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Effect of Thermal Modification on Physicochemical and Functional Properties of Myofibrillar Proteins from Tilapia, Oreochromis mossambicus (Peters, 1852)

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The effect of thermal modification on the physico-chemical and functional properties of myofibrillar proteins (MFP) extracted from Tilapia, Oreochromis mossambicus (Peters, 1852), heated over a temperature range of 3 to 55ºC was evaluated. The protein solubility in solution decreased with increase in temperature, with a steep decline at 40ºC. The decrease in protein solubility was proportional to increase in both cis parinaric acid and 8-anilino-1-naphthalene sulphonic acid hydrophobicity. Ca^{2+} ATPase activity decreased as the temperature increased, correlating with increase in surface hydrophobicity and decrease in solubility. Total and reactive sulphydryl group, showed an inverse relation to viscosity and turbidity of the MFP suggesting aggregation/unfolding of the proteins. Emulsion activity index significantly increased ($p<0.05$) as the temperature increased upto 40ºC. The emulsion stability of the proteins increased gradually with temperature while foam expansion decreased upto 35ºC, thereafter increasing steadily. Foaming properties of the fish protein also improved tremendously on thermal modification.

Keywords : Tilapia, thermal modification, myofibrillar proteins, Ca^{2+} ATPase activity, sulphydryl groups, functional properties

Modification of food proteins imparts better nutritional and functional properties which enhance digestibilityand overall consumer acceptance of the food. Egg proteins are boiled to increase their digestibility, while myofibrillar proteins are treated with salt before thermal denaturation to enhance their gelation functionality. Several studies are reported on chemical and enzymatic modification combined with thermal modification in milk, legume seed proteins etc (Erdem, 2006; Klepacka et al., 1997). Most proteins in their native conformation exhibit specific functional activity but their partially denatured state shows unique functional properties different from those in the native state.

The influence of extraction conditions such as temperature, pH (Mohan et al., 2007) and ionic strength (Devi, 2006) induce physicochemical changes in proteins resulting in the reorganization of both the secondary and tertiary configuration. This exposes the cryptic hydrophobic amino acid residues forming intermediary conformations with enhanced functionality and surface activities (Kinsella & Whitehead, 1987; 1988). Moderate heating improves functional properties due to the partial unfolding and exposure of hydrophobic sites (Phillips et al., 1994, Li-Chan et al., 1985). Molecular properties of proteins like surface hydrophobicity and net charge contribute significantly to the functional properties such as

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solubility, emulsification, film and foam formation while steric factors viz., molecular size and flexibility contribute to viscoelasticity, coagulation and fat binding.

Interdependency of protein structure and function in food applications has to be elucidated so that rational decisions can be made in selecting the best proteins for specific applications or for determining the modifications required for improving a particular functional property. This study aims to understand the conformational changes taking place in myofibrillar protein extracted from brackish water fish tilapia, Oreochromis mossambicus (Peters, 1982), on exposure to different temperature regime, and its effects on various functional properties.

Materials and Methods

The tilapia purchased in prime condition from a local fisherman at Cochin, India, was iced and transported to the laboratory. Fishes in post rigor state were washed in cold water to remove slime and dirt. The fillet and the boneless meat were minced in a pre cooled kitchen mixer/grinder (Kenstar, India) and the mince was used for myofibrillar protein extraction. Care was taken to maintain the temperature between 2 to 4°C.

The minced fish meat was homogenized twice at 9000 rpm (Kinematica, Switzerland, Model PT 3000) in a low ionic strength buffer (0.02 M sodium bicarbonate, pH 7.5) and centrifuged at 11200 g (Remi R24, India) for 15 minutes at 0°C to remove the water soluble proteins. The residue was homogenised in sodium chloride - bicarbonate buffer (0.8 M NaCl containing 0.02 M sodium bicarbonate, pH 7.5) for 1 minute at 9000 rpm and was centrifuged at 11200 g for 15 minutes at 0°C to collect myofibrillar proteins (MFP) (King & Poulter, 1985). The residue was extracted again with the same buffer as above and the supernatants were pooled to collect total MFP.

The extracted MFP of concentration (599 mg/100 ml) was taken in seven conical flasks (Borosil, India) of identical dimensions for exposure at seven different temperatures (3, 25, 35, 40, 45, 50 and 55°C). The flasks were kept in a water bath, pre-set to the treatment temperature. The temperature of contents in the flask was measured using a mercury thermometer and, once the protein solution attained the set temperature, it was held for 10 minutes and then the flasks were removed to ice bath and allowed to stand for two hours so as to facilitate complete precipitation of denatured proteins. The protein solutions were then centrifuged at 11200 g for 10 minutes in a refrigerated centrifuge (Remi R24, India) at 3°C.

Concentration of heat treated protein solution was estimated by Biuret reaction (Gornall et al., 1949) using alkaline copper sulphate reagent. The colour developed was measured at 540 nm using a spectrophotometer (Spectronic 20 Genesis, Rochester, NY, USA). Bovine serum albumin was used as the standard.

Ca^{2+} ATPase activity of myofibrillar proteins (1mg/ml) was assayed to evaluate the extent of denaturation (Jiang et al., 1987). Inorganic phosphate liberated was estimated using the method of Fiske and Subbarow (1925). Surface hydrophobicity of the proteins was estimated using fluorescent probes CPA (cis Parinaric acid) (Molecular Probes, Eugene) and ANS (8-Anilino-1-napthalene sulphonic acid) (Sigma, USA) (Kato & Nakai, 1980) using a spectroflurophotometer (Shimadzu Model RF-540, Kyoto, Japan). Excitation and emission spectra were measured at 374 and 485 nm respectively for ANS and 325 and 420 nm respectively for CPA. The initial slope (S_o) of fluorescence intensity against protein concentration (0.05-0.25ìg/3ml) was taken as the index of protein hydrophobicity. The reactive sulphydryl group in the MFP (1mg/ml) was analysed as

per the method of Sedlak and Lindsay (1968). Total sulphydryl content was analysed according to the method of Opstvedt et al. (1984). The absorbance was read at 412 nm in a spectrophotometer (Spectronic 20 Genesis, Rochester, NY, USA). Viscosity of MFP (4.5 mg/ml) was determined using a viscometer (Model DV III Ultra, Brookfield, USA) at shear rate 200 s^{-1}. The turbidity of the protein solution (1 mg/ml) was measured at 500 nm using the same spectrophotometer.

The ability of the MFP to form emulsions was estimated as emulsion activity index (EAI) according to the methods of Pearce & Kinsella (1978) as per modification of Cameron et al. (1991). Protein (4.5 mg/ml) was homogenized (13500 rpm) with sunflower oil (ratio 3:1) using an emulsion probe (PT-DA 3012/2EC) attached to a Polytron homogeniser (Model PT 3000, Kinematica, Switzerland). The turbidity of the diluted emulsion was measured at 500 nm and EAI was calculated as reported earlier (Mohan et al., 2006; Ramachandran et al., 2006).

Foaming was done using modified method of Wild and Clarke (1996). MFP (4.5 mg/ml) was whipped using a mixer (Kenstar, India) for one minute at 8000 rpm. Foam expansion (FE) and foam stability (FS) were determined as per Mohan et al. (2006) and Ramachandran et al. (2006).

SDS-PAGE of the heated MFP was done using Protean II xi Cell (Bio-rad, USA) as per Laemmli (1970). Protein (25 µg) samples were loaded on to each well and run at a constant voltage (200 V). Gels after staining and de-staining were scanned using gel documentation system (GelDoc EQ, Bio-rad, USA). Mobility of protein bands was calibrated with molecular weight markers (Sigma Chemicals, US). Densitometric analysis was carried out using the software 'Quantity One Ver. 4.5.0 1-D Analysis' (Bio-rad Laboratories Inc., CA, USA).

Statistical analysis was done using windows based SPSS 12.0 version. One way ANOVA was performed and the level of significance was studied at 5%.

Results and Discussion

Protein solubility is a delicate balance between repulsive and attractive intermolecular forces, which are dependent on native structure of protein, extraction medium and extraction conditions. The solubility of tilapia proteins decreased gradually (Fig. 1) from 599 mg at 3°C to 561 mg at 35°C and thereafter decreased sharply at 40°C (23%). With increase in temperature, the ionic bonding (including hydrogen bonds) holding the 3D structure of protein gets disrupted leading to protein-protein interaction affecting protein solubility. The thermal influence on protein leads to opening up of the native structure exposing the hydrophobic patches to the exterior, which effectively displaces water molecules from protein surfaces promoting aggregation (Kuntz & Kauzmann, 1974). Further heating above 40°C up to 55°C slightly increased the protein solubility (4%). Disintegration of the aggregates as a result of proteolytic action at higher temperatures (Yongsawatdigul & Park, 1999) could have contributed to solubility at these temperatures. The common fractions associated with tilapia MFP

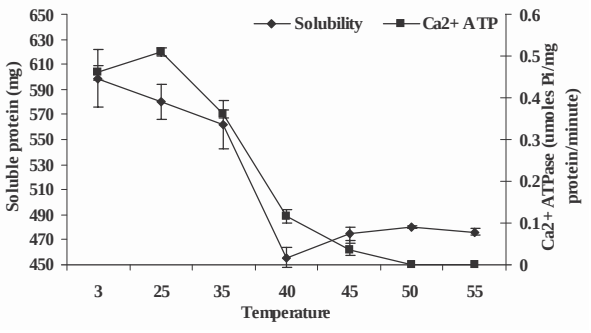

Fig. 1. Solubility and Ca^{2+} ATPase activity of the MFP treated at various temperatures. Values are ± SD, n = 3, $p < 0.05$

include those with molecular weights of approximately 205, 116, 84, 54, 46, 36, 24, and 20 kDs (Fig. 7)

Fish myosin is generally unstable and rapidly forms aggregates with concomitant decrease in ATPase activity (Sankar & Ramachandran, 2005, Ochiai & Chow., 2000). Ca^{2+} ATPase activity, the measure of integrity of myosin head (Roura et al., 1990; Nagai et al., 1999), showed a significant decline with increasing temperature (Fig. 1) except at 25ºC where a 10% increase (p d" 0.05) (0.51 µmoles Pi/mg protein/min) was noticed compared to that at 3ºC. At 40ºC, the activity was only 0.11 µmoles Pi/mg protein/min, with a loss of over 75% compared to the initial value, and at 50ºC, a complete loss of activity was noticed (Fig. 1). Sankar & Ramachandran (2005) reported that Ca^{2+} ATPase activity remained the same up to 40ºC in Labeo rohita and Cirrhinus mrigala, but in Catla catla the ATPase activity decreased above 20ºC indicating the species specificity of fish myosin. The Ca^{2+} and Mg^{2+} ATPase activities of carp myofibrillar proteins were influenced by the habitat, particularly water temperature (Misima et al., 1990). An inverse relationship between habitat temperature and ATPase activity has been reported by Johnston et al. (1973) which showed better activity for tilapia MFP at 25ºC rather than at 3ºC.

The probes CPA and ANS have different affinities for hydrophobic patches on the surface of a protein. CPA binds at aliphatic amino acid patches while ANS shows affinity to aromatic patches (Zayas, 1997). CPA and ANS hydrophobicity did not vary significantly up to 35ºC (Fig. 2) but increased with further increase in tempera-

holding the native conformation of protein molecules thus disrupting the tertiary structures. The hydrogen bonds that stabilize the helical structure break resulting in greater interaction with water and thereby forming new hydrogen bonds with amide nitrogen and carbonyl oxygen of the peptide bonds. The presence of water further weakens nearby hydrogen bonds by causing an increase in the effective dielectric constant near them. As the helical structure is broken, hydrophobic groups are exposed to the solvent. CPA hydrophobicity did not show significant change when exposed to 40 – 50ºC, but increased to 45.3 at 55ºC. The CPA fluorescence pattern (Fig. 2) indicates the stability of myosin rod portion up to 35ºC and alterations in the conformation of MFP at higher temperatures as suggested by Samejima et al. (1981).

ANS hydrophobicity increased steeply from 35 – 40ºC (Fig. 2) and it continuously increased upto 55ºC. This correlates well with the Ca^{2+} ATPase activity of the protein, indicating that the myosin head of tilapia muscle proteins is very stable at higher temperatures compared to other fish myofibrillar proteins. ANS hydrophobicity together with Ca^{2+} ATPase activity indicates that the conformation of head was stable up to 35ºC, after which the myosin head region kept unfolding with increase in temperature.

The concentration of reactive sulphydryl groups in unheated sample is comparable to that reported in other fishes (Devi, 2006; Mohan et al., 2006; Mohan et al., 2008;

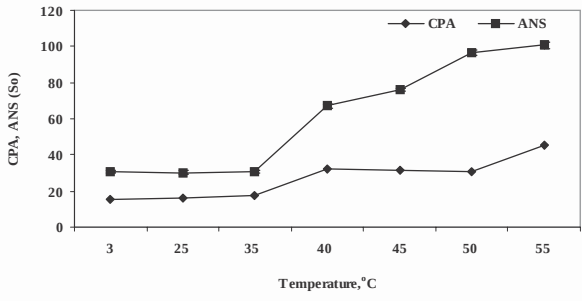

Fig. 2. Surface hydrophobicity of heat treated MFP using CPA and ASN as flouroscent probes. Values are ± SD, n = 3, p < 0.05

Ramachandran et al., 2006). The concentration of reactive sulphydryl (RSH) groups decreased throughout the temperature range studied, indicating disulphide bond mediated aggregation (Fig. 3). The decrease was insignificant between temperature 3 and 35°C, but further increase in temperature decreased the RSH significantly ($p<0.05$) particularly at 40°C. Heat treatment results in exposure of buried sulphydryl groups as

of the protein showed similar trend as the RSH (Fig. 3). Heating of protein solution

sponding increase in disulphide bonds (Opstvedt et al., 1984). Disulphide cross

of sulphydryl groups and sulphydryl disulphide interchange (Cecil & Mc Phee, 1959), and it has been demonstrated during heating of fish proteins (Itoh et al., 1980).

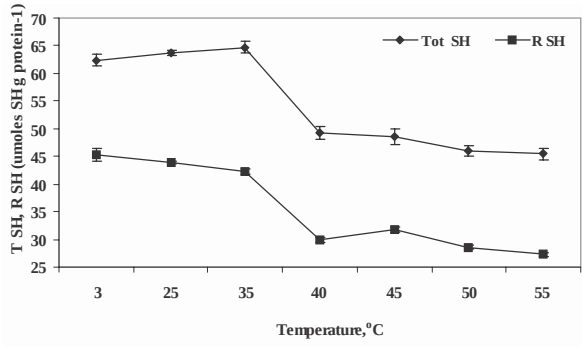

Fig. 3. Total and reactive sulphydryl groups of heat treated MFP. Values are ± SD, n = 3, $p < 0.05$

The initial viscosity of tilapia protein solution (4.5 mg/ml) was 12 cP which is higher than that in many other fishes (Mohan et al., 2006; 2008; Ramachandran et al., 2006). Viscosity of the MFP decreased (Fig. 4) up to 50°C after which it remained almost stable. Since viscosity is directly related to the concentration of soluble proteins remaining in solution, the stability of viscosity could be attributed to the stability of the protein in solution at higher temperature. Roura et al. (1990) attributed the decrease in viscosity of whiting actomyosin with increase in surface hydrophobicity during storage in ice to the alteration in the protein structure and exposure of more hydrophobic patches. Similar phenomenon was noticed in proteins on exposure to different temperature regime in this study.

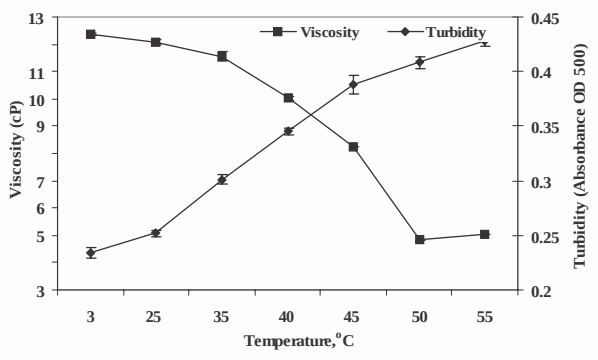

Fig. 4. Viscosity and turbidity of heat treated MFP. Values are ± SD, n = 3, $p < 0.05$

Turbidity changes of heat-treated protein solutions, a measure of light scattering properties induced by aggregation of proteins, indicates the extent of protein-protein

aggregates (Acton et al., 1981). Aggregation was initially slow till 25°C but increased progressively throughout the heat treatment. Increase in absorbance of heat treated fish myosin correlates with the formation of more and larger myosin aggregates (Yongsawatdigul & Park, 1999, Gill et al., 1992). Heating actomyosin beyond 40°C increased the turbidity in L. rohita and C. mrigala while in C. catla turbidity increased from 10°C itself (Sankar & Ramachandran, 2005). The findings are in line with earlier studies, confirming species specificity in the thermal stability of muscle protein (Gill et al., 1992; Chan et al., 1992; Visessanguan et al., 2000).

EAI of the tilapia muscle proteins increased significantly ($p<0.05$) upto 40°C after which the increase was insignificant

(Fig. 5). Protein solubility and surface hydrophobicity are the two major factors affecting emulsion activity (Xiong, 1997). Emulsifying properties can be improved by physical modification which involves partial denaturation as in controlled heating (Mitidieri & Wagner, 2002; Damodaran, 2005). The solubility of MFP decreases on exposure to temperature up to 40°C, during which time the surface hydrophobicity increased significantly ($p<0.05$). Emulsion stability decreased initially up to a temperature of 35°C; thereafter it increased steadily particularly at 40°C ($p<0.05$) (Fig. 5). Exposure of more hydrophobic patches as indicated in the CPA and ANS hydrophobicity, could have contributed to this phenomenon. Emulsion stability is influenced by the rheological properties of the adsorbed protein film.

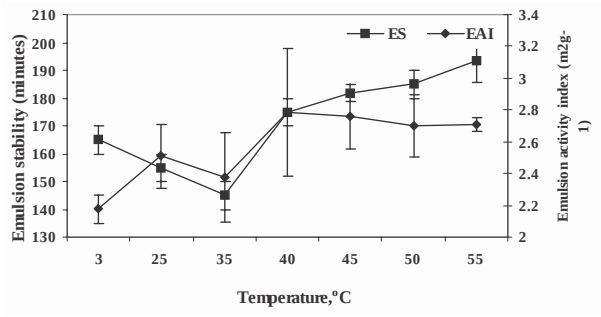

Fig. 5. Emulsion activity index and emulsion stability of heat treated MFP. Values are ± SD, n = 3, p < 0.05

The foam expansion decreased up to 35°C (Fig. 6) and thereafter increased gradually with increase in temperature. The MFP at 3°C did not show good foam stability (Fig. 6). Temperature modification of the protein structure increased the foam stability substantially. Proteins, by moving to the lipid - protein interface, partially unfold and form new intermolecular associations with neighbouring protein molecules to form cohesive films that are essential for foam formation (Kinsella & Phillips, 1989). Foam stability (FS) depends up on the extent of protein - protein interaction within the film matrix (Phillips et al., 1994). The heat induced alteration in the hydrophobic environment contributed to the improved FS of the protein. Physical modification of the protein solution by controlled heating is reported to improve the stability of foams in whey protein isolates (Damodaran, 2005; Bals & Kulozik, 2003). Foaming properties of proteins are enhanced by exposure of hydrophobic groups, increased surface hydrophobicity and decreased surface tension as a result of partial unfolding of myosin at higher temperatures (Zayas, 1997).

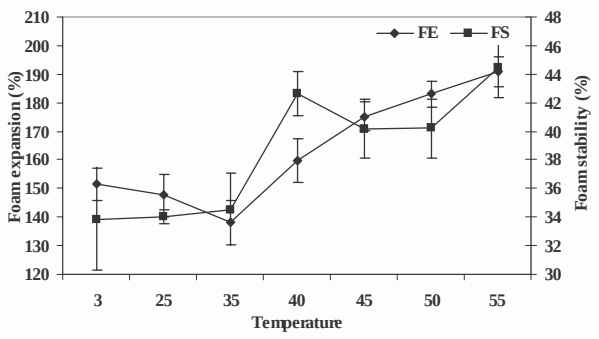

Fig. 6. Foam expansion and foam stability of heat treated MFP. Values are ± SD. n = 3. p < 0.05

The intensity of myosin heavy chain and other major MFP bands such as actin and myosin light chains did not show any significant variation in their band intensities (Fig. 7). Thus the major myofibrillar proteins remained unaffected due to heat treatment up to 50°C. However, heat treatment has brought about conformational changes without affecting the peptide bonds. Fractions between 116 kDa to 84 kDa showed many low intensity bands especially towards the right side of the electrophoretogram (40 to 55°C). This indicates that some of the high molecular weight proteins are susceptible to hydrolysis at higher temperatures. It could be noted (Fig. 8) that bands 3 to 6 show significant ($p<0.05$) increase in density with increase in temperature beyond 40°C and

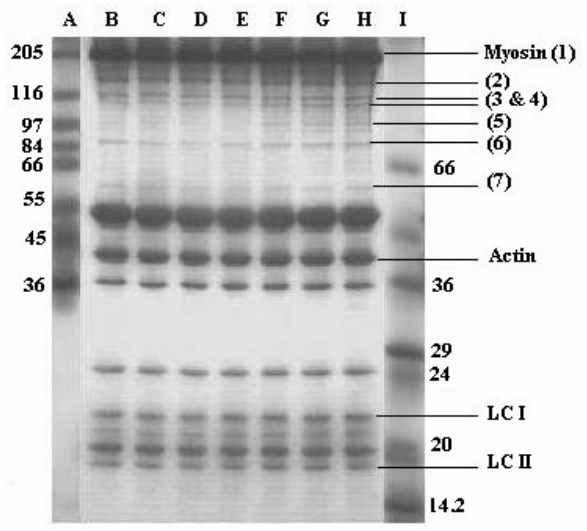

Fig. 7. The electrophoretogram of the MFP treated at various temperatures. Lane A - High molecular weight marker, B – 3°C, C – 25, D – 35, E – 40, F – 45, G – 50, H – 55 and I – low range molecular weight markers.

these protein fractions could have contributed to the improved foaming and emulsion properties of the tilapia MFP proteins. The electrophoretic pattern also supports the fact that tilapia myosin is more stable to higher temperatures compared to other fish species. This higher stability of myosin could be the reason for better solubility (only 27% from 3 to 55ºC) observed in this study.

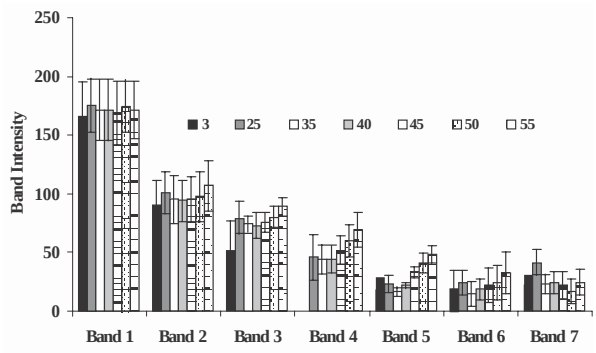

Fig. 8. The band intensities of certain high and medium molecular range proteins from the SDS PAGE shown in figure 7. The bands numbered in the X axis correspond to the bands numbered in the SDS PAGE. Values are ± SD. n = 3. $p < 0.05$

Functional characteristics of food proteins are influenced by temperature, time and rate of heating. Alteration in these conditions affects both the microscopic and macroscopic structural attributes. Heat treatment leading to conformational changes such as increased surface hydrophobicity, decreased Ca^{2+} ATPase activity and decreased sulphydryl content, improved the overall functionality of the protein viz., the emulsion activity, emulsion stability, foam expansion and foam stability. The SDS PAGE showed that the major myofibrillar proteins remained unaltered as indicated by densitometry analysis, suggesting that heat treatment up to 55ºC does not hydrolyse them but at the same time brings about change in their conformation, positively affecting the functionality of the protein.

Modified proteins with better digestibility and improved functionality are more acceptable for product diversification as in value addition. Consumer preference for low calorific/low fat food has brought the modified proteins into the limelight, as fat substitutes. Flavour binding, water binding, emulsifyng and foaming properties are some of the characteristics expected to be improved by temperature modification of the proteins.

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