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Effect of Storage Temperatures on the Formation of Disulfides and Denaturation of Tilapia Hybrid Actomyosin (*Tilapia nilotica* × *Tilapia aurea*)

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Effects of frozen storage temperatures on the formation of disulfides and denaturation of actomyosin (AM), extracted from tilapia hybrid (*Tilapia nilotica* × *Tilapia aurea*) dorsal muscle, were investigated. The activities of Ca-ATPase and Mg(Ca)-ATPase, Ca sensitivity, and AM solubility in 0.6 M KCl decreased at a higher rate at −20 °C than at −40 °C. The insoluble proteins increased at a much higher rate at −20 °C than at −40 °C. The total SHs of sample at −20 °C significantly decreased during freezing and subsequent storage. However, the total SHs at −40 °C slightly decreased during the early 2 weeks of storage, and no significant changes were observed during prolonged storage. This suggested that more disulfides formed in samples at −20 °C than at −40 °C.

Among the factors affecting the stability of fish muscle proteins, the nature of fish muscle and freezing and storage temperatures are considered to be the most important factors (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; Matsumoto, 1980). The stability of muscle proteins of tilapia hybrid during frozen storage at various temperatures was greater than that of milkfish (Tsai et al., 1989). The inactivation rate constant (K_D) of actomyosin (AM) Ca-ATPase of frozen mackerel at −15 °C was 5-fold that at −40 °C (Fukuda, 1986). The stability of AM of cod, halibut, plaice, and rosefish was greater when stored at −23 °C than at −12 and −18 °C (Dyer and Morton, 1956; Dyer et al., 1956). Jiang (1977) and Jiang et al. (1985) reported that mullet and amberfish muscle proteins were much more stable when stored at −40 °C than at −20 °C. No significant changes in extractability, sedimentation constant, and intrinsic viscosity of AM were found in fish muscle frozen by liquid nitrogen (Dyer, 1951; Segran, 1956; Suzuki et al., 1964, 1965; Noguchi and Matsumoto, 1970). However, protein denaturation occurred during frozen storage when the storage temperature was not low enough (Suzuki et al., 1965). The freeze denaturation of AM was found to be dominantly caused by formation of disulfide, hydrogen, and hydrophobic bonds during freezing and storage (Jiang et al., 1988a). More disulfides formed and denaturation occurred in milkfish AM frozen at −20 °C than at −35 °C (Jiang et al., 1988b).

This study aimed to investigate the effects of storage temperature and added reductants ($NaBH_4$ and $NaNO_2$) on the formation of disulfides, solubility, and ATPase activity of freeze–thawed tilapia hybrid AM.

MATERIALS AND METHODS

Preparation of the Actomyosin. Actomyosin was extracted from tilapia hybrid (*Tilapia nilotica* × *Tilapia aurea*) dorsal muscle according to Noguchi and Matsumoto (1970). To investigate the effects of storage temperatures on AM, 30 mL of extracted AM (4.80 mg/mL) was placed in plastic tubes, stoppered, frozen, and stored at −20 and −40 °C for 12 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.

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Table I. Effect of Storage Temperature on the Solubility[a] of Tilapia Hybrid Actomyosin

fraction[b]	treatment[c]	storage time,[d] weeks 0	2	4	6	8	12
I	A	3.59 b (74.8)	3.15 b (65.6)	3.13 b (65.1)	2.49 b (51.9)	2.44 b (50.8)	2.31 b (48.1)
	B	3.63 a (75.6)	3.48 a (72.5)	3.30 a (68.8)	3.25 a (67.7)	3.20 a (66.7)	3.11 a (64.8)
II	A	0.19 a (4.0)	0.43 a (9.0)	0.27 a (5.6)	0.10 a (2.1)	0.17 a (3.5)	0.15 a (3.1)
	B	0.14 b (2.9)	0.19 b (4.0)	0.15 b (3.1)	0.13 a (2.7)	0.11 b (2.3)	0.11 b (2.3)
III	A	1.02 a (21.2)	1.22 a (25.4)	1.40 a (29.2)	2.21 a (46.0)	2.19 a (45.7)	2.34 a (48.8)
	B	1.03 a (21.5)	1.13 b (23.5)	1.35 b (28.1)	1.42 b (29.6)	1.49 b (31.0)	1.58 b (32.9)

[a] The solubility was expressed as milligrams of soluble protein per milliliter. The concentration of unfrozen protein was 4.80 mg/mL. [b] Key: I, salt-soluble; II, urea-soluble; III, insoluble fraction. [c] Key: A, frozen and stored at −20 °C; B, frozen and stored at 40 °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.

Determination of Sulfhydryls (SHs) of Actomyosin. The total SHs of AM were determined according to Buttkus (1971). To 1.0 mL of AM 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 was 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 was incubated at 40 °C for 15 min. The absorbance at 412 nm was measured to calculate the total SHs according to Ellman (1959). The total SHs were expressed as moles per 5×10^5 g of protein.

Solubility. 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 was stirred for 20 min at 25 °C. The supernatant obtained after centrifuging this mixture at 15000*g*, 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, the residue was defined as insoluble fraction III (Hamada et al., 1977; Jiang et al., 1988a).

Protein concentration was measured by the biuret method modified by Umemoto (1966). The solubility was expressed as milligrams per milliliter and the ratio of the quantity of soluble protein to that of original AM.

Ca-ATPase Activity. To 1 mL of AM 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 ATP was added, the rate of release of inorganic phosphate at 25 °C within 3 min reaction was measured. Five milliliters 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(EGTA)-ATPase Activity. Mg(EGTA)-ATPase activity was determined by the same method as that for Ca-ATPase except for the medium. To 1 mL of AM solution (1–5 mg/mL) were added 1.0 mL of 0.02 M $MgCl_2$, 1.0 mL of 5 mM ethylene glycol bis(2-aminoethyl ether)tetraacetic acid (EGTA), 1.0 mL of 0.2 M Tris–maleate buffer (pH 7.0), 0.5 mL of 0.1 M $CaCl_2$, 5.0 mL of deionized water, and finally 0.5 mL of 20 mM ATP solution (pH 7.0). The Mg(EGTA)–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. Mg(Ca)-ATPase activity was determined by the same method as that for Ca-ATPase except for the medium. To 1 mL of AM 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), 0.5 mL of 0.1 M $CaCl_2$, 5.0 mL of deionized water, and finally 0.5 mL of 20 mM ATP solution (pH 7.0). The Mg(EGTA)-ATPase activity was defined as micromoles of inorganic phosphate liberated per milligram of protein within 1 min for the reaction at 25 °C.

Table II. Effect of Storage Temperature on the Rate Changes of Solubility, Total SHs, and Ca-ATPase Activity of Tilapia Hybrid Actomyosin during 12 Weeks of Storage

temp, °C	rate change[b] salt-sol, μg/mL per week	insol protein, μg/mL per week	total SHs[a]	Ca-ATPase act.[a]
−20	−110 a	124 a	−1810 a	−26 a
−40	−42 b	47 b	−680 b	−18 b

[a] The change rates of total SHs and Ca-ATPase activity were expressed as moles/5×10^5 mg per week and P_i nmol/min per mg per week, respectively. [b] Values in the same column bearing different letters differ significantly ($P < 0.01$).

Ca Sensitivity. Ca sensitivity was expressed as follows: Ca sensitivity (%) = [1 − Mg(EGTA)-ATPase activity/Mg(Ca)-ATPase activity] × 100%.

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

RESULTS AND DISCUSSION

Effect of Storage Temperatures on the Solubility of Actomyosin. Immediately after freezing, the ratio of salt-soluble fraction I of actomyosin stored at −20 and −40 °C significantly decreased to 74.8% and 75.6%, respectively, when compared wtih the unfrozen sample ($P < 0.01$) (Table I). The quantity of 8 M urea-soluble fraction II of AM at −20 °C was significantly higher than that at −40 °C ($P < 0.01$). No significant difference in the insoluble fractions (III) was found between samples frozen at −20 and −40 °C (Table I). According to a previous study (Jiang it al., 1988a), the insoluble fractions (III) were considered to result from the formation of disulfides. During freezing, the quantity of insoluble proteins of both samples was much higher than that of urea-soluble proteins. These data indicated that denaturation occurred by formation of disulfide, hydrogen, and hydrophobic bonds in all samples; more disulfides formed than other bonds.

During storage, the quantity of salt-soluble fraction I of all samples decreased with the duration of storage (Table I). The decreasing rate of salt-soluble fraction I was significantly higher at −20 °C than at −40 °C (Table II). The quantity of urea-soluble fraction II of AM stored at both −20 and −40 °C increased during the first 2 weeks of storage but decreased during prolonged storage (Table I). The increase in the urea-soluble fraction indicated formation of hydrogen and hydrophobic bonds during storage. After 2 weeks of storage, the decrease in urea-soluble proteins might be due to formation of the disulfides, which consequently caused incomplete disruption

Table III. Effect of Storage Temperature on the Total SHs of Tilapia Hybrid Actomysin[a]

storage temp, °C	storage time,[b,c] weeks						
	unfrozen	0	2	4	6	8	12
-20	51.3 a A	47.9 b B	43.9 b B	35.9 c B	35.8 c B	34.6 c B	24.7 d B
	(100)	(87.9)	(80.6)	(65.8)	(65.8)	(63.5)	(45.3)
-40	51.3 a A	51.0 a A	49.7 b A	45.3 c A	44.4 c A	43.9 c A	43.2 c A
	(100)	(93.5)	(91.2)	(83.1)	(81.5)	(80.6)	(79.3)

[a] The total SHs are expressed as moles/5 × 10^5 mg 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 present ratios relative to the unfrozen sample.

Table IV. Effect of Storage Temperature on the Ca-ATPase Activity of Tilapia Hybrid Actomysin[a]

storage temp, °C	storage time,[b,c] weeks						
	unfrozen	0	2	4	6	6	12
-20	0.45 a	0.39 b	0.13 c	0.11 c	0.04 d	0.03 d	0.02 d
	(100.0)	(87.6)	(29.7)	(24.1)	(10.1)	(7.8)	(5.4)
-40	0.45 a	0.41 a	0.26 b	0.22 b	0.20 bc	0.18 c	0.16 c
	(100.0)	(90.9)	(59.2)	(50.1)	(44.5)	(39.5)	(35.8)

[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] Values in the parentheses were the percent ratio relative to the unfrozen actomyosin.

of hydrogen and hydrophobic bondings by 8 M urea. The insoluble proteins of both samples increased during storage (Table I) at a rate that at -20 °C was almost 2.6-fold that at -40 °C (Table II). These data suggested that protein denaturation during freezing and subsequent storage was mainly caused by formation of disulfide, hydrogen, and hydrophobic bonds; more disulfides formed in AM stored at -20 °C than at -40 °C.

Effect of Storage Temperatures on the SHs of Actomyosin. No significant change in the total SHs was observed in samples before and after freezing at -40 °C ($P < 0.01$). However, the total SHs of sample stored at -20 °C decreased significantly after freezing ($P < 0.01$). During storage, the total SHs of samples at -20 °C decreased significantly ($P < 0.01$); that at -40 °C decreased during the first 4 weeks of storage, and no significant change was obtained during prolonged storage (Table III). Increase in insoluble fractions and decrease in the total SHs (Tables I and III) indicated formation of the disulfides in both samples at -20 and -40 °C; and more disulfides formed at -20 °C than at -40 °C during freezing and subsequent storage (Tables I and III).

Effect of Storage Temperatures on ATPase Activity. The Ca-ATPase activity of AM at -20 °C decreased significantly after freezing and further decreased during storage (Table IV). However, during the freezing process, no significant change in Ca-ATPase activity of samples at -40 °C was observed (Table IV). The decreasing rate of Ca-ATPase activity was significantly higher at -20 °C than at -40 °C (Table II). The decrease in Ca-ATPase was reported to be highly related to the oxidation of SHs (Buttkus, 1971; Hamada et al., 1977). The decrease in the total SHs and increase in the insoluble fractions during frozen storage (Tables I and III) indicated that the loss of Ca-ATPase activity might be due to the oxidation of -SH on the active site of AM. A change in the Ca-ATPase activity of AM stored at both temperatures was almost concordant with changes in the total SHs and insoluble fraction.

The initial activities of Mg(EGTA)-ATPase of all samples were very low. The Mg(EGTA)-ATPase activity of both samples increased gradually during storage (Table V). The Ca sensitivity of unfrozen samples was quite high but decreased during the first 4 and 8 weeks of storage at -20 and -40 °C, respectively, and then decreased rapidly during prolonged storage. It appeared that the loss of Ca sensitivity of AM occurred in samples stored at -20 °C sooner than it did at -40 °C.

Table V. Effect of the Storage Temperature on Mg(EGTA)-ATPase and Mg(Ca)-ATPase Activities and Ca Sensitivity of Actomyosin during Frozen Storage

temp, °C	storage time, weeks	Mg(EGTA)-ATPase act.[a]	Mg(Ca)-ATPase act.[a]	Ca sensitivity,[b,c] %
-20	unfrozen	0.014	0.557	97.5 a
	0	0.020	0.257	92.3 b
	2	0.014	0.132	89.1 b
	4	0.017	0.042	60.6 c
	6	0.027	0.043	36.5 d
	8	0.028	0.043	35.9 d
	12	0.037	0.040	7.5 e
-40	unfrozen	0.014	0.557	97.5 a
	0	0.018	0.312	94.1 ab
	2	0.020	0.243	91.7 b
	4	0.021	0.149	87.2 c
	6	0.024	0.109	78.0 d
	8	0.027	0.087	68.6 e
	12	0.032	0.062	48.3 f

[a] The Mg(EGTA)-ATPase and Mg(Ca)-ATPase activities are expressed as micromoles of inorganic phosphate released within 1-min reaction at 25 °C per milligram of protein. [b] The Ca sensitivity was calculated as follows: Ca sensitivity = (1 - ATPase act. without Ca/ATPase act. with Ca) × 100%. [c] Values in the same column bearing different letters differ significantly ($p < 0.01$).

The loss of Ca sensitivity of myofibrillar proteins during icing was considered to result from the modification of actin–myosin interaction caused by the oxidation of SHs in myosin, instead of the hydrolysis of tropomyosin and troponins by proteases (Seki and Hasegawa, 1978; Seki and Iwabuchi, 1978; Shitamura and Seki, 1978; Seki et al., 1979). In this study, an increase in the insoluble fractions (Table I), a decrease in the total SHs (Table III), and the loss of Ca sensitivity (Table V) suggested that the oxidation of SHs occurred in AM molecules during frozen storage at a much faster rate at -20 °C than at -40 °C.

Effect of Reductants on the Denaturation of Actomyosin. To investigate the effect of reductants on the recovery of solubility of freeze-denatured AM, $NaBH_4$ and $NaNO_2$ were added to AM that had been stored at -20 and -40 °C for 6 and 8 weeks. An increase in the salt-soluble fraction and a decrease in the insoluble fractions of all samples were observed (Table VI). It was considered that the added reductants might be used to reduce the disulfides formed during freezing and frozen storage, leading to the recovery of solubility in 0.6 M KCl as well as a

Table VI. Effect of Reductants on the Solubility of Tilapia Hybrid Actomyosin[a] Held at -20 and -40 °C for 6 and 8 Weeks[a]

storage temp, °C	storage time, weeks		control	$NaNO_2$ added	$NaBH_4$ added
-20	6	I[b]	51.9 b[c]	69.7 a	70.3 a
		II	2.1 b	5.2 a	5.0 a
		III	46.0 a	25.1 b	24.7 b
	8	I	50.8 b	68.8 a	69.5 a
		II	3.5 b	5.9 a	6.7 a
		III	45.7 a	25.3 b	23.8 c
-40	6	I	67.7 b	71.2 a	70.7 a
		II	2.7 b	6.3 a	6.2 a
		III	29.6 a	22.5 b	23.1 b
	8	I	66.7 b	69.6 a	71.3 a
		II	2.3 c	7.0 a	5.2 b
		III	31.0 a	23.4 b	23.5 b

[a] The solubility was expressed as the percent ratio relative to the unfrozen actomyosin. [b] Key: I, salt-soluble; II, urea-soluble; III, insoluble. [c] Values in the same row bearing different letters differ significantly ($p < 0.01$).

decrease in the insoluble proteins. These data suggested that native proteins could be recovered by the addition of reductants to denatured proteins. This study and previous work (Jiang et al., 1988a,b) provide evidence to support the studies by Jiang et al. (1984, 1986) and Lan et al. (1987), who reported that the addition of reductants improved the gel strength of minced fish product prepared from freeze-denatured cuttlefish, cod, mackerel, and lizard fish muscle.

In summary, from the increase in insoluble fractions and the decrease in total SHs of tilapia hybrid AM during frozen storage, the formation of disulfides might be presumed to be important in protein freeze denaturation. The formation of disulfides and the decrease in solubility and Ca-ATPase activity occurred at a much faster rate at -20 °C than at -40 °C. When reductants were added to freeze-denatured tilapia hybrid AM, they increased the recovery of salt-soluble protein and decreased the insoluble fractions.

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