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# Thermal stability of myofibrillar protein from Indian major carps

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**Abstract: The characteristics and stability of natural actomyosin (NAM) from rohu (*Labeo rohita*), catla (*Catla catla*) and mrigal (*Cirrhinus mrigala*) were investigated. The total extractable actomyosin (AM) was higher (7.60 mg ml$^{-1}$) in the case of rohu compared with that from catla and mrigal (5 mg ml$^{-1}$). Although the specific AM ATPase activity was similar (0.43–0.5 μmol P min$^{-1}$ mg P$^{-1}$) among the three species, the total ATPase activity was lower in mrigal (25 μmol g$^{-1}$ meat) compared with the other species (37 μmol g$^{-1}$ meat). The inactivation rate constants ($k_d$) of AM Ca ATPase activity showed differences in the stabilities of actomyosin among these fish, the actomyosin from catla being least stable. The NAM from these species was stable up to 20 °C at pH 7.0. Catla AM became unstable at 30 °C, while rohu and mrigal AM could withstand up to 45 °C. The thermal denaturation with respect to solubility, turbidity, ATPase activity, sulphhydryl group and surface hydrophobicity showed noticeable changes at around these temperatures.**


**Keywords:** myofibrillar protein; Indian fish; carp; thermal denaturation; solubility; turbidity; ATPase activity; SH groups; surface hydrophobicity

## INTRODUCTION

Among the freshwater fish produced in India, the major carp of India—rohu, catla and mrigal–predominate, with a market share of over 90%. These fish are usually consumed fresh. They are sold fresh direct from the farm, or iced and transported to internal markets. During July to September every year when marine fishing is banned, there is strong demand for these fish, but in other seasons they do not fetch a good return for the producers. There are suggestions to use these fish for the production of surimi and other value-added products.

Fish muscle myofibrillar proteins are relatively unstable.[1] The functional properties of protein are directly related to the quality of myofibrillar protein[2,3] and the properties of myofibrillar protein determine the quality of mince-based products.[4] Aggregation and denaturation of myofibrillar protein occur during frozen storage, affecting the texture,[5–7] as well as the gel-forming characteristics of the mince.[8–11] The stability of fish myofibrillar protein is species-dependent[12,13] and there is evidence relating the stability of actomyosin to the habitat temperature.[14,15] There are other constituents in the meat, such as free fatty acids,[16] free amino acids and nucleotides,[17] that affect the stability of protein during frozen storage.

Protein denaturation involves the formation of intermolecular aggregates through hydrogen, hydrophobic[18,19] and disulphide bonds,[20] making denaturation an irreversible process. Changes in turbidity and light scattering,[21,22] solubility[23] and ATPase activity[24–26] have been reported during protein denaturation. The aim of this study was to investigate the stability and properties of natural actomyosin (NAM) from three major freshwater carp of India, ie rohu, catla and mrigal, and to provide information for the storage and processing of these fish.

## MATERIALS AND METHODS

Pre-spawning Indian major carp, ie rohu (*Labeo rohita*), catla (*Catla calta*) and mrigal (*Cirrhinus mrigala*) weighing about 500 g each were brought from a fish farm about 15 km from the laboratory in iced conditions and were processed on reaching the laboratory (within 30 min). Three separate collections were made for each fish. The pre-rigor fish were cleaned, filleted and mince was separated using a Baader-694 (New Bedford, MA, USA) deboning machine. The mince was used for actomyosin extraction. All experiments were done in triplicate.

### Preparation of actomyosin

For actomyosin (AM) extraction[17] a 10 g portion of meat was homogenized with 90 ml of chilled buffer

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solution (0.6 M KCl, pH 7.2) in a Polytron homogenizer Model PT3000 (Kinematica, Switzerland) at 10 000 rpm for 2 min. The extract was centrifuged using a Remi refrigerated centrifuge (Remi R24, Bombay, India), at 5000 × *g*, 0 °C, for 20 min. The supernatant was diluted to a salt concentration of 0.2 M with chilled distilled water and the AM was collected by centrifugation at 5000 × *g*, 0 °C, for 20 min. The precipitate was dissolved in chilled buffer and subjected to the following analyses.

### Protein estimation

Proteins in the extracted solutions were estimated by Biuret reaction[27] using alkaline copper sulphate reaction and measuring the colour developed at 540 nm using a Spectronic 20 spectrophotometer (Spectronic Instruments, Rochester, NY, USA). Bovine serum albumin (Sigma, St Louis, MO, USA) was used as standard.

### Determination of the stability of actomyosin

The heat stability experiment was carried out with a concentration of 5 mg ml$^{-1}$ protein solution and the analytical experiments were carried out using 1 mg ml$^{-1}$ protein solution. About 10 ml portions of the actomyosin solution were put into a series of test tubes and kept on ice. Test tubes were transferred to water baths maintained at different temperatures, viz. 10, 20, 30, 40, 45, 50, 55 and 60 °C. The temperatures of the protein solutions were monitored using mercury thermometers and the tubes were placed in an ice bath immediately on attaining the respective temperatures.

### Turbidity

The AM solution after heat treatment was diluted to 1 mg ml$^{-1}$ with 0.6 M KCl buffer and the absorbance at 660 nm was expressed as absorbance mg$^{-1}$ ml$^{-1}$ of protein.[28]

### Solubility

The heat-exposed natural actomyosin was centrifuged at 7500 × *g* for 15 min. The concentration of the protein in solution was determined by the Biuret method[27] and expressed as soluble protein (mg ml$^{-1}$).

### Ca-ATPase activity

For the ATPase activity assay,[17] 1 ml of actomyosin (1 mg ml$^{-1}$) was added to a mixture of 0.5 ml of 0.5 M Tris–maleate buffer (pH 7), 0.5 ml of 0.2 M $CaCl_2$, 7.5 ml of distilled water and finally 0.5 ml 20 mM adenosine triphosphate (ATP) solution (pH 7). The reaction was stopped by adding 5 ml 150 g kg$^{-1}$ trichloroacetic acid, 3 min after the addition of ATP. The inorganic phosphate released at 25 °C was measured to calculate the ATPase activity, reported as inorganic phosphate liberated mg$^{-1}$ min$^{-1}$. The inactivation constant for ATPase was calculated[29] using the equation $k_d = (\ln C_0 - \ln C_t)/t$ where $C_0$ = Ca-ATPase activity before incubation and $C_t$ after $t$ s of incubation

### Sulphhydryl group

Total sulphhydryl groups[30] were determined in the heat-treated actomyosin solutions after centrifugation using 5,5′dithiobis(2 nitrobenzoic acid) (DTNB, Sigma) and measuring the colour developed at 420 nm using a Spectronic 21 spectrophotometer.

### Surface hydrophobicity

The surface hydrophobicity of both the control and heated actomyosin solutions was determined using *cis*-parinaric acid as fluorescence probe[31] in a Shimadzu spectrofluorophotometer model RF 540 (Shimadzu, Kyoto, Japan). The emission and excitation spectra were measured at 325 and 420 nm, respectively. The initial slope of the fluorescence intensity vs protein concentration was used as an index of the protein hydrophobicity.

### Statistical analysis

Values are means ± SD for triplicates in each group and significance of the differences between mean values were determined by one-way analysis of variance coupled with Duncan's multiple comparison test using Windows-based SPSS statistical software (SPSS Inc, Chicago, Il, USA).

## RESULTS AND DISCUSSION

The actomyosin from the three species of fish in their pre-spawning stage of maturity exhibited marginally different characteristics (Table 1). The extractable NAM contents were different among the fish ($p < 0.01$) and rohu showed the highest content of 76 g kg$^{-1}$ flesh. The actomyosin content depends on the biological cycle and studies in cod have shown that pre-spawning fish have a lower content of actomyosin compared with post-spawned fish.[32]

The $Ca^{2+}$ ATPase activity of the NAM from fish is less stable than with that of mammalian species.[24,26,33] The $Ca^{2+}$ ATPase activities, both specific and total, in major carp were similar. The reactive SH contents varied among the fish, with catla showing the highest content (81 μmol g$^{-1}$ protein) followed by mrigal and rohu (62 and 52 μmol g$^{-1}$ protein, respectively). The values of SH groups of actomyosin from catla were significantly different from those of rohu ($p < 0.001$) and mrigal ($p < 0.01$) and the values for mrigal were also significantly different from those of rohu ($p < 0.001$). In comparison, harp seal muscle,[34] Pacific mackerel and Alaska Pollock,[35] squid[36] and beet[37] had sulphhydryl contents of 63, 76, 70, 79 and 88 μmol g$^{-1}$ protein, respectively. It was also reported in rainbow trout that in the undenatured protein only 90% of the total SH groups were reactive towards DTNB and the remaining 10% were masked in the actomyosin molecule.[35,30] There were no significant differences in the CPA fluorescence values between rohu and calta but the values for mrigal were significantly different from those of rohu and catla ($p < 0.01$). Changes in the actomyosin composition of mature Argentine

**Table 1.** Comparison of actomyosin characteristics from rohu, catla and mrigal[a]

| | Rohu | Catla | Mrigal |
|---|---|---|---|
| NAM content (g kg$^{-1}$ fish flesh) | 75.86 ± 0.44 | 53.70 ± 0.82a | 50.30 ± 0.09a |
| $Ca^{2+}$ ATPase, specific (μmol $P_i$ min$^{-1}$ mg$^{-1}$ protein) | 0.49 ± 0.09 | 0.43 ± 0.01 | 0.5 ± 0.08 |
| $Ca^{2+}$ ATPase, total (μmol $P_i$ g$^{-1}$ meat) | 37 ± 7.83 | 37 ± 3.81 | 25 ± 2.7 |
| Reactive SH groups (μmol g$^{-1}$ protein) | 52 ± 1 | 81 ± 3.61b | 62 ± 2.65a,c |
| CPA fluorescence intensity | 110 ± 7.5 | 110 ± 15.5 | 60 ± 2.65a,d |

[a] Values are means ± SD; $n = 3$. (a) $p < 0.01$ significantly different from rohu; (b) $p < 0.001$ significantly different from rohu; (c) $p < 0.001$ significantly different from catla; (d) $p < 0.01$ significantly different from catla.

hake (*Merlucius hubbsi marini*) are influenced by the metabolic state of the fish and are related to its reproductive cycle.[38]

## Thermal denaturation of actomyosin

Turbidity measurements provide a rough estimate of the aggregation processes of protein molecules on heating.[21,39] In rohu and mrigal the turbidity remained more or less constant up to 40 °C (Table 2) and then increased. In catla, however, the turbidity values gradually increased from 10 °C and went up considerably beyond 40 °C. This suggested the possible aggregation of NAM at 40 °C, and aggregation was extensive from 45 to 60 °C. The increase in absorbance of heated fish myosin correlated with the formation of more and larger myosin aggregates. The turbidity values were comparable to those reported in *Cyprinus carpio*,[27] where the turbidity remained relatively unchanged up to 30 °C and then increased up to 80 °C. The aggregation of myosin molecules increased with temperature in cod myosin up to 50 °C and decreased thereafter, presumably due to the disintegration of aggregates as a result of proteolytic action.[40]

Solubility remained almost unchanged up to a temperature of 40 °C in the case of rohu and decreased slightly in the case of catla and mrigal (Table 3). The decrease was 6 and 14%, respectively, for catla and mrigal. At 45 °C the solubility suddenly dropped to 29, 23 and 18% of the initial value for rohu, catla and mrigal actomyosin, respectively. Heating further to 60 °C increased the solubility in rohu compared with the other two. Myosin rod, which is responsible for the salt solubility of myosin, is denatured very rapidly during heat treatment.[41] Disintegration of aggregates as a result of proteolytic action at higher temperatures[40] could also contribute to solubility at higher temperatures. Myofibrillar proteins start coagulating on heating at 30–40 °C and the coagulation is nearly completed at 55 °C, with decrease in solubility.

The $Ca^{2+}$ ATPase activity decreased with increasing temperatures (Table 4). The $Ca^{2+}$ ATPase activity remained more or less constant up to 40 °C in rohu and mrigal, and then rapidly decreased on heating from 40 to 50 °C. However, in catla there was a loss of ATPase activity (about 27%) above 20 °C, followed by a major drop in activity above 40 °C. Heat treatment above 50 °C resulted in almost complete loss of activity. The differences in the stability of actomyosin ATPase from *Cyprinus carpio* has been

**Table 3.** Changes in the solubility (mg ml$^{-1}$)[a] of natural actomyosin after heat treatment

| Temperature | Rohu | Catla | Mrigal |
|---|---|---|---|
| 10 | 5.09 ± 0.1c | 4.98 ± 0.4b | 5.28 ± 0.1c |
| 20 | 5.15 ± 0.3c | 4.93 ± 0.5b | 5.28 ± 0.1c |
| 30 | 4.97 ± 0.2c | 4.76 ± 0.9b | 4.52 ± 0.2b |
| 40 | 5.09 ± 0.1c | 4.71 ± 0.1b | 4.56 ± 0.2b |
| 45 | 1.48 ± 0.4b | 1.13 ± 0.2a | 0.96 ± 0.1a |
| 50 | 2.56 ± 0.3b | 1.05 ± 0.1a | 0.76 ± 0.2a |
| 55 | 2.84 ± 0.1b | 1.18 ± 0.1a | 1.01 ± 0.1a |
| 60 | 2.96 ± 0.3a | 1.20 ± 0.2a | 1.08 ± 0.1a |

[a] Values are means ± SD; $n = 3$; values in the same row bearing unlike letters differ significantly ($p < 0.01$).

**Table 2.** Changes in the turbidity[a] of natural actomyosin after heat treatment

| Temperature | Rohu | Catla | Mrigal |
|---|---|---|---|
| 10 | 0.22 ± 0.01b | 0.35 ± 0.01c | 0.29 ± 0.02e |
| 20 | 0.23 ± 0.02b | 0.39 ± 0.02c | 0.30 ± 0.01e |
| 30 | 0.23 ± 0.04c | 0.40 ± 0.01c | 0.30 ± 0.05e |
| 40 | 0.25 ± 0.06b | 0.40 ± 0.03b | 0.31 ± 0.04e |
| 45 | 0.34 ± 0.04a | 0.43 ± 0.01b | 0.38 ± 0.01d |
| 50 | 0.36 ± 0.04a | 0.46 ± 0.02b | 0.43 ± 0.02c |
| 55 | 0.40 ± 0.03a | 0.50 ± 0.05a | 0.48 ± 0.01b |
| 60 | 0.42 ± 0.03a | 0.50 ± 0.01a | 0.50 ± 0.02a |

[a] Absorbance mg$^{-1}$ ml$^{-1}$ in 0.6 M KCL at 660 nm. Values are mean ± SD; $n = 3$; values in the same row bearing unlike letters differ significantly ($p < 0.01$).

**Table 4.** Changes in the ATPase specific activity[a] of natural actomyosin after heat treatment

| Temperature | Rohu | Catla | Mrigal |
|---|---|---|---|
| 10 | 0.49 ± 0.1c | 0.43 ± 0.01c | 0.41 ± 0.01c |
| 20 | 0.50 ± 0.1c | 0.44 ± 0.1c | 0.42 ± 0.03c |
| 30 | 0.50 ± 0.1c | 0.32 ± 0.02c | 0.40 ± 0.01c |
| 40 | 0.48 ± 0.02c | 0.31 ± 0.02c | 0.40 ± 0.02c |
| 45 | 0.18 ± 0.03b | 0.20 ± 0.03b | 0.32 ± 0.02b |
| 50 | 0.01 ± 0.01a | 0.07 ± 0.01a | 0.01 ± 0.01a |
| 55 | —a | 0.05a | 0.01a |
| 60 | —a | 0.05a | 0.01a |

[a] Inorganic phosphate liberated at pH 7 (mg ml$^{-1}$ actomyosin min$^{-1}$). Values are means ± SD; $n = 3$; values in the same row bearing unlike letters differ significantly ($p < 0.01$).

related to habitat temperature[27,45] and to the maturity stage of the fish.[42]

When stabilities of actomyosin at 40 and 45 °C were compared (Table 5), the stability decreased in the order rohu, mrigal and catla. However, catla actomyosin was the most unstable at 30 °C. The inactivation constants of all the fish increased with increasing temperature. Considering the $k_d$ values up to 45 °C, rohu actomyosin was found to be the most stable. The $k_d$ values of rohu, catla and mrigal at 40 °C were higher than those reported for milk fish, tilapia, common carp, tuna, sea bream and rainbow trout, which had $k_d$ values of 41.3, 37.5, 58.7, 15.3, 33.8 and 46.1 $\times 10^{-5}$, respectively.[29]

The available SH groups (μmol $g^{-1}$) in rohu increased by up to 5% on heating to a temperature of 40 °C and then dropped suddenly by 57% on heating to 45 °C (Table 6). This was followed by marginal increases of 4, 23 and 37%, respectively, on heating to temperatures of 50, 55 and 60 °C. In the case of catla, a 6% increase was seen on heating to 40 °C. The SH groups dropped by 23% at 45 °C followed by small increases of 2 and 4% on further heating to 55 and 60 °C, respectively. In mrigal the changes in SH groups were similar to those of rohu with an initial increase up to 40 °C (6%), followed by a major drop at 45 °C (43%). The value then increased by 15, 23 and 29% on further heating to 50, 55 and 60 °C.

The surface-reactive sulphhydryl groups increased marginally between 20 and 40 °C and decreased sharply from 40 to 45 °C. The increase seen in sulphhydryl groups on heating the actomyosin from 20 to 40 °C was due to the emergence of SH groups on the surface as a result of the unfolding of actomyosin molecules at higher temperatures. The decrease seen above 50 °C could be related to the formation of disulphides, as reported for the heating of mackerel meat.[35] Similar changes in the SH groups were reported in harp seal muscle proteins.[34] However, there are reports indicating that the exposure of sulphhydryl groups and the formation of disulphide bonds occur at lower temperatures in extracted actomyosins.[27,43]

The decrease in the sulphhydryl groups in trout and rabbit actomyosin during storage at 0,[20] 4[44] and −20 °C[45] clearly indicated the oxidation of SH groups at active sites for ATPase activity in the myosin head. However, subsequent studies[46–49] did not indicate the involvement of the myosin head in the oxidation process, but the sulphhydryl groups in the tail portion of the myosin molecule are assumed to play a role in the oxidation of actomyosin.

The CPA fluorescence intensity (Table 7) in rohu increased by 58% on heating to a temperature of 40 °C and then increased substantially by about 3-fold on heating to 45 °C. This fluorescence increased further with further increase in temperature. In the case of catla the change was rather quick, increasing by 77% on heating to 40 °C, and to almost four times the initial value on heating to 60 °C. In the case of mrigal an increase in fluorescence intensity of 216% was seen on heating to 40 °C and increased 6-fold on heating to 60 °C. The increase in CPA fluorescence intensity was substantial above 40 °C. These results indicated that hydrophobic interactions among the actomyosin molecules occurred above 40 °C and most extensively at higher temperatures, suggesting the emergence of hydrophobic aromatic amino acid residues on the surface of the actomyosin molecule, leading to hydrophobic interaction. Similar increases in ANS-induced fluorescence have been reported above 30 °C during the heat treatment of actomyosin from common carp[27] and during the freezing of fish.[50]

**Table 5.** Effect of the incubation temperature on the rate constants for inactivation[a] of actomyosin $Ca^{2+}$ ATPase of rohu, catla and mrigal

| | $k_d \times 10^{-3}$, $s^{-1}$ | | |
|---|---|---|---|
| Temperature, °C | Rohu | Catla | Mrigal |
| 10 | 0.00 | 0.00 | 0.00 |
| 20 | 0.00 | 0.28 ± 0.1e | 0.00 |
| 30 | 0.00 | 3.38 ± 0.3d | 0.20 ± 0.1e |
| 40 | 0.16 ± 0.1b | 3.87 ± 0.2d | 0.43 ± 0.1e |
| 45 | 7.07 ± 0.9b | 6.17 ± 0.6c | 13.50 ± 2.1d |
| 50 | 30.20 ± 1.0a | 13.90 ± 2.1b | 20.70 ± 1.0c |
| 55 | 25.90 ± 3.5a | 16.10 ± 0.1a | 28.50 ± 2.7b |
| 60 | 28.20 ± 2.4a | 15.70 ± 1.0a | 35.70 ± 2.6a |

[a] $k_d = (\ln C_0 - \ln C_t)/t$ where $C_0$ = Ca-ATPase specific activity before incubation, and $C_t$ = Ca-ATPase specific activity after incubation time, $t$ (s). Concentration of actomyosin 1 mg $ml^{-1}$; pH 7.0. Means of three determinations from each sample were used to calculate $k_d$ values. Values in the same row bearing unlike letters differ significantly ($p < 0.01$).

**Table 6.** Changes in the SH groups (μmol $g^{-1}$)[a] of natural actomyosin after heat treatment

| Temperature, °C | Rohu | Catla | Mrigal |
|---|---|---|---|
| 10 | 52 ± 1c | 81 ± 4b | 62 ± 3d |
| 20 | 51 ± 4c | 85 ± 1b | 62 ± 2d |
| 30 | 52 ± 1c | 86 ± 2b | 67 ± 3d |
| 40 | 55 ± 1c | 86 ± 1b | 66 ± 3d |
| 45 | 22 ± 1b | 81 ± 7a | 35 ± 3c |
| 50 | 24 ± 4b | 62 ± 5a | 26 ± 4b |
| 55 | 34 ± 4a | 60 ± 1a | 21 ± 4b |
| 60 | 39 ± 4a | 59 ± 4a | 17 ± 3a |

[a] Values are mean ± SD; $n = 3$; values in the same row bearing unlike letters differ significantly ($p < 0.01$).

## CONCLUSION

Thermal denaturation studies on the muscle proteins as NAM from the major carp of India indicate the thermal stability of the meat proteins on exposure to different temperatures. Considerable conformational change took place in the myosin head portion above 40 °C in the case of rohu and mrigal. Protein from catla appears to be more labile to denaturation and conformational changes took place even at lower temperatures of 20–30 °C. The unfolding of protein

**Table 7.** Changes in the CPA fluorescence (arbitary units)[a] of natural actomyosin after heat treatment

| Temperature, °C | Rohu | Catla | Mrigal |
|---|---|---|---|
| 10 | 110 ± 8d | 110 ± 16d | 60 ± 3e |
| 20 | 158 ± 7c | 180 ± 5c | 60 ± 8e |
| 30 | 170 ± 18c | 180 ± 13c | 120 ± 13d |
| 40 | 174 ± 10c | 195 ± 4c | 190 ± 13c |
| 45 | 320 ± 16b | 350 ± 5b | 210 ± 11c |
| 50 | 340 ± 18b | 370 ± 14b | 240 ± 19b |
| 55 | 380 ± 26a | 410 ± 16a | 350 ± 4a |
| 60 | 390 ± 9a | 415 ± 14a | 355 ± 6a |

[a] Values are mean ± SD; $n = 3$; values in the same row bearing unlike letters differ significantly ($p < 0.01$).

molecules increased the hydrophobic amino acid residues and the number of SH groups at the surface. The NAM from rohu was the most stable followed by those from mrigal and catla. Interaction between the hydrophobic amino acids and the SH groups led to the aggregation of molecules, which began slowly at 40 °C and became extensive between 45 and 60 °C.

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