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Changes in Rohu (*Labeo rohita*) Actomyosin During Frozen Storage: Protein Denaturation or Coagulation?

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Changes taking place in actomyosin extracted from *Labeo rohita* (Rohu) during storage at -20°C were studied with a view to find out whether these changes were denaturation or coagulation of proteins. Changes in solubility, Ca^{2+} ATPase activity and Mg^{2+} ATPase activity were monitored for a period of 10 weeks. The irreversible loss of solubility and Ca^{2+} and Mg^{2+} ATPase activities indicate that an irreversible aggregation of proteins, probably through covalent disulphide bonds is taking place during frozen storage of rohu actomyosin and this is suggestive of protein coagulation, as distinct from protein denaturation.

Key words : Actomyosin, denaturation, coagulation, ATPase activity.

Early work on the changes that occur in fish myofibrillar proteins during frozen storage have described these changes as protein denaturation (Connell, 1959; 1962; Dyer, 1951). Though various aspects of the changes of myofibrillar proteins of fish muscle during frozen storage were investigated in subsequent years, the primary concept of denaturation of these proteins remained unaltered (Love, 1962; Buttkus, 1970; Arai & Takashi, 1973; Oguni *et al.*, 1975). However, advances in our knowledge of protein chemistry has helped in distinguishing between denaturation and coagulation of proteins. Some demarcation between the changes that occur on denaturation and coagulation of proteins is now possible (Table 1) (Dill & Shortle, 1991; Saini & Kaur, 1996; Zubay, 1989). Under these circumstances an attempt is made to re-evaluate the changes that occur on prolonged frozen storage of fish myofibrillar proteins. The results of the present study and those published earlier are interpreted using these concepts in protein chemistry to validate that coagulation, and not denaturation of rohu actomyosin occurs on frozen storage for 10 weeks.

Materials and Methods

The myofibrillar proteins (actomyosin) of *Labeo rohita* (rohu) was extracted as described earlier (Rao, 1983a, b). The protein concentration was estimated by the biuret method (Wootton & Freeman, 1982) using bovine serum albumin as standard. All chemicals used in the experiments were of reagent grade. Aliquots of 5 ml of extracted actomyosin (6 mg/ml in 0.71 M KCl) were frozen at -20°C and stored at -20°C for 10 weeks. Samples were drawn at regular intervals and thawed at 20°C for 4 h. Thawed sample was homogenized in a hand homogenizer, stirred vigorously in a vortex mixer and analysed. Actomyosin extractability, Ca^{2+} ATPase activity and Mg^{2+} ATPase activity (superprecipitation) were evaluated as per the procedure reported earlier (Rao, 1983b).

Results and Discussion

Changes in solubility, Ca^{2+} ATPase activity and Mg^{2+} ATPase activity during frozen storage of rohu actomyosin are presented in Table 2. Proteins on coagulation form a water insoluble aggregated clot with significant reduction in their viscosity. When rohu actomyosin was stored frozen at -20°C there was a significant decrease in its solubility even after 1 week of frozen storage. As period of frozen storage increased, the solubility of the protein continuously decreased and at the end of 10 weeks of storage the solubility was lower than 10% of the original (Table 2) and the protein solution was in the form of a clot.

Efforts to redissolve the above clot in 0.7 M saline was not successful and almost the entire fraction of frozen stored rohu actomyosin remained undissolved. It was resistant to solubilization by saline or by saline along with a mixture of sodium dodecyl sulphate (SDS) and urea, as reported earlier (Rao, 1985). These experiments confirmed that rohu actomyosin on frozen storage for 10 weeks formed an insoluble aggregated mass. Similar irreversible loss of solubility has been reported in the case of cod myofibrillar proteins also (Connell, 1959; 1962).

The biological activity of actomyosin as measured by Ca^{2+} ATPase and Mg^{2+} ATPase activity decreased during frozen storage and the values were of 1.5% and negative respectively when compared to the original unfrozen freshly prepared rohu actomyosin, after 10 weeks at -20°C (Table 2).

These observations agree with the criteria indicated by Dill & Shortle (1991) for protein coagulation. Similar changes have been reported for carp actomyosin during frozen storage (Tsuchiya *et al.*, 1975) and for Atlantic croaker actomyosin, on heating (Liu, *et al.*, 1982). These changes have been identified as coagulation of protein.

Table 1. Characteristic changes taking place during denaturation and coagulation of protein

Denaturation	Coagulation
Destruction in part or in total of the native structure of a protein	Native structure destroyed with random polypeptide aggregation to form a clot
Viscosity of protein solution altered or unaltered	Viscosity of protein solution significantly reduced
Solubility may be affected or unaffected	Protein always form an insoluble clot
Significant special reorientation of water of hydration	Significant permanent loss of water of hydration
Partial or near total loss of biological activity	Near total loss of all biological activity
Reversible or irreversible depending on the type of protein	Always an irreversible process with complete destruction of native protein
No random formation of covalent S-S bonds between polypeptides containing cysteine	Random formation of covalent S-S bonds between polypeptides containing cysteine
Can occur at any pH	Favoured at isoelectric pH

Table 2. Changes in solubility, Ca^{2+} ATPase activity and Mg^{2+} ATPase activity in rohu actomyosin during storage at -20°C

	Storage time (weeks)				
	1	3	5	6	10
Solubility in 0.7 M KCl (% of fresh actomyosin)	56.9	46.5	36.8	24.9	9.5
Ca^{2+} ATPase activity (% of fresh actomyosin)@	35.7	25.9	12.8	7.4	1.5
Superprecipitation (Mg^{2+} ATPase activity)*	+ve	-ve	-ve	-ve	-ve

Values are mean of four separate experiments

@ Ca^{+2} ATPase activity of fresh actomyosin was 1.6 mmoles ip/min/mg protein and was taken as 100

* Superprecipitation (clearing response) was graded based on the property of actomyosin in a homogeneous solution to separate from the mother liquid

It was observed earlier that the rohu actomyosin clot formed on frozen storage could not be solubilized in presence of SDS or urea (Rao, 1985). These reagents are reported to break hydrophobic, hydrogen and ionic interactions in protein, but not disulphide bonds (Connell, 1965). However, use of mercaptoethanol to solubilize the actomyosin clot along with SDS and urea led to its solubility and separation by PAGE (Rao, 1985). Mercaptoethanol can break disulphide bonds formed in frozen stored fish meat (Buttkus, 1974). Therefore it is possible that the actomyosin clot is formed as a result of formation of disulphide bonds. These observations further support the concept of coagulation of frozen stored rohu actomyosin, based on the criteria indicated in Table 1. Other covalent interactions like those between myofibrillar proteins and free fatty acids and their oxidation products can occur which may lead to the insolubility of these proteins. (Anderson & Ravesi, 1970; Connell, 1975). Cations like Ca^{2+} and Mg^{2+} present in frozen fish muscle are believed to play a role to stabilize lipid protein covalent interaction (Braun & Radin, 1969) which is enhanced on freezing (Shenouda & Pigott, 1975). Such interactions were also shown to occur with frozen rohu actomyosin (Rao, 1984). These were significantly prevented if the rohu actomyosin was mixed with chelating agents like EDTA, citrate and glutamate before frozen storage (Rao, 1984). These covalent interactions along with disulphide bond formation lead to the clot formation of frozen stored rohu actomyosin. In view of these facts it is proposed that the irreversible loss of solubility and formation of clot observed in rohu actomyosin may be differentiated from simple denaturation of proteins and may be described as protein coagulation. Further detailed investigations are required to fully characterize these phenomena.

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