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Antioxidant and Antibacterial Effect of Protein Hydrolysis of Yellowfin Tuna Waste on Flesh Quality Parameters of Minced Silver Carp

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Abstract

In this study, hydrolyzed protein as a natural preservative for increasing the shelf life of minced meat of silver carp (*Hypophthalmichthys molitrix*) was used in a refrigerator for 12 days. Hydrolyzed protein was added to the minced meat of fish, at 0.5, 1 and 1.5% concentration. In this regard, microbial, chemical and sensory tests were carried out during the period of storage at intervals of 3 days. The results indicated that samples treated with a hydrolyzed protein of 1% and 1.5% were the best-tested groups to postpone the chemical and microbial degradation indicators and as well as the results of their sensory analysis were more acceptable. In general, the present study showed that hydrolyzed protein by a protamex enzyme from visceral of yellowfin tuna can be used as a natural preservative to increase the shelf life of the minced meat of fish.

Key words: FPH; Antioxidant; Antibacterial; Shelf life; Silver carp

Introduction

Fish processing by-products are fish materials remaining from the initial processing of fish manufacturing process. The percentage of by-products generated from fish processing is around 50% of the starting material by weight and exerts a cost to dispose of the material in the absence of value-added solutions (He *et al.,* 2013). Fish viscera, one of the most important by-products, are a rich source of polyunsaturated fatty acids and protein. However, most of this waste was processed into silage and fishmeal (Yanan *et al.,* 2012). Processing techniques for aquatic product waste are required to convert the underutilized wastes into more marketable, valuable and acceptable forms (Benjakul and Morrissey, 1997). In the recent years, fish protein hydrolysates have been exhibited from displaying a wide range of physiological functions including antihypertensive, immunomodulatory, prebiotic, mineral binding, antithrombotic, hypocholesterolemic, antimicrobial and antioxidative effects (Ryan *et al.,* 2011). Antioxidants and antimicrobials can increase shelf-life of food products. Although synthetic antioxidants and antimicrobials are extensively used as food additives, their safety has been questioned (Martínez *et al.,* 2013). Increasing consumer awareness about food safety and quality, have prompted increased interest in the use of natural antimicrobials and antioxidants as alternatives to synthetic compounds (Che Man and Tan, 1999).

Protein hydrolysates have recently been reported to inhibit lipid oxidation and antimicrobial effects in food systems. The antimicrobial and antioxidant activity of protein hydrolysates mainly relies on peptides present in the hydrolysate. Hydrolysates rich in peptides containing hydrophobic amino acids, such as Leu, Pro, Trp, Ala, and Phe, are believed to possess high antioxidant activity (Bernardini *et al.,* 2011; Wiriyaphan *et al.,* 2012).

Silver carp (*Hypophthalmichthys molitrix*), as one of the most important freshwater fish Carp, has been one of the most widely cultured species all over the world due to its easy

cultivation, fast growth rate, high feed efficiency ratio as well as high nutritional value. Nevertheless, fish are perishable food commodities, which generally spoil faster than other muscle foods (Fan *et al.,* 2008). Deterioration of fish mainly occurs as a result of chemical, enzymatic and bacteriological activities leading to loss of quality and subsequent spoilage (Kaale *et al.,* 2011). However, there have been few studies on the use of FPH as antioxidant and antimicrobial to extend the shelf life of minced fish during refrigerated storage. The aim of this study was to investigate the effect of FPH treatments on the quality changes of minced silver carp (*H. molitrix*) during refrigerated storage.

Materials and Methods

Preparation and treatment of fish samples

Yellowfin tuna (*Thunnus albacares*) viscera were prepared from Darya-Khorak, a seafood processing company in Babolsar, Iran, and immediately transferred to the laboratory. After that, they were minced and stored at -20°C. The protamex enzyme (EC 3.4.21.62; Novozymes A/S, Bagsvaard, Denmark) was used for hydrolysis of viscera.

Preparation of fish protein hydrolysate

Yellowfin tuna viscera were hydrolyzed using protamex as previously described by Safari *et al.* (2009). The minced viscera were heated at 85ºC for 20 min in order to inactivate the endogenous enzyme. The cooked viscera were mixed with distilled water 1:2 ratio, and homogenized for about 2 min. Before the starting of the hydrolysis reaction, an initial 15 min mixing was done during the adjustment of pH (pH 6.5) through the addition of HCl 2M and the bringing of temperature to 41ºC (using a water bath). The hydrolysis procedure started by adding protamex with an enzyme/substrate ratio of 1.5% (w/w).

Hydrolysis reactions were performed for 150 min in a shaking incubator (Ivymen System, Comecta, Spain) with constant agitation (200 rpm). Then, the reaction was stopped by heating up to 90 °C for 10 min. The sample was cooled and centrifuged at 6000 g for 20 min at 4 °C. The supernatant was collected and stored in dark glass bottle at refrigerated storage (4°C) until used.

Amino acid composition analysis

The amino acid profiles of the samples were determined using an HPLC system (Knauer, Germany), after 100 mg samples were subjected to acid hydrolysis with 20 mL of 6 N HCl at 105°C for 24 h (Kobbi *et al.,* 2015). The methionine and cysteine contents were determined after performing acid oxidation while the tryptophan content was determined after alkaline hydrolysis (Sila *et al.,* 2014).

Preparation of fish mince

Fresh silver carp (*Hypophthalmichthys molitrix*) (Average weight= 2kg, *n*=7) was purchased from a fish market (Noor, Iran) and transferred to the laboratory in sealed and foamed polystyrene boxes containing flaked ice. Then, these were eviscerated, beheaded, filleted, skinned, and washed in potable water. Then, using a grinder, the white muscle was minced and 4500 g of white mince was obtained. The minced fish was randomly divided into four batches. The first batch was prepared without FPH (control batch), three of the batches processed with FPH as following; batch 2 (0.5% FPH, w/v), batch 3 (1% FPH, w/v), batch 4 (1.5% FPH, w/v). After packaging, they were stored at 4± 1 °C for subsequent quality assessment. Chemical, microbiological, and sensorial analyses were performed at 3-day intervals to determine the overall quality of the minced fish. Moisture, fat, protein and ash were analyzed as per AOAC (2000).

Determination of pH

Minced silver carp samples (10 g) were homogenized in 90 mL of distilled water using an Ultra Turrax T25 homogenizer (Janke and Kunkel, IKA-Labortechnik GmbH and Co., Staufen, Germany) and the pH was measured using a pH meter (Seven Easy portable, Mettler-Toledo GmbH, Schweizenbach, Switzerland) (Moroney *et al*., 2015).

Determination of peroxide value (PV)

The PV was determined in the total lipid extracts according to the method of Egan and others as described by Pezeshk *et al.* (2011). Results were calculated and expressed as

milliequivalent peroxide per kilogram of the sample by the following formula:

$$PV(meq/kg) = \frac{S \times N}{W} \times 1000$$

S= Consumption of thiosulfate; N= Normality and W= Oil sample weight

Determination of thiobarbituric acid

2-Thiobarbituric acid (TBA) was determined as described in the study of Ghorbannejad and Amooaghaie (2017) with slight modification. Approximately 200 mg of minced sample was homogenized in a 25 mL balloon with 1-bothanol. 5ml of the homogenate was mixed well with 5 ml of TBA reagent in a cabbed-test tube. The mixture was heated in boiling water (95–100°C) for 120 min and then cooled to room temperature.

The fat breakdown compounds reacted with the TBA producing a pink-colored complex, which was measured at 530 nm as following where "As" is the absorbance of the sample against a blank sample of distilled water. TBA was calculated and expressed as mg malonaldehyde/ kg lipid.

TBA= 50 (Control absorption – Treatment absorption) ÷ 200

Determination of free fatty acid (FFA)

Free fatty acid (FFA) content was determined by the method of Lowry and Tinsley (1976) with some modifications as described in Takeungwongtrakul *et al.* (2012), based on complex formation with cupric acetate-pyridine followed by spectrophotometric (715 nm) assessment. FFA content was expressed as mg FFA/g TAG (Triacylglycerol) and calculated using a standard curve prepared from oleic acid.

Microbiological analysis

Fish samples (10 g) were taken and homogenized in 90 mL of 0.85% NaCl solution with a stomacher. Decimal dilutions were prepared. Total viable count (TVC) and psychrophilic bacterial counts (PTC) were determined by the pour plate method, using plate count agar (PCA, Merck, Darmstadt, Germany). Plates for total viable aerobic bacteria were incubated at 37◦C for 2 days and plates of psychrotrophic bacteria were incubated at 7◦C for 10 days. All counts were expressed as log10 CFU/g and performed in duplicate (Ojagh *et al.,* 2010; Pezeshk *et al.,* 2011).

Sensory evaluation

Sensory analysis of minced silver carp was performed by a panel of four experienced panelists (Fernández-Fernández *et al.,* 2002). The minced samples were blind-coded by special codes; the panelists were not informed about the experimental approach. The quality of the minced silver carp was assessed based on the odor, color, taste and general acceptance characteristics using a five-point descriptive scale. The scores 5, 4, 3, 2 & 1 indicate "very good", "good", "normal", "bad" and "very bad" quality, respectively.

Statistical analysis

All data were tested for normality. Results were expressed as mean ± standard error of mean (SEM). Statistical analysis was carried out SPSS (Version 16.0 for Windows). The results were subjected to one-way analysis of variance (ANOVA) and where comparison of parameters was carried out simple correlation and regression analysis was used. Comparison of treatment means was based on Duncan's multiple range test. Differences were considered significant at the $P<0.05$ level.

Results and Discussion

Proximate composition

The proximate composition of minced silver carp showed 72.77% moisture, 7.4% crude fat, 19.11% crude protein and 0.71% ash. The proximate composition of the fresh silver carp reported in different studies (Ehsani and Jasour, 2012; Abdollahi *et al.,* 2014) showed some differences, especially for the lipid content. Such variations in the chemical composition of fish depend on the nutrition, fish size, sex, age, catching season, living area, as well as the other environmental conditions (Pacheco-Aguilar *et al.,* 2000). It changes from one species to another and one individual to another. Therefore notable variations can be observed in the components of fish muscle (Abdollahi *et al.,* 2014).

Amino acid compositions

Amino acid compositions of optimized FPH are shown in Table 1. The function of FPH is mainly dependent on its amino acid composition. The presence of hydrophobic amino acids (His, Tyr, Met, Val, Phe, and Lys) has been shown to contribute greatly to the bioactive properties of FPH (Wieprecht *et al.,* 1997). Chuesiang and Sanguandeekul (2015) reported that tyrosine, methionine, histidine, lysine, and tryptophan are generally accepted as antioxidants in spite of their peroxidant effects. As for protein with a high content of hydrophobic amino acids (His, Tyr, Met, Val, Phe, and Lys), the highest antibacterial activity on pathogenic bacteria is generally observed (Andreu and Rivas, 1998).

Table 1. The amino acid composition of FPH.

Amino acid	FPH
Aspartic	10.54
Glutamic	12.69
Serine	5.22
Histidine	1.45
Glycine	13.75
Threonine	1.24
Arginine	8.54
Alanine	9.14
Tyrosine	2.78
Methionine	8.91
Valine	3.04
Phenylalanine	5.36
Isoleucine	8.35
Leucine	2.35
Lysine	0.49

pH Analysis

The pH trend of minced silver carp during storage at 4 ºC for 12 days is shown in Fig. 1. As shown, initial pH of the control and treated fillets was 5.81 which were in agreement with previous findings (Remya *et al.,* 2017, Rode and Hovda, 2016). Newton and Gell (1981) stated that pH value of fish varies from 5.8 to 7.2 depending on struggling at the time of harvesting but the normal variation is 5.8-6.5. In addition, pH differences found in the fresh fish flesh are generally due to the dissociation of carbonic acid, which leads to the pH increase as the storage time progresses. The pH of fresh fish flesh is almost neutral but in the post-mortem period, decomposition of nitrogenous compounds brings about the increase in pH which affects the quality of the product during storage; especially, the sensorial features such as color, odor, and texture are negatively affected (Volpe *et al.,* 2015). It can be found that pH showed significant differences (P<0.05) with the time. The increase in pH can be due to the formation of alkaline compounds of autolysis and microbial metabolites in the fish muscle (Remya *et al.,* 2017). There were no significant differences (P>0.05) in pH between control and treated groups during the storage period except on 9th day, however, lower values in the treated groups might be related to inhibition of bacterial spoilage and less production of basic amines resulted in antibacterial activity of the FPH (Song *et al.,* 2012).

Peroxide value (PV)

The changes of peroxide value, which is an indicator of the initial lipid oxidation and is one of the most common measures of lipid hydroperoxides, are shown in Fig. 2. For control samples, PV raised from an initial level of 0.36 to 4 meq oxygen/kg lipid at 6 d and declined after 6 d (Fig. 2). This could be due to the breakdown of initial oxidation products into secondary oxidation products and also the reaction of hydroperoxide with protein (Ozogul *et al.,* 2016). PV levels tended to increase toward the end of the storage (Fig. 2). There were significant differences between initial and final hydroperoxide content (P<0.05) for the different experimental groups in this study. During the storage period, the treated groups with 1.5% FPH (III) showed generally lower PV than the control. The presence of higher level of peroxides in the controls might have accelerated oxidation, as lipid hydroperoxides will be decomposed to reactive alkoxyl radicals in the presence of heme iron or trace metals (Behnam *et al.,* 2015). In contrast, the antioxidant activity of FPH in the treated minced reduced lipid oxidation (P<0.05) throughout of storage. Antioxidant activity of FPH mainly relies on peptides present in the hydrolysate. Hydrolysates full of peptides containing hydrophobic amino acids, such as Ala, Pro, Leu, Trp and Phe,

are believed to possess high antioxidant activity. Furthermore, acidic (Glu and Asp) and/or basic amino acids (Lys) play an important role in the chelation of metal ions by carboxyl and amino groups in their side chains. Thus, the amino acid composition of peptides in protein hydrolysates is an important factor in controlling the antioxidant activity of protein hydrolysates (Wiriyaphan *et al.,* 2012). The maximum acceptable limit of PV value for foodstuffs is considered to be 10–20 mEq O_2/kg oil (Raeisi *et al.,* 2016). By the end of preservation period (12th day), the peroxide content of all samples remained within the acceptable range for human consumption.

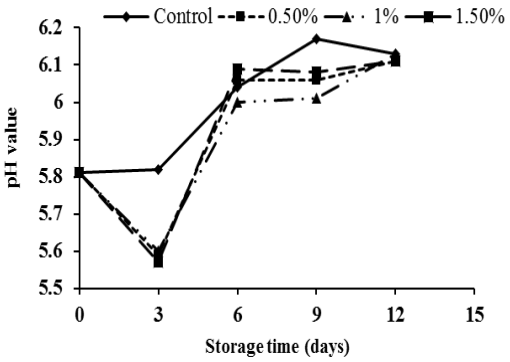

Fig. 1. The pH changes of minced fish sample groups during refrigerated storage: groups I, II, III treated with 0.5%, 1%, and 1.5% FPH, respectively.

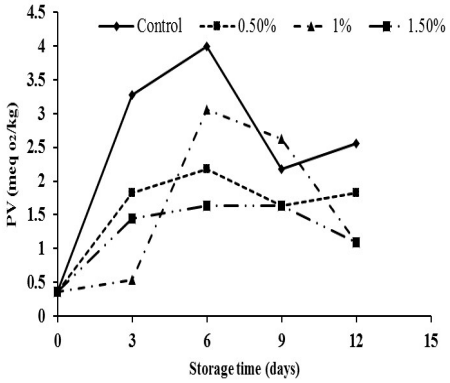

Fig. 2. Changes in PV values of minced fish sample groups during refrigerated storage: groups I, II, III treated with 0.5%, 1% and 1.5% FPH, respectively.

Thiobarbituric acid (TBA)

The TBA value has been extensively used to estimate the extent of lipid oxidation (Shahidi, 1994). TBA value is a measure of the secondary stage of lipid oxidation, which is an index for the malondialdehyde (MDA) content (Chari and Colagar, 2011). MDA is formed through hydroperoxides, which are the initial reaction products between polyunsaturated fatty acids and oxygen (Chari and Colagar, 2011; Fathy *et al.,* 2015). TBA value has been shown to correlate well with the development of rancid odor and flavor (Rode and Hovda, 2016). Changes in TBA in the different treatments during storage are shown in Fig. 3. The initial TBARS of fish samples were found to be 0.02 ± 0.01 to 0.6 ± 0.1 mg MDA/kg. TBA fluctuated for all samples and was higher in the control than those for the treatment groups. Significant differences were observed ($P<0.05$) for TBA values between the untreated (control) and the treated samples during storage.

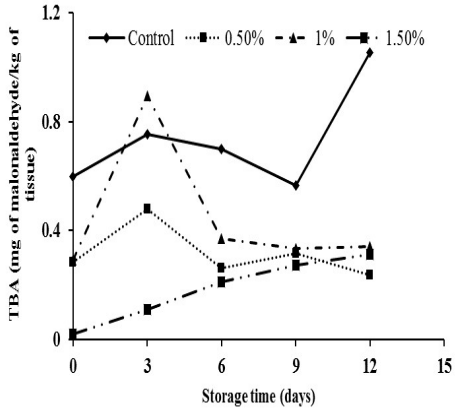

Fig. 3. Changes in TBA values of minced fish sample groups during refrigerated storage: groups I, II, III treated with 0.5%, 1%, and 1.5% FPH, respectively.

The antioxidant activity of FPH was previously reported by Cumby et al. (2008) who mentioned that the antioxidant activity of peptides contained in FPH depends not only on which amino acids are in the sequence, but also on the position that they occupy in the sequence. The antioxidant

activity of a peptide contained in FPH in preventing lipid peroxidation has also been assigned to the peptide's hydrophobicity, due to the hydrophobic amino acids present in the sequence. This hydrophobicity leads to high interactions between the peptide and the fatty acids, resulting in protection against oxidation (Bernardini *et al.,* 2011). According to the literature, TBA values of 1– 2 mg MA kg-1 of the fish flesh are generally regarded as the limit for normal odor or taste (Remya *et al.,* 2017); neither control nor treated groups exceeded such limit in any occasion of examination even after demonstrated objectionable odors and taste.

Free fatty acid value (FFA)

Free fatty acid value FFA is an index of progress in lipid hydrolysis and has been used to establish the degree of deterioration of food products (Abdollahi *et al.,* 2014). FFAs are triacylglyceride products formed by the action of autolytic lipases (Shalini *et al.,* 2015). In this study, FFA content in minced silver carp samples during the storage period is given in the Fig. 4.

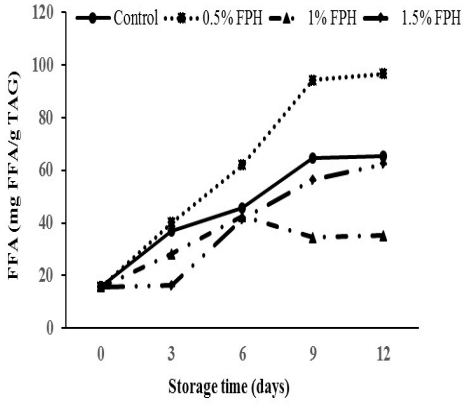

Fig. 4. Changes in FFA values of minced fish sample groups during refrigerated storage: groups I, II, III treated with 0.5%, 1%, and 1.5% FPH, respectively.

The amount of FFA in all treatments increased from 15.73 (mg FFA/g TAG) in the fresh sample until it reached a maximum at the end of the storage period.The increase in FFA shows hydrolytic oxidation caused by bacterial or internal enzymes (Pereirade Abreu *et al.,* 2011). Significant differences were observed (P<0.05) for FFA values between the untreated (control) and the treated samples during storage. Treated samples (groups II and III) had significantly lower FFA during the storage period as compared with the control. Lower FFA level can be related to the antioxidant/antibacterial properties of FPH (Cumby *et al.,* 2008; Lin *et al.,* 2013). Abdollahi *et al.* (2014) reported increasing FFA from silver carp during refrigerated storage in a similar these findings. During of the storage, significantly lower differences (P < 0.05) were noticed in the antioxidant values of treated samples (1.5% FPH and 1% FPH) when compared with control and groups I (I = 0.5% FPH). Generally, most of the amino acids responsible for the antioxidant activity, including Val, Met, Tyr, Leu, Phe, Lys, Arg, and His were detected in the hydrolysate. Particularly, histidine shows strong radical scavenging activity due to the decomposition of its imidazole ring. For protein hydrolysates, an enhancement in hydrophobicity enhances their solubility in lipids and therefore increases their antioxidative activity (Dong *et al.,* 2008; Jumeri and Kim, 2011).

Microbiological analyses

The changes in the value of TVC during the refrigerated storage are presented in Fig. 5A. In the present study, the initial value of TVC was 2.49 log10 CFU/g. Most of the available literature on freshwater fish reports bacterial counts of 10^2–10^6 CFU/g (Mexis *et al.,* 2009). Such value in the control and I (0.5% FPH) groups reached about 6.5 log10 CFU/g at 12 days after refrigerated storage. A TVC value of 7 log CFU/g (ICMSF 1986) is considered the upper acceptable limit for freshwater and marine species (Pezeshk *et al.,* 2011).

The increase of TVC in the fish flesh during storage has been shown by Fan *et al.* (2008). By the day 12 of storage, however, TVC in minced silver carp for groups II and III was still below 6 log10 CFU/g. FPH effectively delayed the microbial growth. The lowest TVC was obtained where 1.5% FPH (III group) were used during the whole storage period (P<0.05) and reached 5.39 log10 CFU/g at the end of the storage trial (12th day).

The gram-negative PTC is the important group of microorganisms responsible for spoilage of aerobically stored fresh fish at chilled temperatures (Ojagh *et al.,* 2010). The initial value of PTC was 3.48 log10 CFU/g, which increased to 7, 7, 6.06 and 6 log10 CFU/g, respectively in groups I, II, III and control on the 12th day of storage (Fig. 5B).

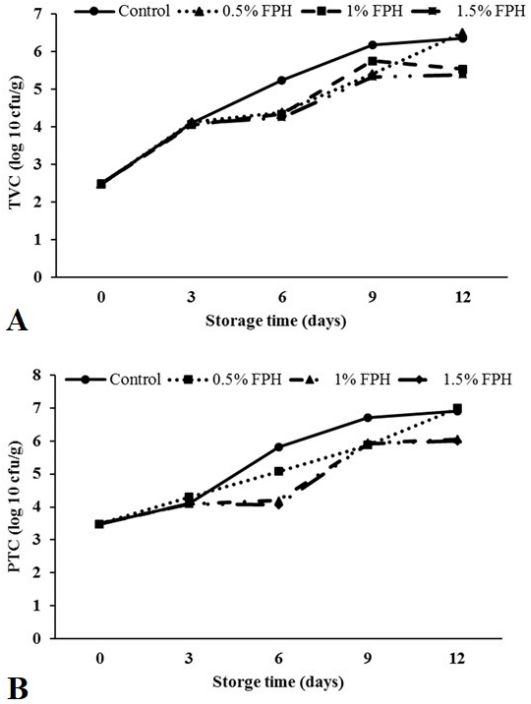

Fig. 5. Changes of the TVC and PTC in the minced fish sample groups during refrigerated storage: A) Changes of the TVC; B) Changes of the PTC; groups I, II, III treated with 0.5%, 1% and 1.5% FPH, respectively.

It is clear from the results that lowest count was detected in samples treated with 1% and 1.5% FPH (groups I and II, respectively) (P < 0.05). From the results, the TVC and PTC were lower in samples treating 1% and 1.5% FPH than those treating 0.5% FPH and control. The antimicrobial properties of peptides in FPH have been reported in the literature (Song *et al.,* 2012; Lin *et al.,* 2013; Sila *et al.,* 2014). The peptide forms a pentacyclic conjugated structure and can accept free electrons and anion radical (Shai, 2002). The bacterial cell surface structure is complex, with hydrophilic and hydrophobic sites. Hydrophilic sites mainly contain uncharged groups, such as carboxyl, phosphoric acid, and hydroxyl groups. By contrast, hydrophobic sites mainly contain plasma membrane phospholipid molecules. The conjugated structure of the ferrous chelating peptide can change the spot surface of the cell membrane, therewith affecting the complete structure of the cell membrane. This phenomenon may be one of the key factors affecting the biological activities of the ferrous chelating peptide (Lin *et al.,* 2013). According to the amino acid composition of the hydrolyzed protein (Table 1), the presence of hydrophobic amino acids (His, Tyr, Met, Val, Phe, and Lys) in the present study can be expository to this phenomenon.

Sensory quality

Sensory analysis is expressed as an art that depends on measuring the impression of food quality by observation, hearing, tasting, and touching (Raeisi *et al.,* 2016). It is fast, simple, and provides immediate quality information (Pezeshk *et al.,* 2015). The results of the sensory evaluation (odor, color, taste, and general acceptability) of minced silver carp treated with FPH are presented in table 1. All samples were fresh and had high sensory scores about 5 at the beginning of storage period which means that they were of very good quality. Fish quality significantly decreased in all samples (P<0.05) during storage. However, significant differences (P<0.05) were observed between the control and treated groups. Groups II and III were

mostly preferred by the panelists. From the data, the sensory acceptability limit was reached at days 6, 6, 6 and 12 of storage period for the control, I (0.5% FPH), II (1% FPH) and III (1.5% FPH) samples, respectively. The results showed that the use of highest concentrations of FPH (group III) improved the sensory quality of minced silver carp. These results were also supported by the results of chemical and microbial quality analyses. In agreement with the current study, previous studies have shown good correlations among chemical and microbial quality with sensory properties (Ojagh *et al.,* 2010, Pezeshk *et al.,* 2011). 1% FPH and 1.5% FPH had the effect on the delay of bacterial and oxidative decay of samples under refrigerated storage, by extending its shelf life for 3 more days.

Table 2. Results of sensory analyses of minced silver crap during storage at 4 ± 1°C

Sensory parameter	Treatment	Storage time (days)				
		0	**3**	**6**	**9**	**12**
Odor	C	5a	4.75a	4a	2.33a	NA
	I	5a	4.75a	2.33b	2.66a	2.66a
	II	5a	5a	2.66b	2.66a	2.66 a
	III	5a	5a	3.66a	2.66a	2.66a
Color	C	5a	4.75a	3.66a	2.33a	NA
	I	5a	4.75a	3.33a	3a	3a
	II	5a	4.75a	4a	2.66a	3a
	III	5a	5a	4a	3a	3a
Taste	C	5a	4.25a	3a	2b	NA
	I	5a	4.5a	2.66a	2b	NA
	II	5a	4.5a	3.66a	3.66a	3.66a
	III	5a	4.25a	3.33a	3.66a	4a
General acceptabilty	C	5a	4.25a	3a	2.33b	NA
	I	5a	4.5a	3a	2.66b	2b
	II	5a	4.5a	3.66a	2.33b	2.66b
	III	5a	4.25a	3.66a	3.33a	3.66a

[a-b] means in the same column with different letters are significantly different from the others ($P < 0.05$); NA – not analyzed.

Conclusions

The results of the study demonstrated the effectiveness of fish protein hydrolysate on microbial growth (TCA and PTC) and lipid oxidation (PV, TBA, and FFA) and extend the shelf life of minced silver carp during storage at 4 °C for 12 days. Groups treated with 1% and 1.5% FPH (II and III) were preferred by the panelists because of their better color, odor, and taste during the storage period. Considering the results, it seems that FPH can be used as a safe and effective natural antioxidant and antibacterial compound in seafood and meat industry.

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