Journal of Scientific & Industrial Research
Vol. 77, February 2018, pp. 111-119

Characterization of Fish Protein Hydrolysate from Red Meat of *Euthynnus affinis* and its Application as an Antioxidant in Iced Sardine

U Parvathy[1]*, K M Nizam[2], A A Zynudheen[2], G Ninan[2], S K Panda[2] and C N Ravishankar[2]

[1]Mumbai Research Centre of ICAR-CIFT, Vashi, Navi Mumbai-400703, India
[2]ICAR-Central Institute of Fisheries Technology, Kochi-29, India

Received 10 October 2016; revised 09 August 2017; accepted 10 January 2018

Effective utilization of the fishery waste to produce value-added products has emerged as a priority area for the global seafood industry development. Tuna (*Euthynnus affinis*) red meat proteins which are generally discarded as waste in canning industry was hydrolysed using papain enzyme (0.5% w/w), and applied as antioxidant in dressed sardine during ice storage. Tuna protein hydrolysate (TPH) comprised of about 89.9 ± 0.6% protein, 1.35 ± 0.15% moisture, 2.71 ± 0.06% fat and 4.03 ± 0.02% ash. A proportional increase in the degree of hydrolysis of tuna protein with time was observed from 14.96% at 15 minutes reaching 22.98% at 45 minutes of hydrolysis. DPPH scavenging activity and reducing power improved with hydrolysis and was found to be 56.82 ± 0.74 % for 2 mg/ml protein and 0.614 ± 0.009 for 10 mg/ml protein, respectively at 45 minutes of hydrolysis. Sardine samples got darkened with storage period as indicated by decreased L* values. Similarly a reduction in hardness value was observed in ice stored sardine samples, more prominent in control compared to treated samples. TBA studies indicated that dip treatment in 0.5% TPH solution significantly reduced ($p < 0.05$) the oxidation in ice stored dressed sardine compared to control revealing the application of protein hydrolysate as a natural antioxidant in foods.

Keywords: Tuna Red Meat, Tuna Protein Hydrolysate, Enzyme Hydrolysis, Papain, Ice Storage

Introduction

Proteins are important in food processing and product development, as they are accountable for various functional properties that influence consumer acceptability. Fish contains high quality proteins with a well-balanced amino acid profile and fish by-product contains the same valuable protein as the fish muscles[1]. Recovery and modification of these by-product proteins and using these as functional ingredients in food systems is a very exciting and potential option[2,3] and application of enzyme technology is very promising in this regard[1]. Better utilization of fish proteins can be done by concentrating it by hydrolysis and this improves the digestibility, maintains a high essential amino acid content and also generates many improved functions for food, bioactive properties, pharmaceutical applications etc[1,4,5]. Fish hydrolysate, in its simplest form, is the fish proteins that are broken down into peptides and this degradation is carried out either chemically (using acids or bases) or biologically (using enzymes). Tuna canning generates as much as 70% solid wastes of which tuna red muscle accounts for about 12%[6] which can be effectively utilized for the production of tuna protein hydrolysates (TPH). Use of natural antioxidants are gaining more importance in recent times to prevent lipid peroxidation in the food industry[7,8]. Fish protein hydrolysates have been found to retard lipid oxidation and are capable to be used as natural antioxidants in foods and biological systems on account of the bioactive peptides present in them. Sardine, a highly nutritious marine fish on account of its richness in poly unsaturated fatty acids especially omega-3 fatty acids has numerous health benefits. On the contrary, presence of these highly unstable fatty acids results in problems such as fat oxidation making these fishes rancid thus affecting the keeping quality. Use of natural antioxidants such as protein hydrolysates can get control of this problem. Hence a study was conducted with the aim of utilizing the proteins present in tuna red meat by enzyme hydrolysis, its characterization and application as a natural antioxidant in dressed sardine during ice storage.

Materials and methods

Raw materials and chemicals

Fresh Tuna fish (*Euthynnus affinis*) was procured from the local fish landing center at Cochin, India.

*Author for Correspondence
E-mail: p.pillai2012@gmail.com

Tuna red meat was separated from the white meat manually for preparing protein hydrolysate. Papain Enzyme (Sigma-Aldrich) was used for hydrolyzing the tuna red meat protein. Fresh sardine samples, procured from the fish landing center, at Kalamukku, Vypin, Cochin, were dressed and used for assessing the antioxidative property of tuna protein hydrolysate (TPH). All chemicals used for the study were analytical grade.

Preparation of tuna protein hydrolysate

Procured tuna were layered between ice and brought to the laboratory. Red meat was separated from white meat manually and minced thoroughly using an electric grinder. The mince was mixed with water (1:1) (w/v) and subjected to 100 °C for 30 minutes to achieve complete inactivation of endogenous enzymes present in the substrate. Further, the temperature was lowered and maintained at 55 °C and pH 6.5, (optimum temperature and pH of the enzyme, respectively) and the sample was hydrolysed using papain @ 0.5 % (w/w) for 45 minutes. An enzyme substrate ratio of 0.5% (w/w) was used for hydrolysis, as it is an optimized ratio for good functional and bioactive properties[9,10].Aliquots were drawn at regular intervals (15, 30 and 45 minutes) to determine the degree of hydrolysis as well as antioxidative property. On completion of hydrolysis, the solution was immediately heated to 90 °C for 20 minutes to arrest the hydrolytic process. The hydrolysate was then course filtered followed by centrifugation @ 8000g at 10 °C for 20 minutes and the obtained supernatant was dried using spray drier (Basic Technology Private Limited, Kolkata, India) to get hydrolysate powder which was further stored in an air tight glass container.

Determination of proximate composition and pH

Proximate composition of tuna red meat and tuna protein hydrolysate (TPH) were estimated[11]. Total nitrogen and crude protein contents were estimated by Micro kjeldahl method. About five gram sample was blended with 45 ml distilled water and pH of the homogenate was measured using a pH meter (Cyberscan 510, Eutech Instruments, Singapore).

Degree of hydrolysis and yield

Degree of hydrolysis was estimated and computed[12]:

$$\text{DH \%} = \frac{\text{10 \% TCA soluble Nitrogen in sample}}{\text{Total Nitrogen in the Sample}} \times 100$$

Yield of TPH was calculated from the ratio of the amount of spray-dried hydrolysate powder to the amount of red meat used for hydrolysis.

$$\text{Yield \%} = \frac{\text{weight of the spray dried hydrolysate (g)}}{\text{weight of tuna red meat used for hydrolysis}} \times 100$$

Colour

The colour of TPH powder was evaluated using Hunter Lab colorimeter (MiniScan XE Plus Hunter Associates Lab inc., Reston, Virginia, USA) to produce numeric results indicative of the color of the sample by measuring L* (the degree of lightness: black (0) to white (100)), a* (degree of redness (+)/greenness (-)) and b* (the degree of yellowness (+) or blueness (-)).

Determination of functional properties

Fat absorption capacity (FAC)

FAC was determined[13] with slight modifications wherein about one gram TPH was taken in a pre-weighed centrifuge tube and thoroughly mixed with 5 ml sunflower oil. Further it was centrifuged @ 3000g for 30 minutes at 25 °C (Heraeus® Multifuge™ X1R, Thermofisher Scientific, Germany). The supernatant was drained off at 45° angle immediately and the centrifuge tube weighed again.

$$\text{FAC \%} = \frac{W3-W2}{W1} \times 100$$

W_1 = Wt of dry sample alone, W_2 = Wt of tube + dry sample and W_3 = Wt of tube + Sediment.

Foaming properties

Foaming capacity and stability of the protein hydrolysate were determined with slight modifications[14]. Twenty milliliters of 0.5% sample solution were homogenized at a speed of 11,000 rpm, using a homogenizer (IKA® T18™ basic, Germany) to incorporate the air for 2 min at room temperature. The whipped sample was immediately transferred into a 25 ml cylinder and the total volume was recorded. The foaming capacity was calculated according to the following equation:

$$\text{FC \%} = \frac{V2-V1}{V1} \times 100$$

where V_2 is the volume after whipping (ml) and V_1 is the volume before whipping (ml). The whipped sample was allowed to stand at room temperature for 3 min and the volume of whipped sample was then recorded. Foam stability was calculated as follows:

$$\text{FS \%} = \frac{V2-V1}{V1} \times 100$$

where V_2 is the volume after standing (ml) and V_1 is the volume before whipping (ml).

Emulsifying properties

10 ml of vegetable oil and 30 ml of 1% protein solution were mixed and homogenized using a

homogenizer (IKA® T18™ basic, Germany) at a speed of 20,000 rpm for 1 min. An aliquot of the emulsion (50 µl) was pipetted from the bottom of the container at 0 and 10 min after homogenization and mixed with 5 ml of 0.1% sodium dodecyl sulphate (SDS) solution. The absorbance of the diluted solution was measured at 500 nm using a spectrophotometer (SPECTRONIC Genesys 5, USA). The absorbance measured immediately (A_0) and 10 min (A_{10}) after emulsion formation was used to calculate the emulsifying activity index (EAI) and the emulsion stability index (ESI) as follows[15]:

$$\text{Emulsifying Activity Index (EAI)} = \frac{2 \times 2.303 \times A_0}{0.25 \times \text{wt of protein}}$$

$$\text{Emulsion Stability Index (ESI)} = \frac{A_{10} \times \Delta t}{\Delta A}$$

Δt =Time and $\Delta A = A_0 - A_{10}$

Determination of antioxidative activity

DPPH (2,2-Diphenyl-1-picryhydrazyl)radical scavenging activity

Protein hydrolysate was dissolved in distilled water to obtain a concentration of 2 mg protein/ml. To 1.5 ml of sample solutions, 1.5 ml of 0.1mM DPPH was added and mixed vigorously. After incubating for 30 min, the absorbance of the resulting solution was measured at 517 nm using a spectrophotometer (SPECTRONIC Genesys 5, USA)[16]. The control was conducted in the same manner, except that distilled water was used instead of sample. In similar manner, DPPH radical scavenging activity of 0.1mM Ascorbic acid was also evaluated. DPPH radical scavenging activity was calculated according to the following equation:

$$\text{DPPH radical-scavenging activity} = 1 - \frac{\text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

Reducing power

The sample solution (0.5 ml, 10 mg protein/ml) was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 ^{o}C for 20 min. An aliquot (2.5 ml) of 10% trichoroacetic acid was added to the mixture, followed by centrifugation at 3000 rpm (Heraeus® Multifuge™ X1R, Thermofisher Scientific, Germany) for 10 min. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 2.5 ml of 0.1% ferric chloride and the absorbance was read at 700 nm. Increased absorbance of the reaction mixture indicates increasing reducing power[17].

Preparation and quality evaluation of FPH treated sardine

Fresh sardine was dressed by beheading and gutting and further washed and divided into four lots. The first lot, considered as control (Sample A) was dipped in chilled water for 10 minutes, and others (Sample B, C and D) were dipped in 0.1 % FPH, 0.5 % FPH and 0.5 % ascorbic acid, respectively for 10 minutes. After draining-off, each samples were packed in labelled polythene bags, and kept layered between flake ice in insulated box and stored in chill room. Samples were drawn periodically for quality assessments viz., Biochemical (pH, TBA[18], PV[19]), physical (colour (Hunter Lab Colorimeter, Miniscan® XE Plus Hunter Associates Lab Inc., Reston, Virginia, USA) and texture (Lloyd Instruments Ltd, Hampshire, UK)), microbiological[20] and sensory analysis[21] by six trained panellists using a 9-point hedonic scale, throughout the study.

Statistical analysis

All experiments were done in triplicate. The data were subjected to analysis of variance (ANOVA) and the differences between means were evaluated by Duncan's multiple range test. SPSS statistic programme (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL,) was used for data analysis.

Results and Discussion

Proximate composition

Tuna contain high amounts of protein of around 27% and is rich in essential amino acids and thus can be considered as an ideal protein source. In the present study, tuna red meat which was used as the raw material for the preparation of TPH contained 28.25 ± 0.45% protein. Earlier studies revealed that the protein content of most tuna species ranged between 15 – 30% and reports indicated a protein content of 26.92 ± 0.27 % in yellow fin tuna dark muscle[22]. TPH comprised of about 89.9 ± 0.6% protein, 1.35 ± 0.15% moisture, 2.71± 0.06% fat and 4.03 ± 0.02% ash. Many researchers have reported that the protein content of fish protein hydrolysates ranged from 60% to 90% of total composition[23,24] and that the high protein content was due to solubilization of proteins during hydrolysis and removal of insoluble solid matter by centrifugation. The lipid content reduction in fish protein hydrolysates was most likely due to the removal of lipids along with insoluble protein fractions during centrifugation[1]. Similarly the low moisture content was on account of the spray drying adopted for the final product.

Degree of hydrolysis (DH) and Yield

DH is most commonly used to describe the extent of hydrolysis of food proteins. During the hydrolytic process, it was observed that DH increased with time from 14.96 % at 15 minutes of hydrolysis to 18.56 % and 22.98 % at 30 minutes and 45 minutes of hydrolysis, respectively. Similar findings were observed in herring and its by-products where DH increased steadily with hydrolysis time[3] and higher the DH, lower was the average length of peptide chain[1]. An optimum DH was achieved with a hydrolysis temperature of 50 °C at 135 minute in catla fish visceral protein[25].

In the present study, the yield of spray dried TPH was only about 4.03% which may be on account of lose in spray drying operation. Yields of fish protein hydrolysates from different enzymes were dependent on DH %. The yield of FPH from striped cat fish frame meat increased from 7.03±0.83% to 9.85±0.25% with increase in enzyme concentration[26]. Reports indicated yields ranging from 3.6-5.5% from raw minced herring fillets and defatted herring hydrolysed by alcalase[12].

Colour

Colour has been shown to influence the overall acceptability of new food products[27], including new protein ingredients in food formulations and is affected by various factors like raw materials used, hydrolytic conditions, drying methods adopted etc. TPH exhibited a creamy-yellowish colour. Analysis of colour using the colourimeter (Hunter Lab Colorimeter, Miniscan® XE Plus, Virginia, USA) indicated an L*, a*, b* value of 90.97 ± 0.05, -0.83 ± 0.01, and 16.14 ± 0.01, respectively. A light yellowish colour with an L*, a*, b* value of 95.38 ± 0.10, -1.30 ± 0.01 and 14.54 ± 0.02, respectively was reported for acid fungal protease hydrolysed salmon fish waste protein[28].

Functional properties

Fat absorption capacity

Fat absorption capacity (FAC) of proteins correlates with surface hydrophobicity[1]. The ability of protein hydrolysates to absorb oil is an important functionality that influences the taste of the food product[29]. The FAC of the hydrolysate sample observed was 2.24 ± 0.12 g oil/g protein. An FAC of 5g oil/g protein was observed in cod by-product hydrolysate[30] whereas hydrolysate from the blue whiting processing by-products gave an FAC of 3g oil/g protein[31]. The FAC of red salmon protein hydrolysates increased during hydrolyzation within a certain time range whereas it dropped on further hydrolyzation[32].

Foaming properties

Foaming properties are the physicochemical characteristics of proteins to form and stabilise foams[33]. The foaming capacity and foaming stability at 3 minutes for 0.5% TPH solution was observed to be 20.30 ± 1.08% and 10.67 ± 2.31%, respectively (Table 1). Good foaming properties was reported for capelin protein hydrolysates at low DH (12%)[13]. An increase in foaming ability from 23.33% to 70% was noticed[33] when concentration of round scad protein hydrolysate increased from 0.1 to 3% and that was reported to be due to the formation of stiffer foams[34].

Emulsifying properties

Protein hydrolysates are good emulsifiers due to their improved amphiphilic nature, as they expose more hydrophilic and hydrophobic groups that enable orientation at the oil–water interface for more effective adsorption[35]. Higher amount of larger molecular weight peptides or more hydrophobic peptides contribute to the stability of the emulsion and excessive hydrolysis result in loss of emulsifying properties. Emulsifying Activity Index (EAI) is a function of oil volume fraction, protein concentration and the type of equipment used to produce emulsion[15]. In the present study, EAI of TPH was found to be 6.8 m^2/g and Emulsion Stability Index (ESI) was 40 minutes. Protein hydrolysates should consist of at least 20 amino acids to possess good emulsifying capacity and studies indicated EAI values of 37, 24 and 13 m^2/g for 0.1%, 0.5% and 1% round scad protein hydrolysate, respectively[1].

Table 1 — Functional and Antioxidative properties of tuna protein hydrolysate

Properties	Values
Fat Absorption Capacity	2.24 ± 0.12 g oil/g sample
Foaming Capacity	20.30 ± 1.08%
Foaming Stability at 3 min.	10.67 ± 2.31%
EAI	6.8 m^2/g
ESI	40 minutes
DPPH radical-scavenging activity at 15 min.	50.64 ± 0.57%
30 min.	54.85 ± 0.33%
45 min.	56.82 ± 0.74 %
Reducing power at 15 min.	0.492 ± 0.005
30 min.	0.569 ± 0.006
45 min.	0.614 ± 0.009

Antioxidative activities

DPPH (2, 2-Diphenyl-1-picryhydrazyl) radical-scavenging activity

This assay evaluates the free radical scavenging capacity of the sample and TPH (2mg/ml) exhibited a DPPH scavenging activity of about 50.64 ± 0.57% at 15 minutes of hydrolysis which increased to 56.82 ± 0.74 % at 45 minutes of hydrolysis (Table 1). The DPPH scavenging activity of 0.1 mM ascorbic acid was observed to be 60.62 ± 1.72%. A DPPH radical-scavenging activity of 59.9% was observed for round scad protein hydrolysate[33]. Antioxidative activity of protein hydrolysates depends on the proteases[36] and hydrolysis conditions employed[36,37]. During hydrolysis, a wide variety of smaller peptides and free amino acids are generated, depending on enzyme specificity and these free amino acids and small peptides affect the antioxidative activity[16].

Reducing power

Reducing power is a measure of the iron-reducing capacity and samples with higher reducing power have better abilities to donate electron and free radicals to form stable substances, thereby interrupting the free radical chain reactions[38]. Reducing power of TPH (10 mg/ml) was 0.492 ± 0.005, 0.569 ± 0.006 and 0.614 ± 0.009 at 15, 30 and 45 minutes, respectively (Table 1). Factors like degree of hydrolysis, nature of substrate and type of enzyme influences the reducing power of hydrolysate[35].

Quality changes of sardine during ice storage

Colour

Analysis of colour of sardine samples during ice storage indicated that the intensity of colour (lightness) reduced significantly ($p < 0.05$) during storage (Table 2) but the difference was not prominent between the samples. It could be inferred that with storage period, the fish got darkened. The a* (redness) and b* (yellowness) values also decreased significantly ($p < 0.05$) during storage and significant difference ($p < 0.05$) between the samples were also observed. The rate of decrease in a* was more for Sample A (control) indicating more oxidation. The loss of redness in mahi-mahi red muscle due to oxidation was measured by the decrease in a* value[39] and a decrease in b* values during refrigerated storage of shrimp waste protein hydrolysate treated croaker fish fillet were observed[40].

Texture profile analysis (TPA)

Texture is the sensory interpretation and expression of the structure or interior construction of products linked to their response to stress and haptic attributes[41]. Compared to sensory evaluation, textural measurements by instrumental methods are better and more precise as they reduce the variations during measurements arising from human factors[42]. The sardine samples were instrumentally analysed mainly for three basic parameters viz., hardness, chewiness, and springiness which decreased significantly ($p < 0.05$) for all samples during storage (Table 2). The decrease in hardness was more for sample A compared to treated samples and the rate of decrease was more significant initially for all samples during storage. Variations in chewiness were also proportional to changes in hardness. This is in agreement with data on texture changes of raw cultured and wild sea bream stored in ice up to 23 days measured by compression using a flat-ended cylinder[43]. The textural parameters of the dorsal part of seabream fillet were measured and it was observed that except springiness, all parameters decreased significantly ($p < 0.05$) within five days of post-mortem storage[44]. Reports suggest that a decrease in fish hardness during storage is on account of proteolysis caused by endogenous and microbial enzymes[45].

pH

pH values can be used as measurements for quality deterioration and shelf life prediction in fishes. Normally fresh fishes indicate a neutral pH in the range of 6.5-7.5. The pH value of sardine samples showed a slight increasing trend (6.2-6.5) without any significant difference ($p > 0.05$) between the samples throughout the storage period. An increase in pH reflects the production of alkaline bacterial metabolites in fish during storage[46]. A decrease in pH from 6.7 to 6.2 for both control and FPH treated mahi-mahi red muscles samples were observed towards the end of 87 h storage time[39] followed by an increasing trend which may be on account of the lactate formed from glycolysis during the initial period of fish storage.

TBA and peroxide value

Fatty fish are particularly vulnerable to lipid oxidation which can create severe quality problems such as unpleasant (rancid) taste and smell, and also it may produce alterations in texture, colour, and nutritional value, even on storage at subzero temperatures. Maximum level of TBA value indicating the quality of the fish frozen, chilled or ice stored is 5 mg of malonaldehyde/kg[47]. On 5th day, the

Table 2 — Changes in texture and colour of dressed sardine during ice storage (A: Control, B: 0.1% TPH treated, C: 0.5% TPH treated, D: 0.5% Ascorbic acid treated) (n=3). Different superscripts for same parameter in the same column (upper case) and same row (lower case) indicate significant difference ($p < 0.05$)

Parameters	Sample	Storage Days						
Texture		0	3	5	7	9	11	15
Hardness1 (N)	A	24.70 ± 1.29^{Ae}	21.20 ± 1.49^{Ad}	19.67 ± 1.53^{Ad}	15.92 ± 0.29^{Bc}	15.12 ± 2.05^{Ab}	12.13 ± 0.47^{Aa}	10.47 ± 1.45^{Aa}
	B	24.70 ± 1.29^{Ae}	24.49 ± 1.43^{Be}	18.78 ± 2.46^{Ad}	15.63 ± 0.49^{Ac}	14.71 ± 1.53^{Ab}	12.60 ± 1.76^{Aa}	11.54 ± 0.90^{Aa}
	C	24.70 ± 1.29^{Af}	25.75 ± 1.72^{Bf}	23.89 ± 1.09^{Be}	18.40 ± 0.71^{Cd}	14.65 ± 1.81^{Ac}	13.68 ± 3.30^{Bb}	13.42 ± 1.21^{Aa}
	D	24.70 ± 1.29^{Ac}	26.07 ± 0.21^{Cd}	23.58 ± 3.59^{Bb}	22.67 ± 2.11^{Db}	14.88 ± 0.68^{Aa}	14.63 ± 0.77^{Ca}	14.61 ± 2.47^{Ba}
Springiness (mm)	A	1.26 ± 0.04^{Af}	0.96 ± 0.05^{Ab}	1.07 ± 0.06^{Bd}	1.0 ± 0.03^{Bc}	1.13 ± 0.02^{Ce}	0.95 ± 0.03^{Ab}	0.55 ± 0.05^{Aa}
	B	1.26 ± 0.04^{Ag}	1.06 ± 0.04^{Cc}	1.14 ± 0.06^{Cf}	1.1 ± 0.04^{Ce}	1.03 ± 0.04^{Ab}	1.07 ± 0.08^{Cd}	0.82 ± 0.06^{Ba}
	C	1.26 ± 0.04^{Ag}	1.14 ± 0.06^{Df}	0.97 ± 0.06^{Ac}	0.94 ± 0.01^{Ab}	1.06 ± 0.13^{Be}	1.03 ± 0.10^{Bd}	0.86 ± 0.05^{Ba}
	D	1.26 ± 0.04^{Ad}	0.99 ± 0.03^{Bb}	1.16 ± 0.02^{Dc}	$0.98\pm.10^{Bb}$	1.00 ± 0.12^{Ab}	1.00 ± 0.04^{Bb}	0.77 ± 0.05^{Ba}
Chewiness (Nmm)	A	11.13 ± 0.56^{Ae}	8.80 ± 0.72^{Bd}	5.63 ± 0.53^{Ac}	3.68 ± 0.19^{Ab}	2.78 ± 0.36^{Aa}	2.76 ± 0.38^{Aa}	1.84 ± 0.18^{Aa}
	B	11.13 ± 0.56^{Af}	8.26 ± 0.26^{Ae}	7.04 ± 0.06^{Bd}	6.31 ± 0.52^{Bc}	3.98 ± 0.53^{Bb}	2.46 ± 0.61^{Aa}	2.03 ± 0.70^{Aa}
	C	11.13 ± 0.56^{Ag}	9.35 ± 0.50^{Cf}	7.59 ± 1.20^{Ce}	5.88 ± 0.45^{Bd}	3.98 ± 0.08^{Bc}	3.72 ± 0.33^{Cb}	2.38 ± 0.13^{Aa}
	D	11.13 ± 0.56^{Ae}	10.26 ± 0.34^{Dd}	9.73 ± 0.21^{Dd}	8.37 ± 0.44^{Cc}	4.60 ± 1.21^{Cb}	3.14 ± 0.27^{Ba}	3.29 ± 0.40^{Ba}
Colour								
L*	A	44.69 ± 0.01^{Ae}	44.48 ± 0.55^{Be}	38.51 ± 0.49^{Bd}	32.48 ± 0.06^{Ac}	27.8 ± 0.33^{Ab}	28.24 ± 0.87^{Bb}	25.26 ± 0.24^{Aa}
	B	44.69 ± 0.01^{Af}	40.20 ± 0.08^{Ae}	37.2 ± 0.10^{Ad}	33.98 ± 0.07^{Bc}	28.11 ± 0.96^{Ab}	28.71 ± 0.12^{Bb}	24.39 ± 0.10^{Aa}
	C	44.69 ± 0.01^{Af}	41.16 ± 0.90^{Ae}	36.61 ± 0.08^{Ad}	36.94 ± 0.03^{Cd}	29.78 ± 0.05^{Bc}	26.62 ± 0.04^{Ab}	26.21 ± 0.10^{Ba}
	D	44.69 ± 0.01^{Af}	49.77 ± 2.75^{Cg}	41.74 ± 0.34^{Ce}	34.54 ± 0.09^{Bd}	31.42 ± 0.04^{Cc}	28.94 ± 0.06^{Bb}	25.83 ± 0.09^{Ba}
a*	A	5.89 ± 0.02^{Ae}	4.28 ± 0.03^{Ad}	3.59 ± 0.02^{Ac}	2.79 ± 0.04^{Ab}	2.12 ± 0.02^{Aa}	2.12 ± 0.04^{Aa}	2.07 ± 0.02^{Aa}
	B	5.89 ± 0.02^{Ad}	4.48 ± 0.13^{Bc}	4.53 ± 0.03^{Cc}	3.19 ± 0.02^{Bb}	3.01 ± 0.05^{Ca}	3.00 ± 0.01^{Ca}	2.97 ± 0.02^{Ca}
	C	5.89 ± 0.02^{Ag}	4.31 ± 0.03^{Ae}	4.35 ± 0.03^{Bf}	2.80 ± 0.03^{Ad}	2.33 ± 0.02^{Bc}	2.21 ± 0.02^{Bb}	2.16 ± 0.02^{Ba}
	D	5.89 ± 0.02^{Af}	5.29 ± 0.02^{Ce}	4.79 ± 0.02^{Dd}	4.32 ± 0.04^{Cc}	4.27 ± 0.04^{Dc}	4.17 ± 0.03^{Db}	3.55 ± 0.05^{Da}
b*	A	13.63 ± 0.79^{Ae}	9.90 ± 0.05^{Bd}	7.4 ± 0.02^{Bc}	6.48 ± 0.06^{Cb}	6.40 ± 0.08^{Ca}	6.15 ± 0.03^{Ca}	6.03 ± 0.05^{Ca}
	B	13.63 ± 0.79^{Ae}	9.8 ± 0.03^{Bd}	6.37 ± 0.03^{Ac}	5.86 ± 0.06^{Bb}	5.39 ± 0.04^{Ba}	5.24 ± 0.03^{Ba}	5.11 ± 0.03^{Ba}
	C	13.63 ± 0.79^{Ae}	9.92 ± 0.04^{Bd}	7.8 ± 0.05^{Bc}	7.33 ± 0.03^{Dc}	6.82 ± 0.03^{Db}	6.22 ± 0.03^{Ca}	6.15 ± 0.03^{Ca}
	D	13.63 ± 0.79^{Ae}	8.53 ± 0.05^{Ad}	6.19 ± 0.04^{Ac}	4.95 ± 0.09^{Ab}	4.29 ± 0.02^{Aa}	4.13 ± 0.02^{Aa}	4.04 ± 0.03^{Aa}

control sample TBA increased upto 5.14 mg/kg malonaldehyde whereas samples B, C and D reached the acceptability limit on 7th, 9th and 11th day respectively (Figure 1).TBA increase was significant ($p < 0.05$) during storage and there was a significant reduction in the extent of TBA increase in treated samples compared to control. TBA values of pink salmon fillets glazed with hydrolysed Pollock skin were significantly ($p > 0.05$) lower than non glazed fillets[48]. Dey and Dora (2014) also reported that shrimp waste protein hydrolysate solution dip effectively arrested oxidation of fat in croaker muscle[40]. The recommended value of peroxide for fresh fin fish is 10-20 meq kg^{-1} of oil and the value beyond this would result in the fish smelling and tasting rancid[49]. The peroxide value, an indication of oxidative rancidity was found to increase significantly ($p < 0.05$) during storage for all the sardine sample lots (Figure 2). Similar to TBA studies, the rate of increase in PV was more in Sample A (control) compared to treated ones. Sample C and D showed a lower and similar trend of increase in PV. Mahi mahi samples treated with whole hydrolysate and <10 kDa fractionate showed a significantly low PV value of 20 µ mol/kg of muscle at 88 h storage[39,50].

Microbiological quality

The total aerobic mesophilic count has been the commonly used microbiological indicators of the quality of foods. Fresh fishes contain total bacterial count in the range of $10^3 – 10^6$cfu per gram of sample. As recommended by International Commission on Microbiological Specification for Food[51], the upper

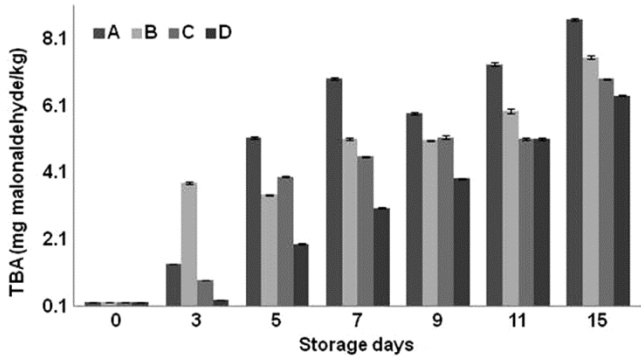

Fig. 1 — Changes in TBA of dressed sardine during ice storage (A: Control, B: 0.1% TPH treated, C: 0.5% TPH treated, D: 0.5% Ascorbic acid treated) (n=3)

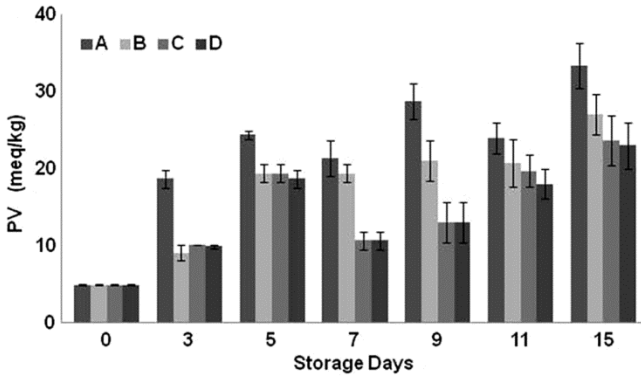

Fig. 2 — Changes in Peroxide value of dressed sardine during ice storage (A: Control, B: 0.1% TPH treated, C: 0.5% TPH treated, D: 0.5% Ascorbic acid treated) (n=3)

acceptability limit of total viable bacterial count in fresh fish is 7 $\log_{10}$ cfu/g flesh and levels exceeding this is regarded as microbially spoiled fish muscle not fit for human consumption. All the samples showed a significant increase ($p < 0.05$) in aerobic plate count (Figure 3) during storage with no significant difference between the samples and none of the lots reached the microbial count limit during the storage period.

Sensory analysis

Sensory assessment has always played a key role in quality and freshness evaluation in the fish industry. They are better at recognizing complexities and are more discriminatory than other techniques. Scoring is the most commonly used scientific method for assessing food freshness. Sensory evaluation of different attributes like appearance, odour, flavor, colour and texture using a 9-point hedonic scale was carried out. For fish samples, a sensory score of 4 is regarded as the limit acceptable for human consumption[52]. All the samples showed a significant

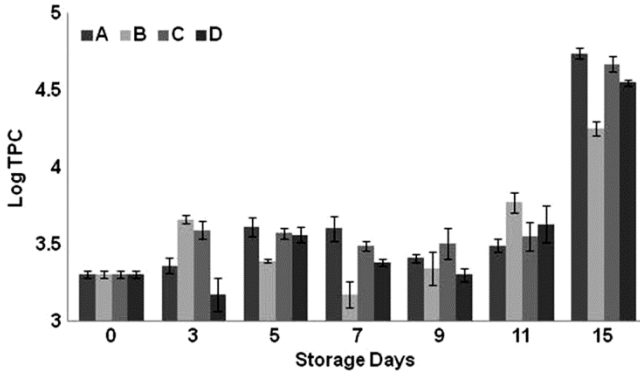

Fig. 3 — Changes in Total plate count of dressed sardine during ice storage (A: Control, B: 0.1% TPH treated, C: 0.5% TPH treated, D: 0.5% Ascorbic acid treated)

decrease ($p < 0.05$) in the sensory score during storage period and the difference was significant ($p < 0.05$) between the samples also. Sample C and D had a better acceptability during storage and all the samples reached the sensory acceptability limit on the 11th day of storage study i.e., it decreased from 8.65 (initial) to 4.08, 4.25, 4.67 and 5.0 for samples A, B, C and D, respectively. The higher sensory scores for treated samples during storage may be correlated to the oxidative changes where the rate of increase of TBA and PV were less compared to control lot. Similar results of gradual but declining sensory acceptability were observed in control and dill extract treated mackerel fillets during refrigerated storage[53].

Conclusion

Fish protein hydrolysate is well established for its antioxidant properties on account for the bioactive peptides they possess. The present study revealed a higher antioxidative activity for protein hydrolysate derived from tuna red meat. Its application in dressed sardine and subsequent ice storage indicated a significant reduction in the extent of oxidation in TPH treated sardine samples compared to control while there were no significant changes between the samples with respect to the microbial counts. Hence the current work concluded the potential of protein hydrolysate as a natural antioxidant dip in reducing the oxidation in ice stored dressed sardine.

Acknowledgement

The authors thank the Indian Council of Agricultural Research (ICAR), New Delhi for the financial support. Authors also thank all staff of Fish Processing Division, ICAR-CIFT for the support rendered during the study.

References

1 Kristinsson H G & Rasco B A, Fish protein hydrolysates: Production, biochemical and functional properties, *Crit Rev Food Sci*, **40(1)** (2000) 43-81.

2 Halim N R, Yusof H M & Sarbon N M, Functional and bioactive properties of fish protein hydolysates and peptides: a comprehensive review, *Trends Food Sci Tech*, **51** (2016) 24-33.

3 Sathivel S, Bechtel P J, Babbitt J, Smiley S, Crapo C, Repond K D & Prinyawiwatkul W, Biochemical and functional properties of herring (*Clupeaharengus*) byproducthydrolysates, *J Food Sci*, **68** (2003) 2196-2200.

4 Mahmoud M I & Cordle C T, Protein hydrolysates as special nutritional ingredients, in *Novel Macromolecules in Food Systems*, edited by Doxastakis G & Kiosseoglou V, *Elsevier Science Press*, Amsterdam, (2000), 181-215.

5 Hartmann R & Meisel H, Food-derived peptides with biological activity: from research to food applications, *Curr Opin Biotech*,**18** (2007) 163-169.

6 Guerard F, Guimas L & Binet A, Production of tuna waste hydrolysates by a commercial neutral protease preparation, *J Mol Catal B-Enzym*, **19–20** (2002) 489-498.

7 Lin C C & Liang J H, Effect of antioxidants on the oxidative stability of chicken breast meat in a dispersion system, *J Food Sci*, **67** (2002) 530-533.

8 Niki E, Assessment of Antioxidant Capacity *in vitro* and *in vivo*, *Free Radical Bio Med*, **49(4)** (2010) 503-515.

9 Tanuja S, Viji P, Zynudheen A A & Joshy C G, Composition, functional properties and antioxidative activity of hydrolysates prepared from the frame meat of Striped catfish (*Pangasianodon hypophthalmus*), *Egypt J Biol*, **14** (2012) 27-35.

10 Parvathy U, Zynudheen A A, Panda S K, Jeyakumari A & Anandan R, Extraction of protein from yellow fin tuna (*Thunnus albacares*) waste by enzymatic hydrolysis and its characterization, *Fish Technol*, **53** (2016) 115-124.

11 AOAC, *Official Methods of Analysis*, **19th edn** Association of Official Analytical Chemists, Washington DC (2012).

12 Hoyle N T & Merritt J H, Quality of fish protein hydrolysates from herring (*Clupea harengus*), *J Food Sci*, **59** (1994) 76-79.

13 Shahidi F, Han X Q & Synowiecki J, Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*), *Food Chem*, **53** (1995) 285-293.

14 Sathe S K & Salunkhe D K, Functional properties of the Great Northern Bean (*Phaseolus vulgaris L*) proteins: Emulsion, foaming, viscosity and gelation properties, *J Food Sci*, **46(1)** (1981) 71-81.

15 Pearce K N & Kinsella J E, Emulsifying properties of proteins: Evaluation of a turbidimetric technique, *J Agr Food Chem*, **26** (1978) 716-723.

16 Wu H C, Chen H M & Shiau C Y, Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*), *Food Res Int*, **36** (2003) 949-957.

17 Oyaiza M, Studies on products of browning reaction: antioxidative activity of products of browning reaction prepared from glucosamine, *J Nutr*, **44** (1986) 307-315.

18 Tarladgis G B, Watts M B & Younathan T M, A distillation method for the quantitative determination of malonaldehyde in rancid foods, *J Am Oil Chem Soc*, **37** (1960) 44-50.

19 AOAC, *Official Methods of Analysis*, **14th edn** Association of Official Analytical Chemists, Washington D C, (1989).

20 Surendran P K, Thampuran N, Nambiar V N, Lalitha K V & Toms C J, *Laboratory techniques for microbiological examination of sea food*, **4th edn** Central Institute of Fisheries Technology, Cochin, (2013) 19-25.

21 Meilgaard M, Civille G V & Carr B T, *Sensory evaluation techniques*, **4th edn** CRC Press, Boca Raton, USA, (2006) 464.

22 Sánchez-Zapata E, Amensour M, Oliver R, Fuentes-Zaragoza E, Navarro C, Fernández-López J, Sendra E, Sayas E & Pérez-Alvarez J A, Quality Characteristics of Dark Muscle from Yellowfin Tuna *Thunnus albacares* to Its Potential Application in the Food Industry, *Food Nutr Sci*, **2** (2011) 22-30.

23 Choi Y J, Hur S, Choi B D, Konno K & Park J W, Enzymatic hydrolysis of recovered protein from frozen small croaker and functional properties of its hydrolysates, *J Food Sci*, **74** (2009) C17–C24.

24 Chalamaiah M, NarsingRao G, Rao D G & Jyothirmayi T, Protein hydrolysates from meriga (*Cirrhinus mrigala*) egg and evaluation of their functional properties, *Food Chem*,**120** (2010) 652-657.

25 Bhaskar N, Benila T, Radha C & Lalitha R G, Optimization of enzymatic hydrolysis of visceral waste proteins of Catla (*Catlacatla*) for preparing protein hydrolysate using a commercial protease, *Bioresource Technol*, **99** (2008) 335-343.

26 Tanuja S, Aiswarya H, Zynudheen A A & Joshy C G, Functional and antioxidative properties of fish protein hydrolysate (FPH) produced from the frame meat of striped catfish *Pangasianodon hypophthalmus* (Sauvage, 1878) using alkaline protease alcalase, *Indian J Fish*, **61(2)** (2014) 82-89.

27 Bueno-Solano C, López-Cervantes J, CampasBaypoli O N, Lauterio-García R, Adan-Bante N P & Sánchez-Machado D I, Chemical and biological characteristics of protein hydrolysates from fermented shrimp by-products, *Food Chem*, **112(3)** (2009) 671-675.

28 Nurdiani R, Dissanayake M, Street W E, Donkor O N, Singh T K & Vasiljevic T, *In vitro* study of selected physiological and physicochemical properties of fish protein hydrolysates from 4 Australian fish species, *Int Food Res J*, **23(5)** (2016) 2029-2040.

29 Foh M B K, Issoufou A, Foh B M, Kamara M T & Xia W, Functionality and antioxidant properties of tilapia (*Oreochromis niloticus*) as influenced by the degree of hydrolysis, *Int J Mol Sci*, **11** (2010) 1851-1869.

30 Slizyte R, Dauksas E, Falch E, Storro I & Rustad T, Characteristics of protein fractions generated from hydrolysed cod (*Gadusmorhua*) by-products, *Process Biochem*, **40(6)** (2005) 2021-2033.

31 Geirsdottir M S, Sigurgisladottir S, Hamaguchi P Y, Thorkelsson G, Johannsson R, Kristinsson H G & Kristjansson M M, Enzymatic hydrolysis of Blue whiting (*Micromesistius poutassou*); functional and bioactive properties, *J Food Sci*, **76(1)** (2011) C14–C20.

32 Sathivel S, Smiley S, Prinyawiwatkul W & Bechtel P J, Functional and nutritional properties of red salmon (*Oncorhynchus nerka*) enzymatic hydrolysates, *J Food Sci*,**70(6)** (2005) 401-406.

33 Thiansilakul Y, Benjakul S & Shahidi F, Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterusmaruadsi*), *Food Chem,* **103(4)** (2007) 1385-1394.

34 Lawal O S, Functionality of African locust bean (*Parkia biglobossa*) protein isolate: Effects of pH, ionic strength and various protein concentrations, *Food Chem,* 86(3) (2004) 345-355.

35 Klompong V, Benjakul S, Kantachote D & Shahidi F, Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type, *Food Chem,* **102(4)** (2007) 1317-1327.

36 Jun S-Y, Park P-J, Jung W-K & KimS-K, Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein, *Eur Food Res Technol,* **219** (2004) 20-26.

37 Jao C L & Ko W C, 1, 1-Diphenyl-2- picrylhydrazyl (DPPH) radical scavenging by protein hydrolysates from tuna cooking juice, *Fisheries Sci,* **68** (2002) 430-435.

38 Juntachote T & Berghofer E, Antioxidative properties and stability of ethanolic extracts of holy basil and galangal, *Food Chem,* **92(2)** (2005) 193-202.

39 Dekkers E, Raghavan S, Kristinsson H G & Marshall M R, Oxidative stability of mahi-mahi red muscle dipped in tilapia protein hydrolysates, *Food Chem,* **124** (2011) 640-645.

40 Dey S S & Dora K C, Antioxidative activity of protein hydrolysate produced by alcalase hydrolysis from shrimp waste (*Penaeus monodon* and *Penaeus indicus*), *J Food Sci Technol,* **51(3)** (2014) 449-457.

41 Coppes Z, Pavlisko A & Vecchi S D, Texture measurements in fish and fish products, *J Aquat Food Prod Technol,* **11(1)** (2002) 89-105.

42 Cheng J H, Sun D W, Han Z & Zeng X A, Texture and structure measurements and analysis for evaluation of fish and fillet freshness quality: A review, *Compr Rev Food Sci Food Saf,* **13** (2014) 52-61.

43 Alasalvar C, Taylor K D A & Shahidi F, Comparative quality assessment of cultured and wild sea bream (*Sparus aurata*) stored in ice, *J Agr Food Chem,* **50** (2002) 2039-2045.

44 Ayala M D, Abdel I, Santaella M, Martinez C, Periago M J, Gil F, Blanco A & Albors O L, Muscle tissue structural changes and texture development in seabream (*Sparus aurata*) during post-mortem storage, *LWT-Food Sci Technol,* **43(3)** (2010) 465-475.

45 Schubring R, Texture measurement on gutted cod during storage in ice using a hand held instrument, *Inf Fischwirtsch.,* **49(1)** (2002) 25-27.

46 Abbas K A, Mohamed A, Jamilah B & Ebrahimian M A, Review of Correlations between Fish Freshness and pH during Cold Storage, *Am J Biochem Biotechnol,* **4(4)** (2008) 416-421.

47 Nunes M L, Cardinal M, Mendes R, Morào Campos R, Bandarra N M, Lourenço H, and Jerome M, Effect of season and storage on proteins and lipids of sardine (Sardine pilchardus) minces and surimi, in *Quality Assurance in the Fish Industry,* edited by Huss H H, Jakobsen M & Liston J, *Elsevier*, Amsterdam (1992).

48 Sathivel S, Jiaqi H & Peter J B, Properties of Pollock (*Theragra chalcogramma*) skin hydrolysates and effects on lipid oxidation of skinless pink salmon (*Oncorhynchus gorbuscha*) fillets during four months of frozen storage, *J Food Biochem,* **32** (2008) 247-263.

49 Connell J J, Quality deterioration and extrinsic quality defects in raw material, in *Control of fish quality*, **4th edn**, edited by Connell JJ, Fishing News Books Ltd, Surrey, England, (1995).

50 Raghavan S & Hultin HO, Model system for testing the efficacy of antioxidants in muscle foods, *J Agr Food Chem,* **53(11)** (2005) 4572-4577.

51 ICMSF: International Commission on Microbiological Specification for Foods, *Microorganisms in foods 2 Sampling for microbiological analysis: principles and specific applications*, **2nd edn** *Blackwell Scientific Publications*, Ltd, Oxford, (1986) 181-196.

52 Ojagh S M, Rezaei M, Razavi S H & Hoseini S M H, Effect of chitosan coatings enriched with cinnamon oil on the quality of refrigerated rainbow trout, *Food Chem,* **120** (2010) 193-198.

53 Kannaiyan S K, Gunasekaran J, Kannuchamy N, Thachil M & Gudipati V, Antioxidant and antibacterial activities of dill extracts and their preservative effect on mackerel fillets during refrigerated storage, *Indian J Nat Prod Resour,* **6(2)** (2015) 106-113.