



Changes in Soluble Protein and Actomyosin during Chilled and Frozen Storage of Tilapia (*Oreochromis mossambicus*)

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Abstract

The changes in characteristics of protein from tilapia (*Oreochromis mossambicus*) as a function of storage in ice and frozen condition were evaluated. The results showed that actomyosin constituted about 57% of total soluble protein which decreased by 4% after 12 days of storage in ice due to insolubilisation. During frozen storage, however, the actomyosin decreased by 5% after one month of storage at -20°C and by 12% in 4 months. The Ca²⁺ATPase activities decreased by 43% after 4 days of storage in ice and by 69% after 2 months frozen storage. Mg²⁺ and EDTA ATPase activities were not significantly altered during ice storage but showed a decrease during frozen storage. Total sulfhydryl groups of actomyosin fraction were less (23 μM SH g⁻¹ AM) initially and then increased. Turbidity of total soluble protein increased by two folds during ice and frozen storage. SDS-PAGE pattern of protein substantiates the insolubilisation of proteins during storage in ice and at -20°C.

Keywords: Tilapia, physicochemical characteristics, denaturation, chilled and frozen storage, SDS-page

Introduction

Fish is one of the sources of good quality animal protein readily available to human population throughout the world and its demand is always growing. Inland resources have great potential to meet the ever-increasing demand for fish. Black tilapia (*Oreochromis mossambicus*) is considered as a

“weed fish” in fish farms and is characterized by its fast growth and adaptability to different environments. The production of farmed Tilapia is increasing in recent times and is mainly marketed in fresh form (Eltholth et al., 2015).

Changes in texture and quality of fish are generally related to quantity and quality of protein and it has direct relation to denaturation of protein (Shenouda, 1980; Xiong, 1997). Denaturation proceeds through any non-proteolytic modification of the unique structure of a native protein giving rise to definite changes in chemical, physical or biological properties (Shenouda, 1980) through modification in hydrogen bonds, hydrophobic interactions as well as salt linkages. Further, the stability of muscle protein during refrigerated and frozen storage depends on species, storage condition (Suyama & Konosu, 1987), environment temperature (Arai, 1974), free amino acid and nucleotide content in the meat (Jiang et al., 1987). Changes in turbidity and light scattering (Zeigler & Acton, 1984), solubility and ATPase activity (Taguchi et al., 1986) has been reported during protein denaturation.

Myofibrillar proteins, contributing to 55–65% of total protein in fish are the important protein fraction responsible for the physico-chemical properties of the protein in a food system. Myosin is one of the important myofibrillar proteins and is a relatively unstable molecule. The Ca²⁺ and Mg²⁺ ATPase activities reflect the integrity and the functionality of myosin molecule in the actomyosin complex (Roura & Crupkin, 1995; Montechia et al., 1997). The domains associated with ATPase activity are localized at the head portion of myosin and at some sites of actin tropomyosin-troponin complexes. Any structural changes in protein due to denaturation leads to alteration in the ATPase activities (Sano et al., 1994) and measurement of

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ATPase activity provides a direct measure of protein denaturation.

Fish myosin molecule is made up of approximately 4500 amino acid residues, of which, 40 are cysteine molecules (Pearson & Young, 1989). Sulfhydryl (SH) groups and disulphide bonds which play an important role in the ATPase activity of myosin, maintain the structure of native proteins and play important roles in their functional properties (Opstvedt et al., 1984) through interaction between myosin and actin during the process of muscular contraction (Needham, 1973). SH groups increase when proteins unfold and there are reports indicating the increase in number of SH groups during protein denaturation in frozen stored cod muscle (Jiang et al., 1986). Several studies demonstrated proteolytic degradation of fishes during ice and frozen storage such as blue tilapia (*Oreochromis aureus*) (Korhonen et al., 1990), carp (Nakayama et al., 1994) Chinook salmon (Jerrett et al., 1996) and skipjack tuna (Crawford et al., 1970). The present study evaluates the changes in the salt soluble proteins, ATPase activities, reactive SH groups and SDS-PAGE protein patterns in tilapia during iced and frozen storage.

Materials and Methods

Farm fresh tilapia were collected from brackish water culture pond and iced within 10 min of catch. On arrival, the fishes were weighed (110 ± 2.5 g and length 15 ± 2.5 cm) and washed thoroughly in chilled water and divided into two lots. One lot was preserved in insulated box with ice (1:1) for 14 days and other lot was frozen in a plate freezer, then packed and held in cold room at -20°C for 4 months. Samples were drawn periodically for different protein stability studies.

Crude protein in the fish muscle was estimated by Micro-kjeldahl method of AOAC (2005) and expressed in percentage. Total soluble protein was extracted as per the procedure of King & Poulter (1985). In short, the homogenised fish muscle was macerated in an ultra turrax T 25 basic homogeniser (IKA- werke, Germany) with 20 volumes of pre-cooled 5% NaCl containing 0.02 M NaHCO_3 buffer (pH 7.5), for 2 min and centrifuged at 10 000 rpm for 20 min in a refrigerated centrifuge (CPR 24, REMI, India) at 4°C . The residue was extracted again and the supernatant after centrifugation was pooled to get total soluble protein. Sarcoplasmic proteins

were separately extracted as per the method of Sankar & Ramachandran (2001) by homogenising the fish muscle with 20 volumes of pre-cooled 0.02 M NaHCO_3 buffer (pH 7.5) as per the method mentioned above.

Actomyosin was extracted according to the procedure of Jiang et al., (1987). The homogenised fish muscle was macerated with 10 volumes of chilled 0.6 M NaCl containing 0.02 M NaHCO_3 buffer (4°C , pH 7.2) for 2 min and centrifuged at 10 000 rpm for 20 min at 4°C . The supernatant was diluted with 2 volumes of chilled distilled water and kept in refrigerator overnight to precipitate the actomyosin. The precipitated actomyosin was separated again by centrifugation at 10 000 rpm for 20 min at 4°C . The actomyosin residue was then reconstituted in the same buffer. The protein content in all the fractions was quantified by the biuret method (Gornall et al. 1949). The actomyosin fraction was then diluted with buffer to 1mg ml^{-1} for different assays.

Stability of the actomyosin fraction during storage was carried out by evaluating the changes in ATPase activities, SH groups, turbidity and SDS-PAGE protein patterns. The simple random sampling was carried out during the study period *viz.*, once in 2 days during iced and once in a month during frozen storage.

Actomyosin (AM) solution diluted with chilled buffer (0.6M NaCl, pH 7.2) was used for the ATPase activity assay (Jiang et al., 1987). The reaction mixture consisted of 1 ml of AM solution, 0.5 ml of 0.5 M Tris-maleate buffer (pH 7.0), 0.5 ml of 0.1 M CaCl_2 or MgCl_2 or EDTA and 7.5 ml of distilled water. Reaction was initiated by the addition of 0.5 ml of 20 mM adenosine 5' triphosphate (ATP) solution (pH 7.0) for 3 min. A reagent blank (without sample) was also prepared. The reaction was then stopped by the addition of 5 ml of 15% trichloroacetic acid (TCA) solution. The liberated inorganic phosphate from ATP was estimated by subsequent reaction with ammonium molybdate to form phosphomolybdic acid in the presence of acid, followed by the reduction to a dark blue complex in the presence of amino naphthol sulphonic acid (ANSA) (Fiske & Subbarow, 1925). The intensity of colour is proportional to the phosphate concentration. The absorbance was read at 412 nm in a spectrophotometer (Spectronic 20 Genesys, USA). The Ca^{2+} , Mg^{2+} and EDTA dependant ATPase enzymes were expressed as $\mu\text{M Pi}$ liberated per mg protein min^{-1} .

Free SH group residues in the actomyosin fraction was quantified by Ellman's reagent, 5,5'-dithiobis (2-nitrobenzoic acid) [DTNB] (Sedlak & Lindsay, 1968). The reaction mixture consisted of 1 ml of diluted actomyosin solution (1 mg ml^{-1}), 1.5 ml of 0.2 M Tris buffer (pH 8.2), 0.1 ml of 0.01 M 5' 5 dithio bis-2-nitrobenzoic acid (DTNB) and 7.9 ml of absolute methanol. A reagent blank (without sample) and a sample blank (without DTNB) were also prepared. A standard was run simultaneously taking cysteine hydrochloride at 0.02 μM to 0.2 mM concentrations. The reaction was allowed for 30 min with occasional shaking and then filtered through Whatman filter paper (No. 2) and the absorbance was read in a spectrophotometer at 412 nm. The sulfhydryl groups were expressed as mM g^{-1} of protein.

Turbidity of the different diluted protein fraction (1 mg ml^{-1}) viz., sarcoplasmic and total soluble proteins were determined by measuring the absorbance at 660 nm in a spectrophotometer and the results were expressed as absorbance mg^{-1} of protein (Sano et al., 1994).

Changes in actomyosin and total soluble protein fraction were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) using 7.5% separating gel and 1% stacking gel. Electrophoresis was run initially at 50 V till the bands enters the running gel and then at 200 V. The gel was then stained with Coomassive blue-R for 30 min and then destained with 7% acetic acid.

Results were presented as means \pm SD and significance of the differences between mean value was determined by one-way analysis of variance

(ANOVA) coupled with the Duncan's multiple range test using windows based statistical software SPSS 10. p-value of less than 0.05 was considered to be significant.

Results and Discussion

Total soluble proteins formed 61% of total protein in the fish in fresh condition which decreased by 23% during storage in ice upto 14 days (Table 1). The decrease was significant on day 2 and day 10 ($p < 0.05$). The sarcoplasmic proteins also decreased from the initial 21 to 13 % on day 14 but the decrease was very gradual upto 7 days ($p < 0.05$) followed by a rapid decrease. The decrease was mainly due to the leaching out of sarcoplasmic proteins in the melt ice. Srikar (1979) reported a decrease in salt soluble protein of croakers and pink perch during ice storage due to the protein aggregation leading to insolubilisation of myofibrillar protein (MFP). Mohan et al., (2006) reported the drop in solubility of sarcoplasmic protein due to leaching/aggregation behaviour of the proteins under the conditions of storage. The decrease in protein extractability could be related to the possible unfolding of MFP exposing the hydrophobic groups to the exterior (Sarma et al., 1999) and the relation between protein insolubility and hydrophobicity has been reported elsewhere (Sankar & Ramachandran, 2005; Sano et al., 1994). Further, formation of cross linking between myosin and actin in the post-rigor muscle could also contribute to the insolubilisation (Offer & Trinick, 1983).

Changes in the actomyosin fraction were marginal with about 4% during ice storage. Actomyosin initially constituted to about 57% of total soluble

Table 1. Changes in protein solubility during iced & frozen storage

Storage, days	TSP	Iced storage SP	AM	Storage, Months	TSP	Frozen storage SP	AM
Fresh	61.58 \pm 2.58 ^a	21.26 \pm 1.53 ^a	35.23 \pm 2.53 ^a	Fresh	61.58 \pm 2.58 ^a	21.26 \pm 1.53 ^{b,c}	35.23 \pm 2.53 ^a
2	56.69 \pm 2.29 ^b	19.88 \pm 1.30 ^{a,b}	33.89 \pm 1.80 ^{a,b}	Frozen	57.89 \pm 2.30 ^b	26.01 \pm 1.60 ^a	30.24 \pm 0.99 ^b
4	56.62 \pm 1.54 ^b	19.77 \pm 1.18 ^{a,b}	32.12 \pm 1.36 ^b	1	52.06 \pm 1.50 ^c	20.64 \pm 0.98 ^{b,c}	30.75 \pm 0.95 ^b
7	56.28 \pm 2.03 ^b	18.85 \pm 0.97 ^b	33.14 \pm 1.14 ^{a,b}	2	51.62 \pm 0.92 ^{c,d}	22.14 \pm 0.78 ^b	24.41 \pm 0.59 ^c
10	50.29 \pm 0.85 ^c	13.66 \pm 0.78 ^c	32.72 \pm 1.09 ^{a,b}	3	50.86 \pm 0.62 ^{c,d}	24.9 \pm 0.76 ^a	23.72 \pm 0.47 ^c
12	48.95 \pm 0.65 ^{cd}	13.97 \pm 0.62 ^c	31.42 \pm 0.96 ^b	4	48.63 \pm 0.51 ^d	19.69 \pm 0.54 ^c	25.01 \pm 0.35 ^c
14	46.95 \pm 0.76 ^{cd}	13.99 \pm 0.43 ^c	31.35 \pm 0.34 ^b	—	—	—	—

Values (Dry weight basis) with different letters within the columns are significantly different ($p < 0.05$). TSP - total soluble protein, SP - sarcoplasmic protein, AM - actomyosin (represented as percentage of total protein)

protein and later increased gradually to 66% by 14th day indicating that the insolubilisation of actomyosin was only 11% against the 34% loss in sarcoplasmic proteins. Similar observations were reported in the extractable actomyosin from pink perch during ice storage (Sarma et al., 1999).

Changes in protein fractions during frozen storage of tilapia (Table 1) showed that the total soluble protein decreased significantly from 61 to 48% during storage, while the sarcoplasmic protein showed only a marginal (7%) decrease. This showed that the leaching of sarcoplasmic protein was lower in frozen storage compared to that in ice storage (34%). At the end of storage, actomyosin constituted only about 51% of total soluble protein, which was very low when compared to ice stored tilapia, indicating a much higher loss in chill storage compared to frozen storage. A 6% loss of actomyosin (percentage of total soluble protein) was noticed during the frozen storage of tilapia.

The Ca²⁺, Mg²⁺ and EDTA ATPase activities of actomyosin fraction were 0.81, 0.50 and 0.58 $\mu\text{M Pi/mg protein/min}$, respectively in the fresh tilapia (Fig. 1). The Ca²⁺ ATPase activity decreased quickly by 43% ($p>0.05$) on day 4 with no further change during the storage period. Ca²⁺ ATPase activity of sardine muscle decreased by 50% on day 6, followed by concomitant degradation of myosin heavy chain (Sekiet al., 1990) but only a slight decrease in Ca²⁺ ATPase activity was reported in lizardfish during ice storage (Benjakul et al., 2003). The decrease in Ca²⁺ ATPase activity is also related to oxidation of SH group at the active site of myosin molecules

(Sompangse et al., 1996). The Ca²⁺-ATPase activities of the extracted NAM from lizardfish mince kept in air or under vacuum decreased with increasing frozen storage time (Leelapongwattanaa et al., 2005)

The Mg²⁺ and EDTA ATPase activities did not decrease as in the case of Ca²⁺ ATPase during ice storage indicating not much structural alteration in myosin-actin complex. Minimum change in Mg²⁺ ATPase activity was noticed in lizardfish upto 15 days and in fresh pacific whiting up to 8 days during iced storage suggesting the integrity of actin-myosin complex (Benjakul et al., 1997). The oxidation of sulfhydryl group, located at light meromyosin results in the inactivation of Mg²⁺-EDTA ATPase activities of actomyosin (Horigome & Yamashita, 1977) which are mostly unaffected in tilapia during storage in ice.

It is known that Ca²⁺-ATPase activity can be used as an indicator for the integrity of myosin molecules (Benjakul et al., 1997) and the globular heads of myosin is reported to be responsible for Ca²⁺-ATPase activity (Benjakul et al., 2003; Ramachandran et al., 2007). During the frozen storage, the ATPase activity did not significantly decrease till 2nd month, but later decreased by 69% ($p<0.05$) and then remained the same (Fig. 2). This loss or decrease in ATPase demonstrated the possible structural changes in myosin molecules between months 2 to 3 in frozen stored tilapia. In contrast to ice storage, the Mg²⁺ ATPase activity showed gradual decrease until month 2 (14%) and then declined sharply to 0.1 $\mu\text{MPi mg}^{-1}$ of protein min^{-1} after month 4 (80%) indicating a major alteration of actin-myosin

Table 2. Changes turbidity of protiens during iced and frozen storage

Storage, days	Iced storage		Storage, Months	Frozen storage	
	TSP	SP		TSP	SP
Fresh	0.24±0.02 ^a	0.09±0.01 ^a	Fresh	0.23±0.01 ^a	0.09±0.01 ^a
2	0.24±0.01 ^{ab}	0.09±0.01 ^{ab}	Frozen	0.25±0.01 ^b	0.09±0.01 ^b
4	0.25±0.01 ^b	0.08±0.01 ^a	1	0.37±0.01 ^c	0.16±0.01 ^c
7	0.33±0.02 ^c	0.11±0.01 ^b	2	0.40±0.01 ^a	0.16±0.01 ^{cd}
10	0.38±0.01 ^{cd}	0.16±0.01 ^c	3	0.46±0.01 ^c	0.16±0.01 ^b
12	0.44±0.01 ^{cd}	0.18±0.01 ^{cd}	4	0.45±0.01 ^c	0.17±0.01 ^c
14	0.44±0.02 ^{cd}	0.21±0.01 ^c	—	—	—

Values with different letters within the columns are significantly different ($p<0.05$). TSP -total soluble protein, SP - sarcoplasmic protein

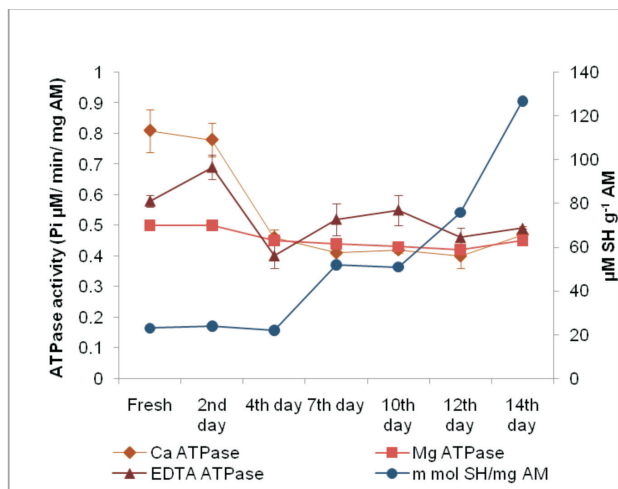


Fig. 1. Difference in ATPase activity and SH groups during iced storage (Days)

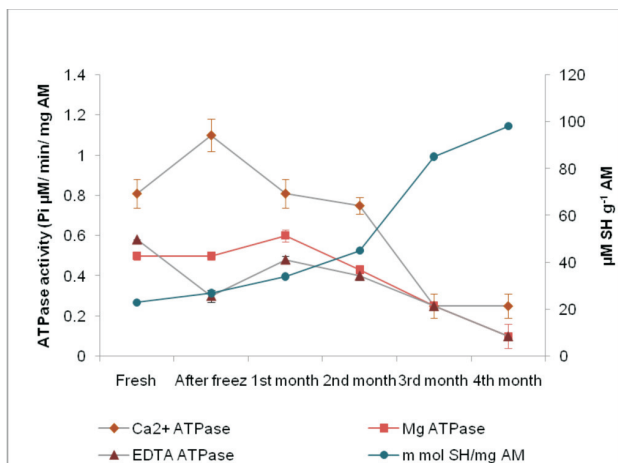


Fig. 2. Difference in ATPase activity and SH groups during frozen storage (months)

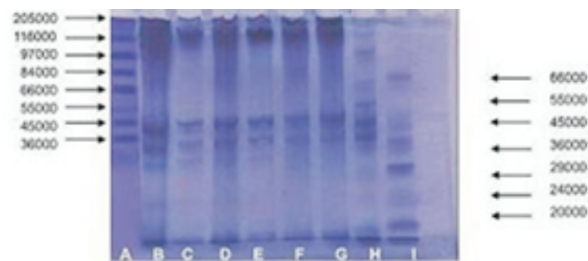
complex caused by Mg²⁺ salts in addition to Ca²⁺ salts. EDTA ATPase activity showed similar result as like Mg²⁺ ATPase activity.

Disulfide cross-linking in proteins may occur due to oxidation of sulfhydryl groups and sulfhydryl-disulfide interchange and disappearance of SH and or appearance of SS bonds, have a detrimental effect on the stability. Total sulfhydryl groups content was 23 μmoles/min/mg AM which increased from 4th day of iced storage (Fig. 1) and the increase was more than 5 times on day 14 indicating protein denaturation. The change in sulphide groups was minimum for the first seven days and then increased at a faster rate. Unfolding of protein during storage in ice resulted in the exposure of hydrophobic

residues to the exterior increasing the surface active SH group in threadfin bream from day 3 of ice storage (Yongsawatdigul & Park, 2002). Mohan et al., (2006) reported SH groups content of 88 μmoles/min/mg in AM from rohu which increased by 20% on iced storage during the first 6 days. Similar increases in SH groups were reported during the storage of farmed Atlantic salmon (Wang et al., 2005) and threadfin bream (Yongsawatdigul & Park, 2002). During frozen storage, however, the increase in SH was rather gradual throughout the storage and increased to 98 μmoles/min/g in 4 months of frozen storage (Fig. 2) indicating that protein unfolding occurred rather slowly during frozen storage compared to ice storage. The actomyosin becomes insoluble during storage and is directly related to protein unfolding as indicated by the increasing SH groups.

Turbidity measurement provides rough estimates of aggregation of protein molecules on denaturation (Acton et al., 1981). The turbidity of sarcoplasmic and total soluble protein increased by two folds during ice storage (Table 2) from the initial values of 0.24 to 0.44 values and 0.09 to 0.21 values,

(i)



(ii)

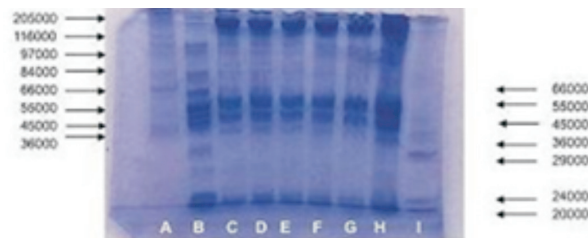


Fig. 3. SDS page profile of Total Soluble Protein (i) and actomyosin (ii) from Tilapia during storage in ice. A - High molecular weight markers, B- fresh, C - 2nd day, D - 4th day, E- 7th day, F - 10th day, G - 12th day, H - 14th day and I - low molecular weight markers

respectively. During frozen storage a similar trend was noticed for sarcoplasmic and total soluble protein (Table 2). Changes in turbidity values of actomyosin take place rather quickly at 30°C in carps (Sano et al., 1994). Sankar & Ramachandran, (2005) reported the changes in turbidity values during the denaturation in Indian major carps during different temperature treatment of proteins. The development of turbidity in protein has been reported during setting of protein at temperatures ranges 35–45°C in myosin solutions due to the size and rate of aggregation (Yarnpakdee et al., 2009; Ding et al., 2014)

In fresh tilapia, total soluble protein showed four distinct bands below 55 kDa (Fig. 3). The bands seen between 97 kDa and 66 kDa did not show much variation upon storage in ice. Also, no change was noticed in myosin (200 kDa) and actin (45 kDa) bands. Similar results in the electrophoretic pattern of hake actomyosin fraction was noticed during 11 days of iced storage (Crupkin et al., 1979), while reduction in myosin heavy chain and actin was

reported in threadfin bream stored in ice up to 12 days (Yongswawatdigul & Park, 2002).

During frozen storage of tilapia, there is not much change in degradation of total soluble protein bands with higher molecular weights of upto 55 kDa (Fig. 4). The disappearance of bands in the range of 97 kDa and 55 kDa was clearly seen in the actomyosin fraction. Disappearance of bands between 45 kDa and 36 kDa and appearance of bands between 36 kDa and 29 kDa in actomyosin (Fig. 4) was also noticed. Disappearance of bands and appearance of new bands during frozen storage was also reported in salt soluble protein during frozen storage of squid (Gomez-Guillen et al., 2003). The actin band (45 kDa) remained more or less uniform throughout the frozen and iced storage indicating the stability of actin during frozen storage.

Decrease in Ca²⁺ ATPase activity on the 4th day of ice storage and in month 3 during frozen storage of tilapia indicated the onset of spoilage. Subsequent increase in SH groups suggested the initiation of oxidation of SH groups leading to protein unfolding. Storage temperature influenced Ca²⁺ ATPase activity as there was a decrease of 43% activity in ice storage and 80% activity in frozen storage. No change in Mg²⁺ and EDTA ATPase activities during ice storage depicts the integrity of actomyosin complex as evidenced through SDS-PAGE pattern, while reduction of Mg²⁺ and EDTA ATPase activities during frozen storage showed disintegration of actomyosin complex leading to formation of small molecular weight peptides. The Mg²⁺ and EDTA ATPase activities are possible indicators for predicting protein disintegration or denaturation of frozen tilapia than Ca²⁺ ATPase activity. Extensive protein denaturation did not take place in iced tilapia, however the increase of SH groups occurred due to protein unfolding rather than protein disintegration.

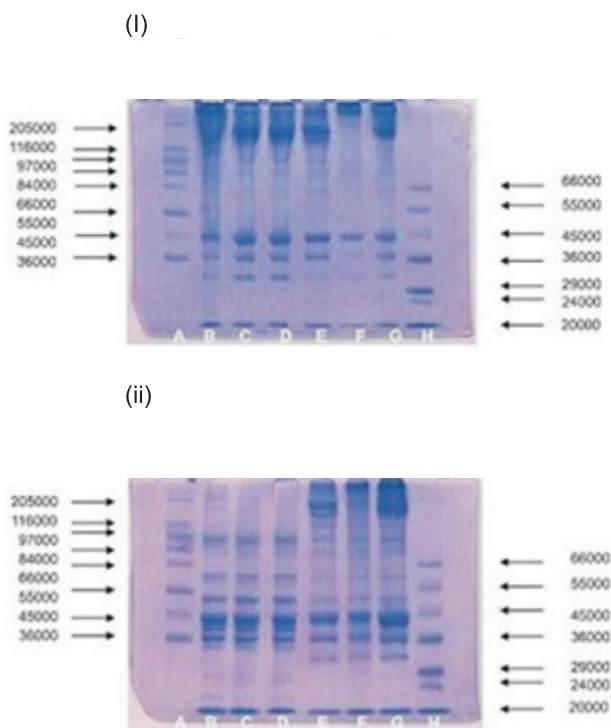


Fig. 4. SDS page profile of Total Soluble Protein (i) and actomyosin (ii) from Tilapia during frozen storage. A - High molecular weight markers, B- fresh, C- immediately after freezing, D - 1st month, E- 2nd month, F - 3rd month, G - 4th month, H -low molecular weight markers

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