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Characterization of tuna dark muscle protein isolate

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Abstract

Fish protein isolate (FPI) was extracted from tuna dark muscle (TDM) using the pH-shift method that improves significantly its sensory quality and nutritional value. The amino-acid composition of the FPI was close to that of TDM, but the total protein content was higher. The solubility of FPI in water was higher than 85% at pH ≥ 6 and pH ≤ 3, but it dropped to about 10% at pH values close to the isoelectric point pI ≈ 5.0. The soluble FPI was present in the form of aggregates that were characterized by light-scattering. The microstructure of FPI solutions on larger length scales was probed by confocal microscopy, large protein flocs formed at pH close to pI. SDS-PAGE indicated that a significant fraction of myosin heavy chains was broken down into smaller chains. The

viscosity decreased with decreasing of pH, which correlated with the decreasing size of the aggregates.

Keywords: tuna, dark muscle, fish protein isolate, extraction, aggregation

Practical application

Tuna dark muscle (TDM) represents a significant fraction of industrially processed tuna. It is currently considered a low value by-product, because it has poor sensory qualities. However, TDM has a high protein content that does not suffer from sensory defects if it is extracted using the pH-shift method. TDM protein isolate therefore has the potential to be a valuable nutritious food ingredient. In order to develop industrial food products with this fish protein isolate it is necessary to characterize it thoroughly.

1 INTRODUCTION

Proteins can be effectively isolated from fish by-products by dissolving the proteins at high or low pH and subsequently precipitating them at intermediate pH where the net charge of the proteins is close to zero (Tang et al., 2020). Fish protein isolate (FPI) obtained in this manner can be used as nutritious ingredients in industrial food products (Tang et al., 2020; Desai et al., 2019 & 2021; Asad Nawaz et al., 2020). Naturally, in order to optimize the usage of FPI for different types of applications, it is necessary to thoroughly characterize its properties in aqueous solution at different pH.

In industrial processing of tuna fish 50-70% of its body mass is considered as by-products (head, skin, dark meat, viscera, bone, etc) of which about 12% is dark muscle (Guérard et al., 2002; Herpandi et al., 2011). It has low commercial value, but is rich in nutrients and its extracted protein displays important technological properties (Sánchez-Zapata et al., 2011). Tuna dark muscle (TDM) contains approximately 27% protein with high nutritional value, but it also contains hemoglobin and

myoglobin that can oxidize and thereby render the muscle dark (Chaijan et al., 2010; Ochoa et al., 2017; Tahergorabi et al., 2015; Tian et al., 2017). In recent years, protein recovery from fish dark muscle has attracted the interest of both scientists and manufacturers as it contributes to improved economic efficiency and towards zero-waste processing. In which the recovery method and characterization of protein are the main concerns.

The amount of protein that is dissolved from fish muscle depends strongly on the pH (Batista, 1999; Geirsdottir et al., 2007). It was found that at pH > 10 or pH < 3.5 more than 80% of the proteins is soluble, whereas at pH close to the pI ≈ 5.2-5.5 it is less than 10% (Kristinsson et al., 2013). Therefore an efficient method to extract fish protein is to solubilize fish meat in a strong base or acid and subsequently to precipitate the proteins close to pI (Davenport & Kristinsson, 2011; Foh et al., 2012; Y. Kim et al., 2003; Lee et al., 2016; Surasani, 2018; Surasani et al., 2018; Surasani et al., 2016; Undeland et al., 2002; Zhong et al., 2015)(Zhen-Xing Tang et al., 2020). This method removes the dark color and the lipids (Chaijan et al., 2010; Sánchez-Zapata et al., 2011) resulting in a fish protein isolate (FPI) that is useful for food applications. Fish muscle can be dissolved both in strong acid and base, but alkali treatment has advantages in terms of the yield and the strength of gels formed by aqueous FPI solutions (Abdollahi & Undeland, 2018; Batista, 1999; Chaijan et al., 2010; Davenport & Kristinsson, 2011; Kaewdang et al., 2016; Y. Kim et al., 2003; Oliyaei et al., 2016; Zhong et al., 2015).

The molecular weight of the protein components of FPI and its distribution have been determined by electrophoresis (Brenner et al., 2009a; Foh et al., 2012; Geirsdottir et al., 2007; Kristinsson & Ingadottir, 2006; Sun et al., 2019; Tian et al., 2017; Yongsawatdigul & Park, 2004). The results show prominent bands corresponding to myosin heavy (≈200 kDa) and light (17-23 kD) chains, actin (≈ 43 kDa) and tropomyosin (≈ 38 kDa). The secondary structure of tilapia protein isolate (TPI) was characterized by Kobayashi et al. (2017) using Fourier Transform Infrared spectroscopy (FT-IR). The presence of α-helical and β-sheet structures was demonstrated by absorption bands near 1648 cm^{-1} and 1660 cm^{-1}.

Brenner et al. (2009b) characterized FPI extracted from cod in dilute aqueous solutions at pH 11 using light scattering techniques. They found that the protein was present in the form of aggregates with a z-average radius of gyration R_g = 150 nm and a hydrodynamic radius R_h = 180 nm. The FPI also contained about 15 wt% much larger aggregates. The dependence of the scattering intensity on the scattering wave vector showed that the aggregates had a self-similar structure characterized by a fractal dimension of 1.8.

The viscosity of FPI in aqueous solution was investigated by Foh et al., (2012) and Geirsdottir et al. (2007). Geirsdottir et al. (2007) observed that the viscosity of herring protein isolate suspensions increased at pH values far from the pI. Foh et al. (2012) found that at pH 7 the viscosity of tilapia protein isolate increased with increasing protein concentration. Brenner et al. (2009a) reported that reduction of the pH from 11 to between 9.5 and 8.5 led to formation of homogeneous gels, whereas reduction to lower pH values down to 3.8 led to formation of heterogeneous gels showing syneresis.

It is clear from this brief review that FPI extracted from white flesh and mixed fish by-products has already been studied quite extensively. However, FPI from dark tuna muscle has been rarely investigated. Sánchez-Zapata et al. (2011) reported on the iron and myoglobin content of tuna dark muscle as well as the water and oil holding capacity. Here we present an investigation of the amino acid composition, structure, solubility, and physico-chemical properties of FPI extracted from tuna dark muscle at pH 12 as a function of the pH. The objective is to establish if, and to what extent, FPI from tuna dark muscle is different from other types of FPI so as to facilitate its use in food products.

2 MATERIALS AND METHODS

2.1 Materials

The fish protein isolate used for this study was extracted from yellowfin tuna *(Thunnus Albacares)* dark muscle provided by Hai Vuong Co., LTD (Khanh Hoa province, Vietnam) using

the alkaline extraction method described by Undeland et al. (2002) with slight modifications. Milled dark muscle was washed twice with deionized water (3:1 ratio) at 4 °C to remove the dark color. Excess water was removed by wrapping the muscle in a filter bag with a pore size of 50 µm and manually pressed. The cleaned muscle was solubilized by adding water at pH 12 at a ratio 1:5. The mixture was homogenized at 4 °C for 2 min at a speed of 3500 rpm using an Ultra-turrax (model: IKA T18, Germany). 2 N NaOH was used to adjust to pH 12 after homogenization. The mixture was stirred for 3 hours at room temperature (20 °C) using an Orbital shaker (model: NB-101M, Korea) and subsequently centrifuged (MF600, Labkorea) at 5000 rpm for 30 min at 4 °C. The supernatant was decanted through a filter bag with a pore-size of 5 µm and the soluble protein was precipitated by reducing the pH to 5.5 using 2 N HCl. The precipitate was thoroughly washed with distilled water at 4 °C. Excess water was removed by manually wrapping and squeezing the protein isolate in a filter bag with a pore size of 5 µm. Finally, the FPI was freeze dried (Lyobeta 35, Spain) and stored at -20 °C. The chemical composition of FPI and TDM is shown in Table 1.

2.2 Sample preparation

Stock solutions of FPI were prepared by dissolving the freeze-dried FPI at a concentration of 30 g/L in deionized water at pH 12 by stirring for a few hours at ambient temperature. Solutions at lower concentrations and lower pH were prepared by dilution with deionized water and addition of small amounts of 0.1 N HCl.

2.3 Methods

2.3.1 Proximate composition

The composition of tuna dark muscle (TDM) and protein isolate (FPI) extracted from TDM was determined using the procedures of the AOAC (2012). The crude protein content, moisture content, crude fat and ash were determined using the Kjeldahl method, method 928.08, method 948.04 and method 920.153, respectively.

Sodium, NaCl, and histamine contents were determined using methods 969.23, 930.23, 997.13 (LOD = 3 ppm), respectively. Heavy metal (As, Hg, Pb, and Cd) contents were determined using methods 986.15, 971.21, 972.23, and 973.34, respectively.

2.3.2 Fourier Transform Infrared (FT-IR) Spectroscopy

FPI powder was characterized using a FT-IR spectrometer (Model Alpha, Bruker, Ettlingen, Germany) equipped with a horizontal attenuated total reflectance (HATR) accessory. The internal reflection crystal was made of zinc selenide, and had a 45° angle of incidence to the IR beam. The sample was mixed with KBr in the ratio of 98:2 (w/w). A total of 32 scans were done in the wavenumber range from 500 to 4000 cm^{-1} with a resolution of 4 cm^{-1}. The spectra were collected at room temperature (20 °C) using approximately 0.5 g of the sample that was placed on the surface of the HATR crystal. Analysis of the spectra was carried out using the OPUS 7.0 data collection software program.

2.3.3 Amino acid composition

The amino acid composition of the samples was determined using the method ISO 13903 (2005). In brief, FPI samples were hydrolyzed for 24 hours in 6 N HCl at 110 °C in a sealed vessel. The samples were then dissolved in deionized water and filtered. About 20 μL hydrolyzed sample or the standard was injected into the amino acid analyzer (Biochrom 30+, Biochrom Ltd., Cambridge, UK) equipped with a high pressure PEEK column (No: H-0563) packed with Ultropac 8 cation exchange resin (No: 132-56). The automated analyzer was configured with post-column ninhydrin derivatization. The ninhydrin derivative of Pro was detected at 440 nm whereas the remaining amino acid derivatives were detected at 570 nm.

2.3.4 Zeta potential

The zeta potential (ζ) of aqueous FPI solutions at 0.1 g/L was determined as a function of the pH by measuring the electrophoretic mobility (U_E) using a zeta potential analyser (Nano Partica SZ-100Z, Horiba Scientific, Japan). The zeta potential was calculated from the electrophoretic mobility by applying Henry's equation (Lunardi et al., 2021):

$$U_E = \frac{2\varepsilon.\zeta.f(\kappa\alpha)}{3\eta} \quad (1)$$

where, ε is the permittivity, *f*(κα) is the Henry coefficient and η is the dispersion viscosity. Following the Smoluchowski approximation *f*(κα) was set at 1.5. All measurements were done in triplicate.

2.3.5 Light scattering

Dynamic and static light scattering measurements were conducted with a commercial apparatus (ALV-CGS3, ALV-Langen) operating with a vertically polarized He-Ne laser with wavelength λ = 632 nm. The temperature was controlled at 20 °C with a thermostat bath to within ± 0.2 °C. The measurements were performed on FPI solutions diluted to below 1 g/L in order to avoid the effect of interactions and at angles of observation between 13° and 140°. The relative scattering intensity (I_r), of the solute, calculated as the intensity minus the solvent scattering divided by the scattering intensity of toluene, is related to the weight average molar mass (M_W) and the scattering wave vector (q) dependent structure factor (S(q)) (Brown, 1993):

$$\frac{I_r}{KC} = M_W\, S(q) \quad (2)$$

where K is an optical constant defined as:

$$K = \frac{4\pi^2 n_S^2}{\lambda^4 N_A}\left(\frac{\partial n}{\partial C}\right)^2\left(\frac{n_{tol}}{n_S}\right)^2\frac{1}{R_{tol}} \quad (3)$$

with N_A is Avogadro's number, $\partial n/\partial C$ = 0.189 mL/g the refractive index increment and R_{tol} = 1.35 × 10^{-5} 1/cm the Rayleigh constant of toluene at 20 °C.

The normalized electric field autocorrelation functions ($g_1(t)$) obtained from dynamic light scattering measurements (Berne & Pecora, 1976) were analyzed in terms of a distribution of relaxation times (τ):

$$g_1(t) = \int A(\tau) \exp(-t/\tau) d(\tau) \quad (4)$$

In dilute solution, the relaxation was caused by translational diffusion of the particles. The diffusion coefficient (*D*) was calculated from the average relaxation rate:

$$D = <\tau^{-1}> / q^2 \quad (5)$$

The z-average hydrodynamic radius (R_h) of the solute was calculated from D extrapolated to q = 0 using the Stokes-Einstein equation:

$$R_h = \frac{kT}{6\pi\eta D} \quad (6)$$

with η the viscosity of the solvent, *k* Boltzman's constant, and *T* the absolute temperature.

2.3.6 Confocal Laser Scanning Microscopy

The morphology of FPI solutions was studied with Confocal Laser Scanning Microscopy (CLSM) in the fluorescence with mode utilizing a Zeiss LSM 800 microscope (Oberkochen, Germany). A water immersion objective lens was used (HCxPL APO 63x NA=1.2 with a theoretical resolution of 0.1 µm in the x-y plane. The proteins were fluorescently labeled by adding 5 ppm rhodamine B that physically bound to the proteins. No effect of labeling was observed at the concentration used. The fluorescent label was excited by a laser at 543 nm and the fluorescent light was detected between 543 and 900 nm. The solutions were inserted between a concave slide and a coverslip and hermetically sealed.

2.3.7 Viscosity measurement

The viscosity was measured at 20 °C as a function of the shear rate using a rheometer (Kinexus Pro 50N, Malvern, England) with a cone and plate geometry (diameter 60 mm; angle 1°).

After loading, the samples were pre-sheared at 100 s^{-1} for 1 min. The viscosity was determined as a function of the shear rate between 0.1-1000 s^{-1}.

2.3.8 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

FPI solutions at 5 g/L and different pH were mixed with the same volume of an electrophoresis sample buffer (0.25 M Tris (pH 6.8), 2% SDS, 10% glycerol, 100 mM dithiothreitol, 0.2% bromophenol blue) and heated at 95 ^{o}C for 10 min. The running gel contained 15% polyacrylamide in 0.5 M Tris–HCl (pH 8.8) and 0.3% SDS, whereas the stacking gel contained 5% polyacrylamide in 0.5 M Tris HCl (pH 6.8) and 0.2% SDS. The electrode buffer contained 0.025 mol/L Tris– HCl, 0.192 M glycine and 0.1% SDS at pH 8.3. SDS-PAGE electrophoresis was performed at 15 mA using a Mini Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). Following electrophoresis, the gels were stained with 0.05% Coomassie Blue G250 in 25% v/v ethanol and 10% v/v acetic acid for 4 h. The gel was finally destained with an aqueous solution containing 5% (v/v) methanol and 10% (v/v) acetic acid. Molecular weights of the FPI subunits were estimated using molecular weight standards (Invitrogen, USA) in the range from 3.5 kDa to 260 kDa.

2.3.9 Solubility

Protein solutions at 10 g/L and different pH were centrifuged at 1.3 x 10^{4}g for 30 min at 20 °C by a refrigerated centrifuge (Hettich, Mikro 22R, Germany). The protein concentration of the supernatant was determined by UV absorption at 278 nm with a UV-Visible spectrometer (Libra S50 Bio, Cambridge, England) using an extinction coefficient 1.0 L/g.cm. The soluble protein fraction was determined by dividing the protein concentration in the supernatant by the total concentration. All measurements were done in triplicate.

2.4 Statistical analysis

Statistical analysis of the data was performed using SPSS software (Version 16.0, Chicago, IL, USA). One-way analysis of variance (ANOVA) by the Student's T-test was used for statistical comparisons of means. Standard deviations and differences at $P < 0.05$ are indicated.

3 RESULTS AND DISCUSSION

3.1 FPI powder characterization

3.1.1 Amino acid composition

The amino acid composition of tuna dark muscle and fish protein isolate extracted from TDM is given in Table 2. As expected, the total protein content in FPI was higher (90%) than in TDM (73%), but the amino acid composition of the proteins was similar with glutamic acid, aspartic acid, leucine, and lysine as the most abundant amino acids. The fraction of essential amino acids (EAA) was 44% in TMA and 45% in FPI. The amino acid composition was similar to that reported for muscle tissue of yellowfin tuna by Peng et al. (2013) and for FPI extracted from pangas (*Pangasius pangasius*) processing waste (Surasani et al., 2018).

3.1.2 Fourier-Transform Infrared Spectroscopy

As far as we are aware, the only previous FT-IR study of FPI was reported by Kobayashi et al. (2017) for tilapia protein isolate. These authors focused on the amide I (1600 - 1700 cm^{-1}) and amide II (1500–1600 cm^{-1}) regions. They assigned the amide I band with a maximum at 1639 cm^{-1} to the β-sheet structure and the amide II with a maximum at 1550 cm^{-1} to the α-helix structure. Figure 1 compares the FT-IR spectra of TDM and the FPI powder. Small, but significant differences were observed indicating that the extraction procedure had some influence on the secondary structure of the proteins. The height of the amide II band relative to the amide I band was lower for FPI and a shoulder appeared around 1740 cm^{-1}, indicating that FPI contained relatively less α-helices. The amide II peak for FPI from tuna was higher than for FPI paste from talapia reported by Kobayashi et al., (2017). The higher α-helix content was perhaps caused by the high

concentrations of amino acids (alanine, glutamic acid, lysine and leucine), which generally prefer to adopt an α-helix structure. However, one needs to be careful when comparing these results directly, because the FPI paste investigated by Kobayashi et al. (2017) contained 75% water, whereas the FPI powder studied here contained very little water.

3.2 Characterization of tuna dark muscle protein isolate solutions at different pH

3.2.1 Zeta-potential

The zeta potential (ζ) of FPI as a function of the pH is shown in figure 2. It changed from strongly positive at pH < 4 to strongly negative at pH ≥ 6. The isoelectric point (pI) was close to pH 5.

3.2.2 Solubility

The solubility of FPI in water was determined as a function of the pH between 2 and 12. Figure 3 shows that FPI solutions were transparent between pH 6 and pH 12, but became increasingly turbid at lower pH down to pH 5.0 indicating the formation more and larger aggregates. At pH 5.0 a dense precipitate was formed. Protein aggregates also precipitated at pH 4, but the precipitate was less dense. With further decrease of the pH the turbidity decreased indicating that fewer and smaller aggregates were formed.

The fraction of soluble proteins (F_{pis}) was determined by measuring the protein concentration of the supernatant after centrifugation as described in the section 2.3.9, see figure 4. At pH ≥ 6.0 and pH ≤ 3.0, F_{pis} was higher than 0.85, but close to the pI, F_{pis} decreased to values as low as 0.12. These results are similar to those reported by Foh et al. (2012) for tilapia protein isolate and Sun et al. (2019) for myosin extracted from skeletal muscle of blue round scads. Similar observations were also reported for white muscle from cod, rockfish and Alaska pollock (Brenner et al., 2009a; Y. S. Kim & Park, 2008; Yongsawatdigul & Park, 2004). The low solubility close to pI can be attributed to weak electrostatic repulsions between the proteins.

3.2.3 SDS analysis

The protein composition of soluble FPI was analyzed by SDS-PAGE at different pH above pH 6 and below pH 3. The results show numerous bands spread out over the whole range of molecular weights from 15 kDa to 260 kDa independent of the pH, see figure 5. The distinct band at 15 kDa can be attributed to myosin light chains. Indistinct bands at 37 kDa and 42 kDa correspond to tropomyosin and actin, respectively (Abdollahi & Undeland, 2018; Taktak et al., 2018). However, albumins (from the sarcoplasmic fraction) are also typically found in this region (Felix et al., 2017). Remarkably no distinct band corresponding to myosin heavy chains (MHC) was observed at 250 kDa. This could indicate that polymerization of MHC had occurred resulting in larger proteins that could not enter the gel (Y. S. Kim & Park, 2008; Nunez-Flores et al., 2018). Alternatively, the absence was caused by a high degree of hydrolysis due to the well-known high proteolytic enzyme activity in the dark muscle (Abdollahi & Undeland, 2018; Kristinsson & Ingadottir, 2006; Zhong et al., 2015), which would explain the presence of proteins with an almost continuous range of lower molecular weights.

3.2.4 Size & molar mass

The size and molecular weight of FPI in aqueous solution was determined using static and dynamic light scattering at 20 °C. The z-average hydrodynamic radius (R_h) and weight average molar mass (M_w) were determined as described in section 2.3.5 and are shown as a function of the pH in figure 6. Aggregates with $R_h \approx 230$ nm and $M_w \approx 10^7$ g/mol were formed at the highest pH (11 and 12). The aggregate size and molar mass decreased with decreasing pH down to $R_h \approx 90$ nm and $M_W \approx 1.4 \times 10^6$ g/mol at pH 7-9. At pH 3 the aggregates were again slightly larger than at pH 6, but at pH 2 the size was close to that at pH 7-9. Most FPI precipitated between pH 3 and 6 and could not be analyzed by light scattering. It is curious that the aggregate size increased strongly between pH 9 and pH 11 even though the zeta potential remained approximately constant. As was

mentioned in the Introduction, Brenner et al. (2009b) observed R_h = 180 nm for FPI extracted from cod at pH 11.

3.2.5 Microstructure

The microstructure of FPI at 10 g/L was investigated with CLSM at different pH, see figure 7. Between pH 12 and pH 5.8, where the solutions were transparent, the CLSM images were homogeneous showing only a few microscopic particles. Significantly more microscopic particles were seen at pH 5.7 and at lower pH down to pH 3.5 larger protein flocs were formed (Figure 7). At pH 3 and lower the images were again homogeneous (not shown). The larger flocs sedimented during centrifugation and at some pH values even under gravity as was shown in section 3.2.2.

3.2.6 Viscosity

The viscosity of FPI solutions at 10 g/L at different pH was measured as a function of the shear rate, see figure 8. Significant shear thinning was observed at all pH values. At a given shear rate the viscosity was highest at the highest pH values (11 and 12) and decreased strongly with decreasing pH down to pH 6. At pH 3 the viscosity was again higher, but at pH 2 it was close to that in the pH range 6-9. Comparison with the results on the aggregate size shown in section 3.2.4 shows the pH dependence of the viscosity was correlated with that of the average aggregate size - FPI solutions containing larger aggregates being more viscous. The effect of the concentration of FPI solutions is shown in Figure 9 at pH 2 and pH 12. As expected, the viscosity increased with increasing in protein concentration. The increase was more important for the solution at pH 12 that contained larger aggregates. These results can be explained by the fact that larger aggregates take up a larger volume fraction as has been discussed in more detail for whey protein aggregates by Inthavong et al. (2019).

4 CONCLUSIONS

FPI can be extracted from tuna dark muscle using the pH-shift method. The FPI was rich in essential amino acids and kept the secondary structure of raw material. More than 85% of the FPI proteins could be solubilized at pH ≥ 6 and pH ≤ 3 in the form of aggregates that were significantly larger at pH 10 and 12 than between pH 6 and 9 and below pH 3. At intermediate pH FPI formed large flocs that sedimented under gravity. Soluble FPI solutions were shear thinning with a viscosity that was increased strongly for pH ≥ 10 where the solutions contained larger aggregates. A significant fraction of the myosin heavy chains was broken down into smaller chains. The FPI extracted in this manner has an excellent potential for application in food products for human consumption.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

CONFLICTS OF INTEREST

The authors declared no conflicts of interest.

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TABLE 1 Chemical composition of tuna dark muscle and FPI.

PROXIMATE	**Unit**	**TDM**	**FPI**
Moisture content	wt%	69.40 ± 0.66^{a}	77.30 ± 0.87^{b}
Ash	wt%	1.20 ± 0.04^{a}	1.40 ± 0.04^{b}
Protein	wt%	26.30 ± 0.35^{a}	20.10 ± 0.30^{b}
Fat	wt%	2.54 ± 0.09^{a}	0.66 ± 0.06^{b}
Sodium	mg/kg	577.3 ± 2.85^{a}	435.4 ± 2.00^{b}
Histamine (LOD = 3 ppm)	mg/kg	nd	nd
NaCl	mg/kg	-	1.09 ± 0.05
Arsenic (As)	mg/kg	-	nd
Mercury (Hg)	mg/kg	-	nd
Lead (Pb)	mg/kg	-	nd
Cadmium (Cd)	mg/kg	-	nd

Note: Different superscript letters within the same row represent significantly different ($P < 0.05$).

Abbreviations: nd (not detected); LOD (Limit of Detection)

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496 **TABLE 2** Amino acid composition of tuna dark muscle and protein isolate (dry basis).

Amino Acid	TDM (g/100g)	FPI (g/100g)
Alanine (Ala)	5.04 ± 0.30[a]	5.11 ± 0.62[a]
Arginine (Arg)	4.21 ± 0.14[a]	5.63 ± 0.57[b]
Aspartic acid (Asp)	7.21 ± 0.14[a]	9.24 ± 0.3[b]
Glutamic acid (Glu)	11.14 ± 0.68[a]	16.21 ± 0.63[b]
Glycine (Gly)	3.70 ± 0.20[a]	3.38 ± 0.12[a]
Histidine (His)**	3.57 ± 0.27[a]	2.40 ± 0.13[b]
Isoleucine (Ile)*	3.95 ± 0.30[a]	4.89 ± 0.23[b]
Leucine (Leu)*	6.49 ± 0.24[a]	8.29 ± 0.35[b]
Lysine (Lys)*	7.77 ± 0.33[a]	9.42 ± 0.33[b]
Methionine (Met)*	2.07 ± 0.07[a]	3.00 ± 0.24[b]
Phenylalanine (Phe)*	3.14 ± 0.16[a]	4.16 ± 0.26[b]
Proline (Pro)	2.36 ± 0.26[a]	2.38 ± 0.25[a]
Serine (Ser)	2.98 ± 0.21[a]	3.93 ± 0.17[b]
Threonine (Thr)*	3.07 ± 0.12[a]	4.21 ± 0.15[b]
Tyrosine (Tyr*	2.60 ± 0.36[a]	3.26 ± 0.11[b]
Valine (Val)*	3.48 ± 0.35[a]	5.04 ± 0.22[a]
EAA	32.56	42.26

TAA	**72.79**	**90.55**

Note: Different superscript letters within the same row represent significantly different ($P < 0.05$).

EAA -total essential amino acid, TAA-total amino acid

* Essential amino acids according to (FAO/WHO, 1975)

** Indispensable amino acid in human adult according to FAO/WHO/UNU (1985)

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519 **Legend of Figures**

520 **FIGURE 1** Fourier Transform Infrared Spectroscopy spectra of tuna dark muscle and FPI powder at
521 two different ranges of the wave number: 4000-500 cm^{-1} (a) and 2000-1400 cm^{-1} (b).

522 **FIGURE 2** Zeta potential of FPI as a function of pH.

523 **FIGURE 3** Photos of solutions of 10 g/L FPI taken 3 h after setting the pH at different values
524 indicated in the figure.

525 **FIGURE 4** Fraction of proteins in the supernatant as a function of pH for FPI solutions at 10 g/L.

526 **FIGURE 5** SDS-PAGE pattern of FPI at pH 2 (middle) and pH 12 (right). The left column shows
527 distinct bands of the markers with molar masses indicated in the figure.

528 **FIGURE 6** Average hydrodynamic radius (circle) and molar mass (triangle) of aggregates of
529 soluble FPI in aqueous solution at different pH.

530 **FIGURE 7** CLSM images of FPI solutions at 10 g/L at different pH. The images represent 110
531 x110 µm.

532 **FIGURE 8** pH-dependence of the shear viscosity for FPI solutions at 10 g/L at a shear rate of 1 s^{-1}.

533 **FIGURE 9** Protein concentration dependence of the shear viscosity for FPI solutions at pH 2
534 (circle) and pH 12 (triangle) and at shear rate of 1 s^{-1}. Solid lines are guides to the eye.

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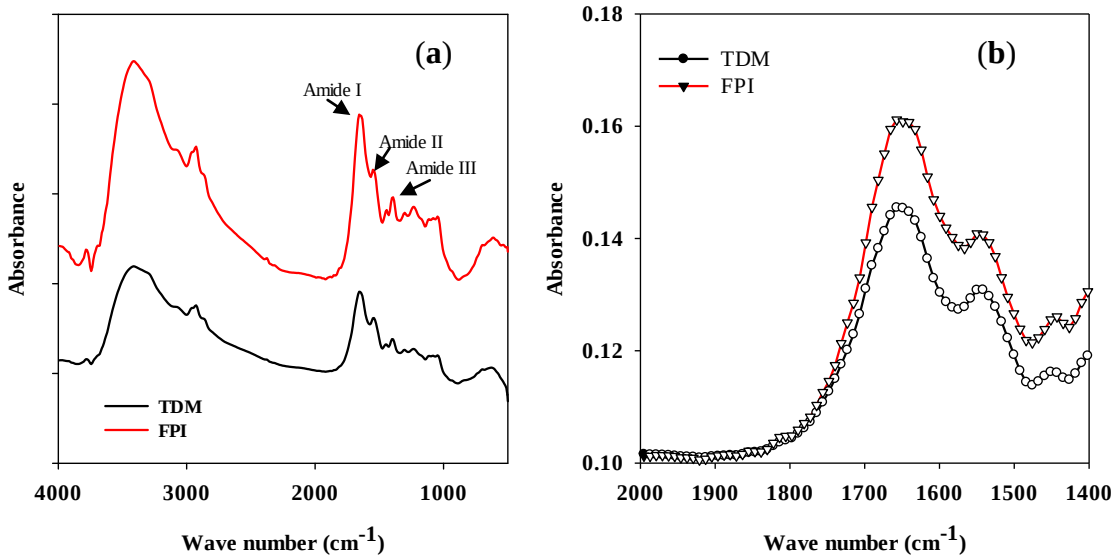


FIGURE 1 Fourier Transform Infrared Spectroscopy spectra of tuna dark muscle and FPI powder at different range of wave number: 4000-500 cm^{-1} (a) and 2000-1400 cm^{-1} (b).

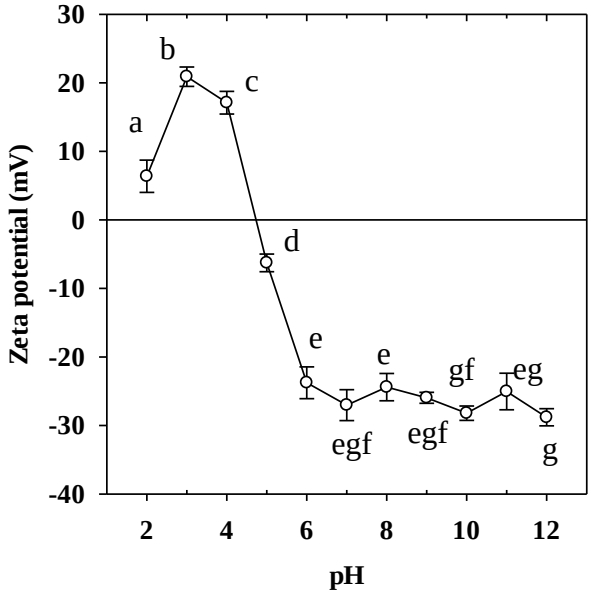


FIGURE 2 Zeta potential of FPI as a function of the pH.

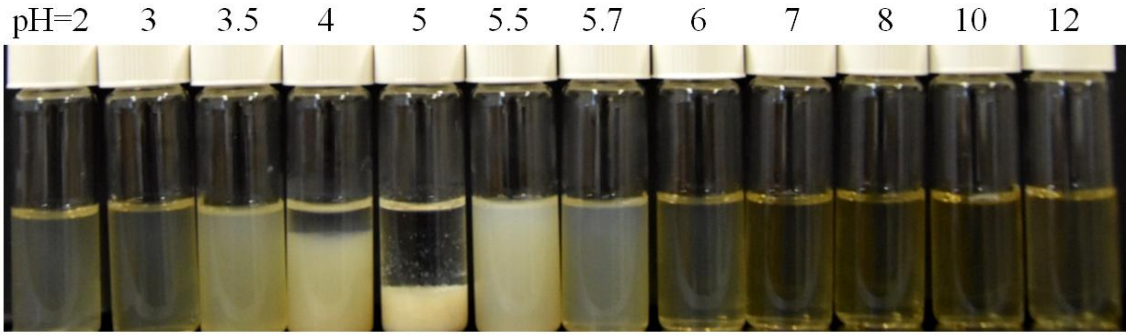


FIGURE 3 Photos of solutions of 10 g/L FPI taken 3 h after setting the pH at different values indicated in the figure.

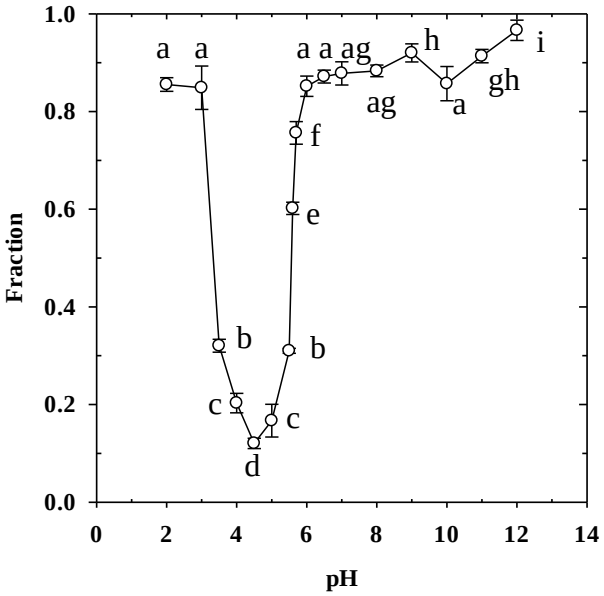


FIGURE 4 Fraction of proteins in the supernatant as a function of pH for FPI solutions at 10 g/L.

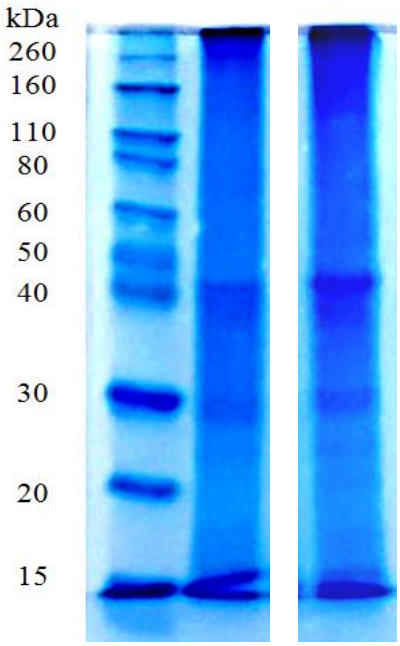


FIGURE 5 SDS-PAGE pattern of FPI at pH 2 (middle) and pH 12 (right). The left column shows distinct bands of the markers with molar masses indicated in the figure.

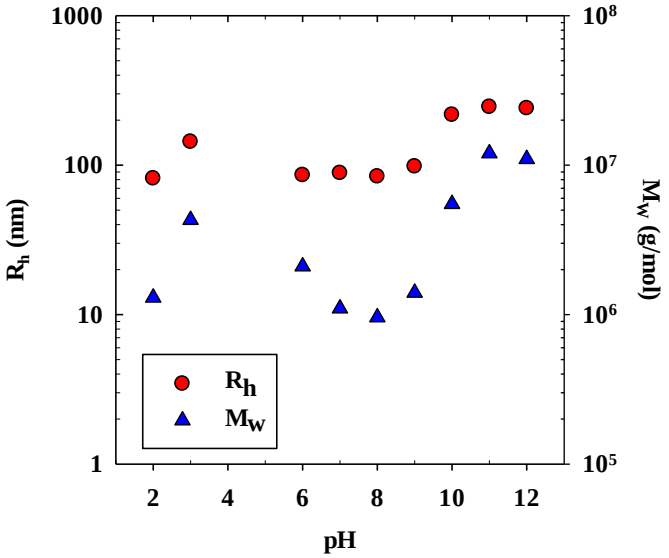
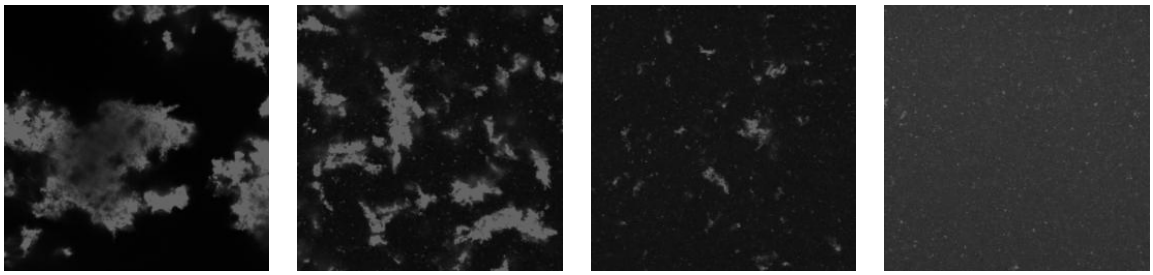
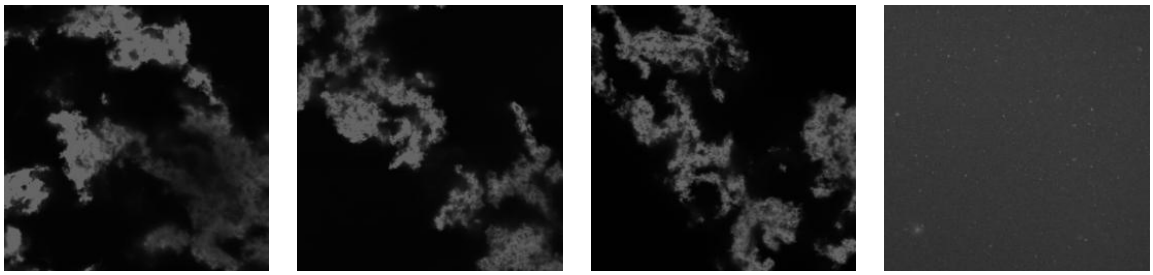


FIGURE 6 Average hydrodynamic radius (circle) and molar mass (triangle) of aggregates of soluble FPI in aqueous solution at different pH.



pH 5.0 **pH 5.5** **pH 5.6** **pH 5.7**



pH 4.5 **pH 4.0** **pH 3.5** **pH 3.0**

FIGURE 7 CLSM images of FPI solutions at 10 g/L at different pH. The images represent 110 x110 μm.

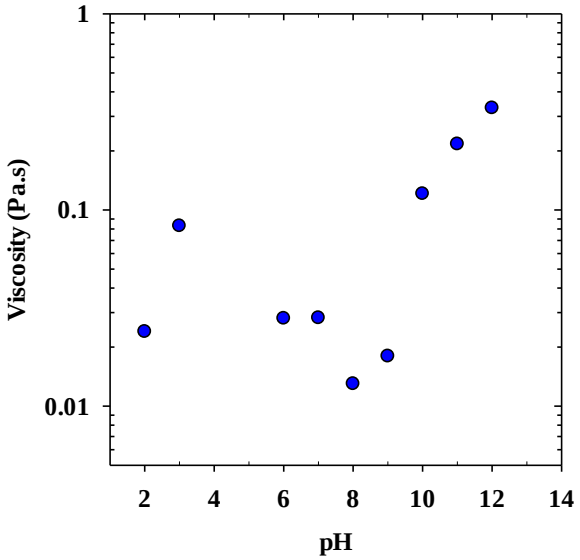
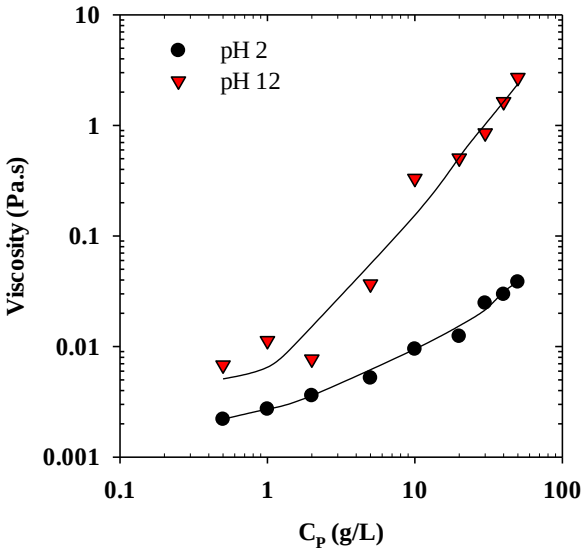


FIGURE 8 pH-dependence of the shear viscosity for FPI solutions at 10 g/L at a shear rate of 1 s^{-1}.



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663 **FIGURE 9** Protein concentration dependence of the shear viscosity for FPI solutions at pH 2
664 (circle) and pH 12 (triangle) and at shear rate of 1 s^{-1}. Solid lines are guides to the eye.

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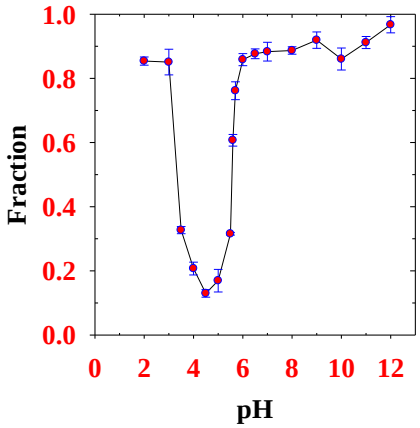
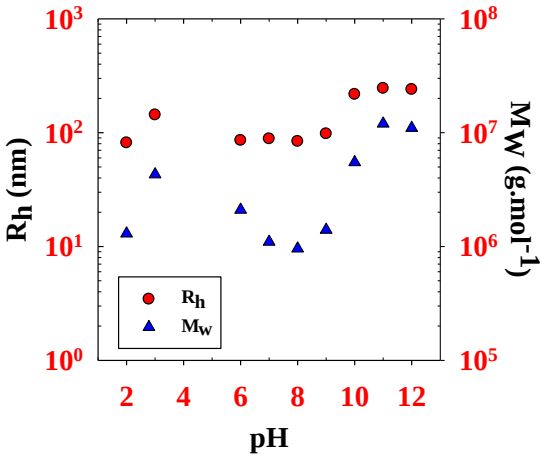
678 **Graphical Abstract**

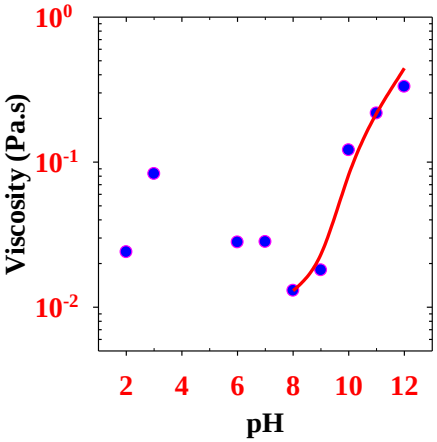
composition



solubility



size



viscosity

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