitory effect of Mg^{2+} on myosin ATPase activity (Lowey et al., 1969), the initial Mg(Ca)-ATPase activity of all samples was very low before freezing. The Mg(Ca)-ATPase activity of both samples decreased to 25% immediately after freezing, with a further decrease during storage (Table VI). Furthermore, the Mg(Ca)-ATPase activity was almost completely lost after 6 weeks of storage at -20 °C. It appeared that the loss of Mg(Ca)-ATPase activity of myosin occurred in samples stored at -20 °C sooner than it did at -35 °C.

Comparison of the Denaturation of Actomyosin (AM) and Myosin. As reported by Jiang et al. (1988a), the total SHs of AM decreased to 65.2% and 85.1%, while that of myosin decreased to 56.2% and 66.3% (Table III) after 8 weeks of storage at -20 and -35 °C, respectively. The Ca-ATPase activity of AM (Jiang et al., 1988a) decreased to 21.7% and 66.5% after 8 weeks of storage at -20 and -35 °C, respectively, while that of myosin decreased to 71.6% after 8 weeks of storage at -35 °C and almost lost completely after 4 weeks of storage at -20 °C (Table V). When the changes in total SHs and Ca-ATPase activity are compared, the myosin seems to be more susceptible to frozen storage, especially at -20 °C. It is suggested that actin, tropomyosin, and troponins played a protective role on the stability of AM.

Studies on the role of SHs in actin are still controversial. Several lines of evidence suggested that the SHs are involved in the polymerization of G-actin (Katz and Mommaerts, 1962; Drabikowski and Gergely, 1963), nucleotide binding (Strohman and Samorodin, 1962; Katz, 1963; Kuehl and Gergely, 1969), and myosin combination (Perry and Cotterill, 1964; Bailin and Barany, 1967). However, some papers reported that modification of two or three cysteine residues of actin with (2-aminoethyl)isothiouronium (Seraydarian et al., 1968), with an azo dye (Lusty and Fasold, 1969) or with iodoacetate or iodoacetamide (Bridgen, 1972) exhibited no effect on the ability of G-actin to polymerize and bind myosin, nucleotide, and calcium ion. Although the SHs of actin might be involved in actin–myosin interaction, it is also possible that SHs ap-

pearing on the surface of F-actin would compete with those on myosin molecules for oxidation into disulfides during frozen storage, resulting in the higher stability of AM.

In summary, from the increase in total $NaBH_4$-soluble and -insoluble fractions and the decrease in total SHs of myosin during frozen storage, it is suggested the formation of disulfides might be important in protein freeze-denaturation. This is partly supported by the findings that the formation of disulfides and decrease in solubility and Ca-ATPase activity occurred at a much faster rate at −20 °C than at −35 °C.

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