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HYDRODYNAMIC AND PHYSICOCHEMICAL PROPERTIES OF PROTEINS FROM THREADFIN BREAM (*NEMIPTERUS JAPONICUS*) FISH MEAT AS AFFECTED BY FREEZING AND FROZEN STORAGE

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ABSTRACT
Hydrodynamic and physicochemical properties of proteins from semi dressed threadfin bream (*Nemipterus japonicus*) fish meat as affected by freezing and frozen storage (-20° ± 2° C) has been evaluated for the period of 200 days. Non-protein nitrogen content has shown a significant ($P < 0.05$) increasing trend with increased frozen storage period indicating degradation of constituents such as free-amino acid, TMA-O and other substances. The significant ($P < 0.05$) reduction in free-SH content clearly demonstrates conformational changes of the protein molecules induced by freeze denaturation. The association-dissociation phenomena of total proteins with increased frozen storage were revealed by the SDS-PAGE patterns. This clearly demonstrates the formation of insoluble aggregates which were further supported by gel filtration profile. Such changes during frozen storage may have an impact on setting and gel forming ability if the same freeze denatured fish protein meat used further. The results noticeably reveal that the freezing and frozen storage of semi dressed threadfin bream have a significant effect on both the hydrodynamic and physicochemical properties of proteins.

INTRODUCTION

Fish and shell fish is an important source of dietary proteins worldwide. The demand for fish and fishery product is continually increasing because of health benefits mainly contributed from proteins and lipids. Fish is a perishable commodity and its utilization for human consumption is a challenging task in tropical country like India. However, there is a great mismatch between supply and demand for fish. World demand for fish for direct human consumption is now estimate to accomplish 153.6 million tonnes. As a result there was an increase in per capita fish consumption as food, to 20.6 kg in 2016 (FAO, 2016). Nearly, 20 million tons of fish in a year are discarded at the sea (Kumolu-Johnson and Ndimele, 2011) and the estimated loss of fish due to spoilage is around 10 to 12 million tons per year that accounts 10 % of total production of fish (Getu *et al*., 2015). Better utilization of the aquatic resources should therefore intend primarily at reducing this enormous loss by improved processing techniques. Perishable nature and shortage of fish supply give cal for innovative processing methods for efficient utilization of available resources. The term "processing" refers to all operations that are carried out to extend the shelf life of fish and fishery products. The common processing methods employed in the preservation of fish and fishery products are icing, freezing and frozen storage, drying, salting and drying, fermentation, smoking, thermal processing, and preparing ready-to-eat (RTE) or ready-to-cook (RTC) fish mince based products. At present, freezing is the most important and an effective technique used to preserve the fish (Pan *et al*., 2010). The freezing process consists of three stages-cooling the product to its freezing point (pre-cooling or chilling stage), removing the latent heat of crystallization (phase transition stage) and finally cooling the product to the final storage temperature (tempering stage). The phase transition part of the freezing process involves the conversion of water to ice through the crystallization process and is the key step in determining the efficiency of the process and the quality of the frozen product (Steffolani *et al*., 2011).

The status of fish species get effected by several factors like feeding behaviour, season, maturation and so on (Khileri *et al*., 2016), and these factors in turn have got a greater influence on the composition fish proteins. After harvesting, the complex alterations which take place in fish muscle proteins due to freezing and frozen storage results in the changes of protein conformation. The chemistry of protein denaturation is an active area of research and much information has been gathered by different workers (Asghar *et al*., 1985; Kumazawa *et al*., 1995; Badii and Howell, 2002; Leygonic *et al*., 2012). The fish protein undergo series of alterations in its properties consequent to different processing methods employed.So an

attempt has been made to correlate frozen storage period and denaturation of fish proteins from threadfin bream. The choice of threadfin bream for evaluating the proteins is that this fish belonging to the genus Nemipterus shall serve as a potential protein source for the benefit of all human kind (Diana and Manjulatha, 2012). With this rationale, the objectives of present investigation were to assess the changes in hydrodynamic and physicochemical properties of proteins from semi dressed threadfin bream fish meat as a function of frozen storage.

MATERIALS AND METHODS

Fish

The fish used for the present investigation were threadfin bream (*Nemipterus japonicus)* which were procured from fish landing centre, Mangalore. The fish were washed in chilled potable water and iced in the ratio of 1:1 in polyurethane boxes. The semi dressed fish were packed in high density polyethylene bags (two fish were packed/bag). The packed fish were then subjected for the freezing process using an air blast freezer (Armfield, Armfield Limited, Ringwood Hampshere, England) at -40º C for 90 min. The frozen samples were stored in the deep freezer (Vestfrost, Holland) at -20º ± 2º C. The samples were drawn periodically at different periods of frozen storage and were subjected for further analyses.

Non-protein nitrogen content

Non-protein nitrogen content was estimated by following the method as described by Velankar and Govindan (1958) and the nitrogen content was estimated by Kjeldahl method (AOAC, 2010). The NPN content was expressed as mg N/100 g meat. The NPN values reported were the mean of triplicate values.

Free sulfhydryl content

Free sulfhydryl content was determined according to the method as described by Ellman (1959). About 2 g of the meat was mixed with extraction buffer (extraction buffer or EB refers to 50mM phosphate buffer, pH 7.5 containing 1M NaCl) in the ratio of 1:15:: mince: EB and homogenised at 9000 rpm for 2 min. The slurry was filtered using a whatman No.1 filter paper (GE Healthcare UK Limited, Amersham place Little Chalfont, Buckinghamshire, UK). An aliquot of 3 ml filtrate was made up to 10 ml using EB to get the sample solution. To the 3 ml of sample, 1 ml of Ellman's reagent was added and mixed thoroughly by vortex mixer (Cyclo mixer, Remi equipments, Mumbai, India). After 30 min of incubation at ambient temperature (27º to 28ºC), the absorbance was measured at 412 nm was using double beam spectrophotometer. The free sulfhydryl content was determined using the following equation,

$$Co = A/E \times D$$

Where, Co- Concentration (mM SH/g meat), A- Absorbance at 412 nm, E- Extinction coefficient (13,600) and D-Dilution factor

The free sulfhydryl content was expressed as mM/g of meat and the values reported were the mean of triplicate values.

Sodium-do-doecyl sulphate poly acryl amide gel electrophoresis (SDS-PAGE)

Sodium-do-doecyl sulphate poly acryl amide gel electrophoresis was carried out using the method as described by Laemmlli (1970). Electrophoresis was carried out using poly acryl amide gel slabs of 10× 8 cm (length x width) in a vertical slab gel electrophoresis apparatus (SE-250 Hoefer-Pharmacia Biotech Inc., San Francisco, CA, USA). The concentration of acryl amide for separating gel was T % -10 and C % = 2.6 while for stacking it was T %-4 and C % = 2.6. For polymerisation of the gel, TEMED was used as the initiator and APS as the catalyst. The gels were cast in a dual gel caster. The thickness of the gel was 0.75 mm. The number of wells in each gel was 10. The electrophoresis was carried out at a constant voltage mode. Initially it was set to 30 V for stacking gel run and later the voltage was set to 90 V. The concentration of protein loaded to gel was 5 μg. The run was continued till the dye front reached the bottom of the gel. The bands were stained with 0.02 % (w/v) coomassie brilliant blue R-250 for overnight. Gels were de-stained with 7.5 % acetic acid - methanol mixture (5 % methanol (v/v) and 7.5 % acetic acid). De-staining was done for 30 ± 10 min till the protein bands were clearly visible. The molecular weight of the protein bands obtained in the sample was approximated by measuring the relative mobility of the standard protein molecular weight markers (high molecular weight markers from Sigma, St. Louis, MO, USA).

Gel filtration profile

Gel filtration profile of total proteins extracted from fish mince was carried out at an ambient temperature using an EB as solvent as suggested by Karthikeyan *et al*. (2006). The gel used was sepharose 6B and packed in a glass column of 1.6 cm 95 cm (diameter x height). The total bed volume of the column was 170 ml. The void volume (V_o) was determined by using blue dextran and found to be 72 ml. Proteins from fish meat sample was extracted with EB as solvent in ratio 1:15. The homogenization and centrifugation was carried out as described earlier. Before loading into the column, protein concentration of the supernatant was determined by the method as described by Lowry *et al*. (1951). The flow rate was adjusted to 30 ml/hr. The fractions of 3 ml were collected manually in a series of test tubes. The concentration of protein fractions were determined by measuring the absorbance at 280 nm using double beam spectrophotometer. A plot of optical density A_{280} nm against elution volume was obtained to get gel filtration profile.

Viscosity

The total proteins were extracted using EB as described previously and viscosity of total protein from the fish meat was carried out using the method as described by Supreetha *et al*. (2015). Viscosity of total proteins from the fish meat was determined using rotational viscometer (Brookfield Viscometer DV-II + Pro, Brookfield Engineering labs, Inc., Middleboro, MA, USA) at a constant temperature of 25p ± 1p C and corresponding viscosity was recorded and expressed in mPa.s.

Statistical analysis

Analyses of free-sulfhydryl and NPN content were done in triplicates. The data were subjected to analysis of variance (One-way ANOVA: Post Hoc Multiple comparison). Mean values were analysed using Tukey's test (Proust, 2007) to find

out the significant differences between the frozen storage periods using the statistical software package (IBM SPSS) version 21.

RESULTS AND DISCUSSION

Non-protein nitrogen content

The changes in non-protein nitrogen (NPN) content with increased frozen storage period were given in Fig. 1A. The increase in NPN content was gradual up to 80 days of storage and thereafter, values increased reaching a value of 353.77 mg/100g meat. The increase in NPN content may be attributed to breakdown of TMA-O and other sarcoplasmic protein fractions. The NPN content of fish sample gives an idea about the level of various non-protein nitrogenous substances like free amino acids and peptides present (Karthikeyan *et al*., 2004). A marginal increase in NPN content of common carp meat during frozen storage has been reported (Ganesh *et al*., 2006). The NPN content of frozen stored milk fish (*Chanos chanos*) showed an initial increase in the value and decreased at the end of 20 weeks of storage (Jiang *et al*., 1988).

SDS-PAGE pattern

The SDS-PAGE pattern of the total proteins from fresh threadfin bream (Fig. 2, Lane B) clearly indicated two myosin heavy chain (MHC) bands in the molecular weight range of 205 kDa and multiple bands in the molecular weight range of 116 to 24 kDa. The number of bands in the pattern clearly indicated large number of proteins in the muscle. Whereas the total proteins from immediately after freezing, clearly shows that the two MHC got aggregated to form a single band (Fig. 2, Lane C) and not much change in the other bands. The electrophoretic mobility of proteins under a reduced condition from ocean perch and flounder did not show any changes in the pattern with increased frozen storage (Ragnarsson and Regenstein, 1989). The protein bands in the molecular weight range of 55 to 25 kDa did not undergo much change in the present investigation. However, at the end of 120 days of frozen storage the SDS-PAGE pattern revealed disappearance of band in the molecular weight range of 29 to 24 kDa. These bands appeared at the end of 160 and 200 days frozen storage which possibly revealed association-dissociation phenomena. To confirm this gel filtration profile of total proteins were analysed during different periods of frozen storage.

Gel filtration profile

The gel filtration profile of total proteins from threadfin bream fish meat during different periods of frozen storage is presented in Fig. 3 A-E. Gel filtration as a tool to monitor association-dissociation-aggregation phenomena have been used to compare fresh and frozen cod muscle (Ohinishi and Rodger, 1980) and common carp meat (Ganesh *et al*., 2006). There were two fractions in the fresh condition and at the end of 200 days of frozen storage the number of fractions found to be 4. The concentration of high molecular weight component progressively reduced with increased frozen storage period. This reduction is possibly due to reduced solubility in extraction buffer which was used as an eluent to study the gel filtration profile. The elution volume at which a high molecular weight gets eluted ranged from 75 to 78 ml during different periods of frozen storage. The emergence of low molecular weight components could be arrived from the dissociation of high molecular weight components or from the constituents of the sarcoplasm. In the present study gel filtration profile clearly indicated association-dissociation-aggregation phenomena.

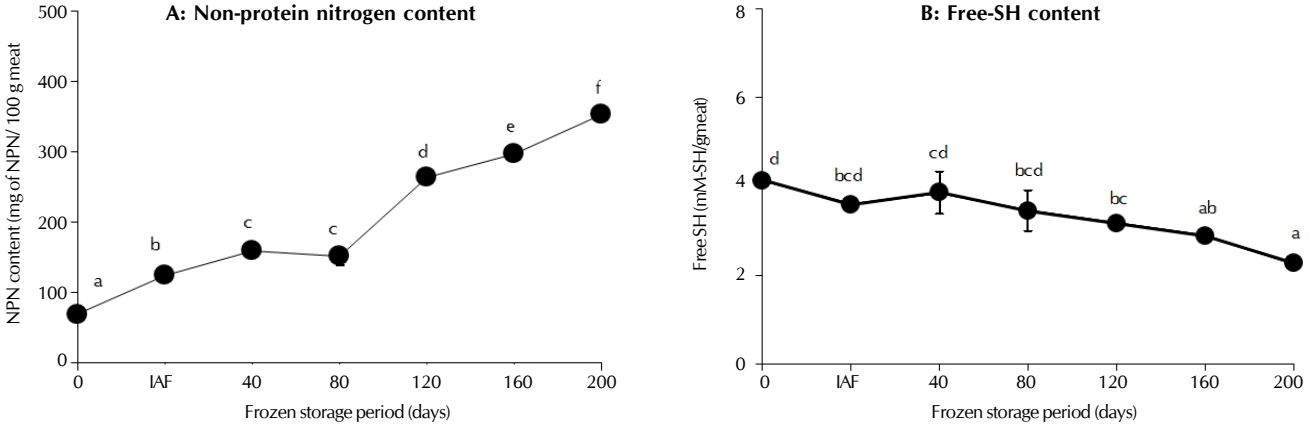
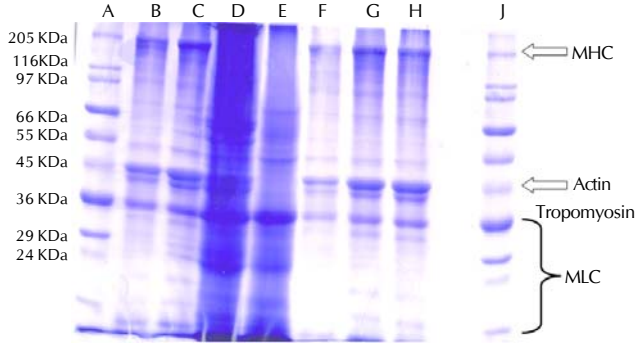
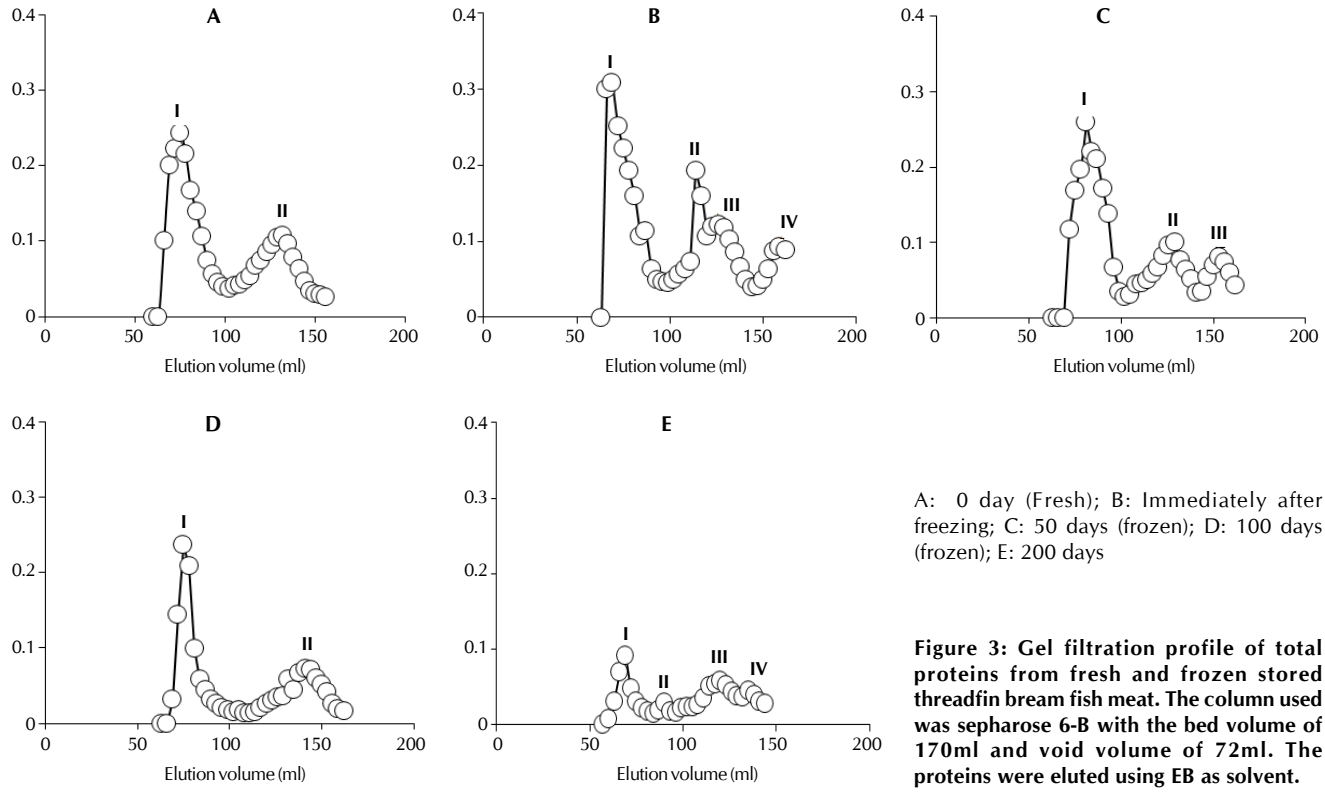


Figure 1: Effect of freezing and frozen storage on the properties of proteins from threadfin bream fish meat



Lane A and J: Molecular weight markers;Lane B: 0 day (fresh fish);Lane C: Immediately after freezing (IAF) fish protein;Lane D: 40 days fish protein;Lane E: 80 days fish protein; Lane F: 120 days fish protein;Lane G: 160 days fish protein; Lane H: 200 days fish protein

Figure 2: SDS-PAGE pattern of total protein from fresh and frozen stored threadfin bream fish meat



A: 0 day (Fresh); B: Immediately after freezing; C: 50 days (frozen); D: 100 days (frozen); E: 200 days

Figure 3: Gel filtration profile of total proteins from fresh and frozen stored threadfin bream fish meat. The column used was sepharose 6-B with the bed volume of 170ml and void volume of 72ml. The proteins were eluted using EB as solvent.

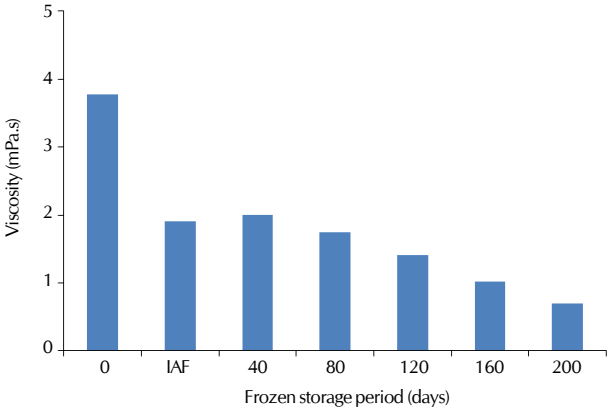


Figure 4: Change in viscosity of semi dressed threadfin bream fish meat with increased frozen storage period at 2mg/ml protein concentration

Viscosity

The viscosity of total proteins from fresh and frozen stored threadfin bream fish meat was measured at a protein concentration of 2 mg/ml in EB as solvent (Fig. 4). The initial viscosity value was 3.78 mPa.s. Immediately after freezing the viscosity decreased to 1.90 mPa.s, which could be due to the changes in conformation of protein caused due to freezing process. The reduction in viscosity values has mainly occurred because of freeze denaturation which reduces the protein axial ratio and could promote dissociation giving raise to low viscosity (Capillas *et al*., 2002). Decrease in viscosity can also be due to the alignment of the protein molecules leading to the reduction in frictional resistance (Venugopal, 2002). Viscosity further provides information as physicochemical interactions among proteins by indicating structural changes that may occur in the protein molecules (Rha and Pradipasena, 1986). Viscosity can be used to determine the degree of protein denaturation and aggregation during frozen storage (Colmenero *et al*., 1988). In the present investigation viscosity values decreased progressively with increased frozen storage and its reduction were attributed to changes in myofibrillar protein conformation.

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