Research Paper

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The Lipid Peroxidation Induced Changes of Proteins in Frozen Fish

Food Science

KEYWORDS : Lipid peroxidation, fish , Thiobarbituric Acid Reactive Substances (TBARS), Salt Soluble Protein (SSP) content, SDS

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ABSTRACT

Progression of lipid peroxidation of tilapia stored at -10 0C was evaluated for 28 days at weekly intervals using the content of Thiobarbituric Acid Reactive Substances (TBARS) as an indicator. The effect of peroxidation on muscle protein was evaluated by Salt Soluble Proteins (SSP) content and subjecting the SSP into gel electrophoresis. The experiment was repeated three times with three replicates for each and data were analyzed.

TBARS levels of fish significantly increased (p = 0.003) during storage period with 0.41 mg/ Kg/week indicating the progression of lipid peroxidation and SSP levels of fish decreased significantly (p = 0.004) even under - 10 0C. A negative correlation (R2=0.90) between TBARS and SSP levels suggests possible deleterious effect of lipid peroxidation on protein. The decrease of the intensity of the electrophoretic bands of Somatolactin, Myosin Heavy Chain, Actinin, Actin and Myosin Light Chain A1 in SSP extract during the storage period.

INTRODUCTION

Many people preferred fish than other animal protein sources due to its high biological value, higher level of Polyunsaturated Fatty Acids (PUFA) and minerals. Marine fish production is progressively decreased due to the increased amount of habitat loss and over exploitation. Therefore the fresh water fishery has become the main way to increase the fish production to meet the ever increasing global demand. Among the fresh water fish species, tilapia has become the most popular fish species in tropical countries due its high adaptability, tolerance limits and prolificacy. It is popular among the low income people of the world due to its cheap price compare to other fish species particularly to marine fish species. The major producing countries of tilapia are China, Egypt, Thailand, Philippines and Indonesia (Fitzsimmons,2010 and Josupeit, 2010).

The higher level of PUFA and minerals enhances the lipid peroxidation leading to rapid deterioration fish. In addition some of the peroxidative products such as Malonaldehyde (MDA), 4-hydroxy-(2E)-hexanal (4-HHE), and 4-hydroxy-(2*E*)-nonenal (4-HNE) like aldehydes are cytotoxic, carcinogenic, and mutagenic (Esterbauer et al., 1991, pp. 81-128). Further these aldehydes and peroxidative products react with amino acids to form protein carbonyls, lysis and aggregation of peptides, resulting in deterioration of muscle proteins (Buttkus, 2006, pp.432-434). However it is well known that the level of PUFAs and minerals is less in freshwater fish, particularly in white muscles fish compared to marine water fish (Li et al.,2006 and Ugoala et al., 2008) suggesting less progression of lipid peroxidation and thereby slow pace of the deterioration of muscle protein.

The peroxidative products including aldehydes such as MDA (most abundant aldehyde) reacts with Thiobarbituric Acid (TBA) in 1:2 ratio to generate red fluorescent and the intensity of that is measured by spectrophotometer to detect and quantify the lipid peroxidation and express as content of Thiobarbituric Acid Reactive Substances (TBARS) (Botsoglou et al., 1994). On the other hand denaturation of protein due to peroxidation decreases the solubility of protein in salt solution (Awad et al., 1969) and the Salt Soluble Protein (SSP) content is use as an index of quality of protein.

The cold storage is the main mode adopted to prevent/ reduce the progression of lipid peroxidation and thereby to increase the shelf life of the fish and fishery products. Among different cold storage conditions, -10 0C has been recommended by FAO (FAOWeb: http://www.fao.org/wairdocs/tan/x5919E/x5919e01.htm/ "Accessed on 25 Feb 2013".) for short term storage. In this context, the progression of lipid peroxidation in tilapia stored at -10 0C for 28 days was evaluated using the content TBARS as an indicator. Further, effect of peroxidation and frozen storage on muscle protein was evaluated by determining the Salt Soluble Protein (SSP) content while that of individual proteins was assessed by SDS-PAGE.

MATERIALS AND METHODS

The Chemicals; Hydrochloric acid, Tris HCl, TBA reagent and Sodium Chloride were analytical grade. Biuret reagent and Albumin Standard were from Fortress, UK. While Sodium Dodecyl Sulfate (SDS), Bromophenol blue, Ammonium persulphate, Acrylamide, Bis-acrylamide and Coomassie brilliant blue were from Sigma, USA. Molecular weight standards were from Bio-Rad,USA

Sample preparation

Freshly harvested tilapia (*Oreochromis niloticus*) were brought to the laboratory in ice. Skin was removed to obtain dorsal epaxial white muscle. The white muscles was separated and prepared four portions, each weighing nearly 25 g. One portion was used for the analysis of the first day (day - 0) and the rest (three portions) was wrapped separately with aluminium foils, put into polythene bags, and stored in a freezer set at - 10 ^{o}C (temperature of the freezer was monitored using a minimum and maximum thermocouple) and analyzed on day 7, 14 and 28 for TBARS and SSP contents and SSP was subjected to SDS-PAGE. The experiment was repeated three times and each indicator was expressed as a mean of three replicate.

Measurement of TBARS content

Five gram of muscle tissue was macerated, mixed with 50 ml of distilled water (d H_2O) and homogenized for 2 min. A 2.5 ml of 4 N HCl and 47.5 ml of d H_2O were added to the homogenate, and pH was adjusted below 1.5 before makeup the total volume to 100ml with d H_2O. Thus prepared homogenate was subjected to distillation until get 50 ml of distillate A 5 ml of 0.02 M TBA was added to 5 ml of distillate and kept in boiling water for 35 minutes. The reaction mixture was cooled in room temperature and the optical density was measured at 538 nm using a spectrophotometer. The TBARS content was determined as follows and expressed in mg/kg of sample weight. (Tarladgis et al., 1960)

$$\frac{7.843 \times \text{absorbancy reading} \times 10\text{g}}{\text{weight of the sample (g)}} = \text{TBARS content (mg/kg of sample)} \quad (1)$$

Determination of SSP content

The SSP content was measured according to the method of Munasinghe et al (2003). In brief, 15 ml 0.8 M NaCl was added to one gram of macerated muscle and homogenized for 10 min in ice. The homogenate was centrifuged at 2600 g for 20 min us-

ing a refrigerator centrifuge set at 4 °C. The supernatant was decant into another tube, mixed well, and 50 µl of it was added to 2.5 ml of Biuret reagent (Prepared according to the manufacturer's instructions, Fortress UK). The mixture was kept for 30 min at room temperature before measuring the optical density at 540 nm using a spectrophotometer. The SSP content was determined by means of a standard curve prepared using known total protein standards (Bio-Rad, USA) and expressed as mg/ g of fish tissue.

Identification of salt extractable proteins

The supernatants of the replicates of SSP extracts were pooled and mixed well. A 200 µl of pooled extract was mixed with equal volume of sample buffer and heated for 10 minutes at 100 oC. Thus prepared samples were stored at -80 oC until electrophoresis. Sodium Doecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out according to Lammeli (1970) (Laemmli, 1970). Samples were loaded at 30 µg of protein per well using 4% staking and 10% separating gels and electrophoresis was carried out at 60 and 120 constant voltages for staking and separating gels respectively. The gels were stained using coomassie blue dye solution (0.1% coomassie brilliant blue, 50% methanol, 10% acetic acid in distilled water) and destained with same solution sans coomassie brilliant blue. The molecular weights of the individual proteins were determined with the help of Lower Range (LR) and Higher Range (HR) molecular weightmarkers (Bio-Rad, USA).

Analysis of data

Data were analyzed using a linear mixed effects model fit by maximum likelihood using R statistical software (R Development Core Team, 2011)

RESULTS

TBARS content

The TBARS content of fish in all three experiments showed a gradual increase during 28 day storage period under -10 °C (Table1). The increment of TBARS content between day 0 and day 28 in the three experiments were significant (P<0.05)

Table 1 – Progression of lipid peroxidation of tilapia stored at -10 0C for 28 days

	Average TBARS (mg/kg of tissue)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Exp 1	2.02±0.03	2.09±0.03	2.35±0.53	3.13±0.2	4.01±0.2
Exp 2	2.01±0.1	2.92±0.09	3.15±0.08	4.14±0.18	4.55±0.04
Exp 3	0.17±0.03	0.26±0.08	0.37±0.35	0.45±0.34	0.52±0.26

Value (mean ± SEM, n=3) with significant level set at P < 0.05

Salt Soluble Protein (SSP) Content

The SSP content of fish in all three experiments showed a gradual decrease during 28 day storage period under -10 °C (Table2). The decrement of SSP content between day 0 and day 28 in the three experiments were significant (P<0.05)

Table 2 – Variation of salt soluble protein content (g/kg of tissue) of tilapia stored at -10 0C for 28 days

	Average SSP (mg/g of tissue)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Exp 1	5.75±0.06	5.19±0.03	5.29±0.03	5.18±0.03	4.97±0.10
Exp 2	8.44±0.14	7.95±0.03	7.90±0.12	7.41±0.11	7.36±0.08
Exp 3	6.23±0.22	5.37±0.13	4.83±0.16	4.67±0.09	3.32±0.24

Value (mean ± SEM, n=3) with significant level set at P < 0.05

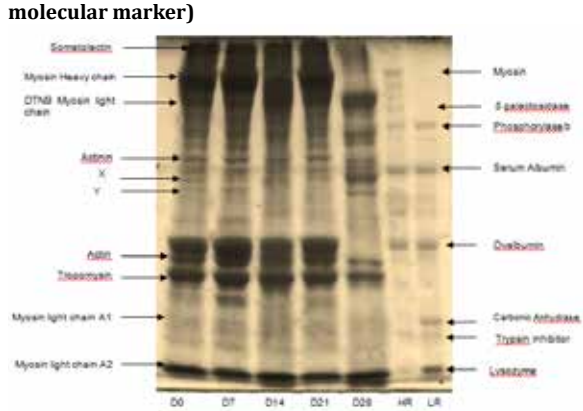
Relationship between TBA content and SSP content

The variation of TBARS and SSP contents of the three experiments showed a significant negative correlation during 28 day storage period. R^2 values of the three experiments were 0.8, 0.98 and 0.91 respectively. The gradient of the inverse relationship was high in experiment 3 (7.22) compared with that of experiment 1 (0.31) and 2 (0.43).

The protein profiles of the salt soluble proteins

A 23 protein bands were observed on day 0 in the SSP extracts and 8 bands were identified (Actin, Somatolactin, Myosin heavy chain, DNTB Myosin light chain, Actinin, Tropomysin, Myosin light chain A1 and Myosin light chain A2) based on their molecular weights (Figure 1). Of these 23 bands Somatolactin (26 kDa), Myosin Heavy Chain (200 kDa), Actinin (95 kDa), Actin (42 kDa) and Myosin Light Chain A1 (21-22.9 kDa) showed gradual decrease of their intensities during the storage period and disappeared in the fourth week of storage. On the other hand, Myosin Light Chain A2, Band X and Y showed a gradual increase of their intensities. However, Tropomysin (35 kDa) remained unchanged in its intensity.

Figure 1: Electrophoretic banding pattern of salt soluble protein extract during 28 day storage period at -10 °C (HR = Higher range molecular marker, LR = Lower range molecular marker)



DISCUSSION

TBARS value of Tilapia has progressively increased during 28 day storage period under -10 ^{0}C showing steady increase of lipid peroxidation of fresh water white muscle fishes, during frozen storage at -10^{0}C. The TBARS value of minced yellowtail meat, white muscle marine water fish stored at 4^{0}C does not show an increase during first 7 days (Munasinghe et al., 2005). However, in this experiment, the tilapia muscle showed an increase of TBARS during the first 7 days of storage even under -10 ^{0}C storage. The reason for this difference is not known at this stage. However, these findings suggest the importance of investigating the progression of lipid peroxidation under different storage temperatures and physical conditions of fish meat. According to the Egyptian Organization for Standardization and Quality

Control (EOS) 2005 (Mohamed Ali, 2011) the food with TBA (malonaldehyde) content (which is equivalent to TBARS in this experiment) more than 4.5 mg. Kg are not suitable for the consumption. In this context it is note worthy that in the experiment 1 and 2 the TBARS values on the day 28 were 4.01 and 4.55 mg/ kg respectively. This indicates the storage of tilapia more than 21 days even under -10°C may not be suitable for consumption.

In all three experiments, the SSP contents showed a steady decrease during the storage period indicating the deleterious effects of lipid peroxidation and frozen storage on tilapia. Most of the good quality fish meat proteins are belongs to SSP content (Huss, 1988) and the SSP content of Japanese red sea bream has continuously decrease with the time at the storage temperature of -18 ^{0}C (Reddy et al. 1996). French et al (1988) observed when Coho salmon (*oncorrhynchus kisutch*) and Sockye salmon (*oncorrhynchus nerka*) stored under 0, -1, -2, -3 and -25^{0}C for 5 days and the highest rate of hydrolysis of individual proteins occurs at temperature of -2 and -3 ^{0}C.

In this study negative correlation (R^2=0.9) between TBA content and SSP content was showed the deleterious effect of lipid peroxidative substances on salt solubility of proteins.

In this experiment 23 protein bands were obtained in gel electrophoresis in Tilapia. However only nine electrophoretic bands have obtained in SSP of Yellowtail minced meat during 10 day of storage time at 4 0C (Munasinghe et al, 2003).Intensity of Somatolactin, Myosin Heavy Chain, Actinin, Actin and Myosin Light Chain A1 has reduced with the increase of storage period indicting deleterious effect of frozen storage on these proteins. The protein profile of SSP in day 28 was different from that of day 0, 7, 14 and 21 indicating severe effect of freezing on SSP beyond 21 days of storage.

TBARS contents of the white muscle fish (Tilapia) stored under -10 0C were gradually increased during 28 day storage period indicating progression of lipid peroxidation of fish white muscle even under freezing temperature. On the other hand, the SSP content was progressively decreased indicating negative effect of freezing on quality of protein. The variation of TBARS and SSP contents were highly correlated suggesting the negative effect of peroxidation on muscle protein.

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