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High-pressure processing (HPP) impacts on indian mackerel meat: insights into quality attributes, β-parvalbumin antigenicity, and proteomic changes

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ABSTRACT

High-pressure processing (HPP) is a non-thermal method that uses high hydrostatic pressure on food products to improve quality and shelf life. However, it can cause structural changes in proteins that alter food characteristics. This study investigated the effects of high-pressure processing (HPP) at 200, 400, and 600 MPa for 5 or 20 min on the quality, protein profiles, and antigenicity of β-parvalbumin, a major fish allergen, in Indian mackerel fillets. It also examined the *in vitro* digestibility of total protein and β-parvalbumin after HPP treatments. Protein profiles of the 600 MPa 5-minute treated and untreated fillets were analysed using 2D-PAGE, and proteins with significant changes were identified via MALDI-TOF/TOF MS. Results revealed that HPP at 400 and 600 MPa significantly increased fillet hardness and whiteness while reducing soluble protein content. Though β-parvalbumin was present in all treated samples, its levels decreased after digestion, becoming undetectable after 15 min. Specific proteins, including β-parvalbumin, actin, and ATP synthase, were significantly altered in the 600 MPa-treated fillets. HPP surpassing 600 MPa enhanced β-parvalbumin digestibility and reduced its antigenicity. These findings highlighted the delicate balance required in optimising HPP for improved digestibility and minimising the antigenicity of β-parvalbumin in Indian mackerel without compromising its quality.

1. Introduction

Fish has been widely consumed as they are a significant source of protein, minerals, and vitamins with low levels of saturated fats. However, proteins such as β-parvalbumin in fish, have been found to cause allergic reactions (Sharp & Lopata, 2014). The prevalence of fish allergy normally ranges between 0 % to 7 % and is also higher in countries where fish is a substantial part of their diet (Moonesinghe et al., 2016; Saptarshi, Sharp, Kamath & Lopata, 2014). The molecular diversity of two protein lineages, α- and β-parvalbumin (PV), the main allergenic fish protein, was previously shown to vary significantly across fish species (Stephen et al., 2017). The structural differences of the epitopes affect differences in its allergenicity, leading to variability in clinical allergic responses. In one study, β-parvalbumins were identified as the major allergens in 33 freshwater and marine fish including the Indian mackerel (Ruethers et al., 2018). The allergenicity of β-parvalbumin has been extensively studied in numerous species of fish and as of 2022, 290 allergenic fish parvalbumin are listed in the allergome database (www.allergome.org). β-parvalbumin can be found in abundance in fish muscle and is resistant to heat (Saptarshi, Sharp, Kamath & Lopata, 2014; Kubota et al., 2016). Furthermore, compared to other meat, fresh fish meat is highly perishable and more prone to spoilage (Dasanayaka et al., 2022). Multiple processing methods have been utilised to preserve fish following harvest.

Conventional thermal treatments, while effective in food preservation, may cause a reduction in the nutritional value and organoleptic properties of food products due to the high temperature and long processing duration (Niu et al., 2020). The undesired effects of thermal

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processing on food have led to the development of non-thermal processing that can preserve food while also maintaining nutritional values and sensory characteristics (Z.H. Zhang et al., 2019). One of the more notable non-thermal processes is high-pressure processing (HPP). HPP employs high hydrostatic pressure to improve safety, organoleptic properties, and quality of food (Khan et al., 2019). Foods processed with HPP are also shown to be able to withstand longer storage duration (Suemitsu & Cristianini, 2019; Pita-Calvo et al., 2018). Compared to thermal treatment, HPP produces fewer changes in the flavour, texture, and colour of processed food (Sun, Sridhar, Tsai & Chou, 2019).

On the other hand, HPP promotes partial unfolding of proteins followed by non-covalent, hydrophobic and hydrogen interactions, causing the denaturation of protein molecules (Cropotova et al., 2020). Since HPP does not alter covalent bonds, vitamins, minerals, and flavour compounds are unaffected by this processing (Chizoba Ekezie, Cheng & Sun, 2018). As HPP induces structural changes in proteins, food allergens may be reduced or eliminated through HPP by modifying allergenic proteins in the food. Multiple studies have highlighted the efficacy of HPP in reducing the immunoreactivity of food allergens (Cepero-Betancourt et al., 2020; López-Pedrouso, Lorenzo, Gagaoua & Franco, 2020; Pazos, Méndez, Vázquez & Aubourg, 2015; Y. Zhang et al., 2019). Furthermore, structural changes in proteins can also alter the appearance of the final HPP products. HPP-pressured chicken and fish meat resulted in increased firmness and whiteness (Cropotova et al., 2020; Cap et al., 2020).

This study explored the effect of HPP on Indian mackerel (*Rastrelliger kanagurta*) as this fish is one of the most highly consumed fish species in Malaysia due to their availability, affordability, and versatility (Ismail, Failler, March & Thorpe, 2022). The Indian mackerel can be found all over South-East Asia either fresh or processed, in the form of salted fish, fish paste or fish crackers. Indian mackerel is an easily acquired source of protein with a protein content of 21 % and contains essential mineral (Tsighe et al., 2018). Fish usually contains polyunsaturated fatty acids (PFAs), which have preventive properties on cardiovascular diseases (Tørris, Småstuen & Molin, 2018). PFAs are present at 37 % in the Indian mackerel and 87 % of the polyunsaturated fatty acid accounts for omega-3 making them a good source of marine PFAs (Bahurmiz, Adzitey & Ng, 2017).

The effects of HPP on the quality, digestibility, and allergenicity of pelagic fish meat such as Indian mackerel are less explored. Despite Indian mackerel being widely consumed in Malaysia, no previous studies have examined the effects of HPP on its protein profile, digestibility, or PV antigenicity. HPP has been shown to improve the digestibility of proteins in meats, processed meats and seafood (Cepero-Betancourt et al., 2020; Y. Zhang et al., 2019; Xue et al., 2020). Nonetheless, limited studies have been done on fish protein and allergen digestibility after HPP treatment. Common HPP conditions for preserving fish quality typically involve pressures of 200, 400, and 600 MPa for 5 min at 25 °C (Prego et al., 2021; Tsironi et al., 2019; Mengden, Röhner, Sudhaus & Klein, 2015). However, these conditions may not be sufficient to significantly reduce the allergenicity of β-parvalbumin, as allergens are generally resistant to short-term processing treatments. Interestingly, an extended holding time of 20 min has been shown to successfully reduce the IgG- and IgE-reactivity of non-specified fish allergens in cod (Y. Zhang et al., 2019). However, Indian mackerel, a pelagic fish, is phylogenetically distant from demersal fish like the cod, suggesting that its allergens, including parvalbumin, have unique allergenic features. The lipid-protein matrix of pelagic fish like Indian mackerel may cause HPP-induced allergen reduction differently than in lean demersal species (Nordhagen et al., 2020). This difference not only limits the applicability of cod-derived data but emphasises a knowledge gap in understanding the effects of HPP on allergenic proteins in lipid-rich fish. In this study, Indian mackerel fillets were subjected to treatments at pressures of 200, 400, and 600 MPa with holding times of both 5 and 20 min, and their effects on the quality, specifically on colour and textural attributes, protein digestibility, and β-parvalbumin antigenicity of the fish fillets were investigated. By focusing on antigenicity (IgG-binding) rather than IgE-reactivity, we provide complementary data on how HPP changes protein epitopes, which is crucial for food processing applications. Antigenicity reduction can still indicate potential allergen mitigation.

In addition, proteomic analysis is an important tool for identifying protein biomarkers responsible for changes in protein profiles as affected by HPP (López-Pedrouso, Lorenzo, Gagaoua & Franco, 2020; Pazos, Méndez, Vázquez & Aubourg, 2015; Carrera, Piñeiro & Martinez, 2020; Dang et al., 2019; Nissa et al., 2021). Changes in these protein biomarkers through food processing have been shown to affect quality attributes in meat and fish (Dang et al., 2019; Nissa et al., 2021; Yu et al., 2020). However, as of now, no literature has been found studying the effects of HPP on the proteomic of Indian mackerels. This study also aims to fill that gap by investigating the impact of HPP treatment on the proteomics of Indian mackerel fillets.

2. Materials and methods

2.1. Indian mackerel samples

Fresh Indian mackerels (*R. kanagurta*) were acquired from a local supermarket and transported on ice in an insulated container to the laboratory at the Faculty of Food Science and Technology, UPM. The fish were caught in the South China Sea, procured in Kuantan, Malaysia, and transported to the market within 24 h. *R. kanagurta* was identified using morphological identification based on the description from W. Fisher and Bianchi (1984) (W. Fisher & Bianchi, 1984). All fishes obtained were beheaded, gutted, and filleted. Approximately 35 g of the fillets were individually vacuum sealed using a vacuum sealer in sterile polyethylene-polyamide plastic bags ready for high-pressure processing (HPP).

2.2. HPP conditions

HPP was performed using QFP 2 L 700 Avure high hydrostatic pressure equipment (Avure Technologies Inc., USA). Fresh individually sealed fish fillets in polyethylene-polyamide plastic bags were placed in the HPP unit and treated at 200, 400 and 600 MPa at 20 °C for 5 min and 20 min, respectively, using water as the transmission medium at 25 °C. Each treatment was performed in triplicate and the unpressurised raw meat was used as a control. Pressure-treated and control fillets were then stored at −20 °C until further analysis.

2.3. Textural analysis

Stable Micro Systems Company texture analyser the TA.XT2i equipped with a 10 mm diameter cylinder Delrin was used to perform textural analysis. The Indian mackerel fillets were analysed by a cylindrical probe producing readings on the texture profile analysis (TPA). Textural analysis was done following the method from Chouhan et al. (2015) (Chouhan, Kaur & Rao, 2015). Treated and untreated Indian mackerel fillets were equilibrated at room temperature for 1 h before the analysis. TPA readings show the hardness (g), springiness, gumminess (g), cohesiveness, and chewiness (mm) of the fish meat. The readings were done in triplicates and raw untreated Indian mackerel meat was used as the control. TPA test was conducted using a 10 mm cylinder Delrin. The test conditions were set as follows: rate pre-test speed: 1.00 mm/s; test speed: 2.00 mm/s; post-test speed: 5.00 mm/s; strain: 75 %; distance: 10 mm, trigger force: 5.0 g trigger type: automatic.

2.4. Colour analysis

Colour analysis on the treated meat was performed using Konica Minolta Chromameter CR-400. The analysis was done in triplicate. The colour analysis readings were taken in the form of CIELAB colour space

divided into three different values: L*, a* and b*. L* value constitutes lightness from white to black, a* value from green to red, and b* value from blue to yellow. The chroma (C_H), and whiteness index (WI) of each sample were calculated following these equations (Sun, Sridhar, Tsai & Chou, 2019; Briones-Labarca et al., 2012):

$$C_H = \left[\left(a*^2 + b*^2 \right)^{\frac{1}{2}} \right] \quad (1)$$

$$WI = 100 - \left[(100 - L*)^2 + \left(a*^2 \right) + \left(b*^2 \right) \right]^{\frac{1}{2}} \quad (2)$$

2.5. Protein extraction

Protein extractions were performed in two different phases as soluble and insoluble protein fractions needed to be extracted separately. The extraction of the soluble fraction of samples was performed following the S.J. Koppelman et al. (2010) method (S.J. Koppelman et al., 2010). Meanwhile, protein extraction was done according to Malva et al. (2018) method for the insoluble fraction (Della Malva et al., 2018). For soluble protein extraction, 10 g of fish meat was homogenised in 10 mL of deionised water. Following that, 20 mL of deionised water and 800 μL of 1 M Tris–HCl (pH 8.0) and 400 μL of protease inhibitor were added to the homogenised mixture with the addition of 1 M NaOH to adjust the slurry to pH 8. The slurry was stirred in a magnetic stirrer on ice for 10 min while maintaining at pH 8. The homogenised mixture was then centrifuged at 4000 rpm for 30 min at 4 °C. Following that, the supernatant containing soluble fraction was collected and filtered through a vacuum filter with Whatman filter paper. The protein extraction was then stored until further use at −20 °C.

The insoluble protein extraction was done after the soluble protein extraction through the precipitation left behind after soluble protein extraction. Lysis buffer of the same volume 1:1 was added to the solid residue in the tube and the mixture was incubated overnight at 4 °C while mixing. Following incubation, the homogenised mixture was centrifuged at 10,000 rpm for 15 min at 4 °C. Following centrifugation, the supernatant containing the insoluble protein fractions was then collected and stored at −20 °C until further use.

2.6. Protein concentration determination

The concentrations of protein extracts were estimated using the Bradford assay (Biorad Laboratories, Hercules, *CA*, USA). BSA (Biorad Laboratories, Hercules, *CA*, USA) concentrations of 0 to 1 mg/mL were used to generate a standard curve. A total of two dilutions (1:50, 1:100) of Indian mackerel protein extract were made and both BSA standard and diluted samples were measured in triplicates. The soluble protein samples were diluted in 0.01 M sodium phosphate buffered saline (0.85 % NaCl) pH 7.5 (PBS), and insoluble samples were diluted in lysis buffer to ensure compatibility with extracted protein following the methods from W. Koppelman et al. (2010) and Malva et al. (2018) (S.J. Koppelman et al., 2010; Della Malva et al., 2018). Samples and BSA standard of 10 μL were pipetted into a 96-well microtiter plate followed by the addition of 200 μL of Bradford reagent. The plate was incubated for 10 min at room temperature. Incubated samples were analysed with an absorbance of 595 nm in MultiSkan GO microplate reader (Thermo Fisher Scientific Inc., USA).

2.7. In vitro digestion of treated and untreated indian mackerel

In vitro digestion of HPP-treated and untreated Indian mackerel samples was performed following the method by Brodkorb et al. (2019) (Brodkorb et al., 2019). *In vitro* digestion was done on untreated (control) and 600 MPa-treated fish fillets for both holding times (5- and 20-minutes). Samples of digested fish meat were taken at a time intervals of 15 min, 30 min, 60 min, 180 min, 210 min, and 270 min. To stop enzyme activities at each time interval, the samples were placed in heat-shock treatment of 100 °C for 5 min. All the simulated digestive fluids were pre-warmed at 37 °C prior to use.

Fish meat (5 g) was pounded in a pestle and mortar to simulate mastication. The pounded sample was then added into an Eppendorf tube containing 5 mL simulated salivary fluid (SSF), 975 μL deionised water and incubated at 37 °C for 2 min while mixing using a tube rotator (LTF Labortechnik GmbH & Co. KG, Germany). After 2 min, 10 mL simulated gastric fluid (SGF) was added followed by 5 M HCl to adjust the oral bolus to pH 3. After pH adjustment, 0.45 mL porcine pepsin solution (2000 U/mL) prepared in water was added and the homogenised mixture was incubated for 2 h while mixing at 37 °C. After 2 h, 10 mL of simulated intestinal fluid (SIF) was added followed by 5 M NaOH to adjust the mixture to pH 7. After pH adjustment, 2 mL of bile solution (10 mM) prepared in SIF was added and the slurry was incubated at 37 °C for 30 min while mixing to achieve full solubilisation of bile. Following that, 3.75 mL of pancreatin (100 U/mL) prepared in SIF was added and the sample was incubated at 37 °C for 2 h while mixing. Protein extraction was performed on digested samples.

2.8. Proteomic analysis

2.8.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to M. Mansor et al. (2023) using 15 % resolving gels with 5 % stacking gel (M. Mansor et al., 2020). A 10 μL volume of samples containing 500 μg/μL of protein in were mixed with 10 μL of sample buffer. The mixture of protein extracts was heated at 95 °C for 5 min and 10 μL were loaded into the followed by 5 μL of Precision Plus Protein Dual Color Standard from Biorad for reference. The loaded gel was run for 10 min and 1 hour at 90 V and 200 V respectively in running buffer (0.25 M Tris-base, 1.92 M glycine and 1 % (w/v) SDS) in the Mini Protean Tetra Cell (Biorad, USA). The gel was then transferred into a container, washed with deionised water, and stained overnight with Coomassie Brilliant Blue. Stained gel was de-stained using a de-staining solution of 25 % (v/v) methanol and 5 % (v/v) acetic acid and viewed using ImageQuant LAS 500 chemiluminescence CCD camera (GE Healthcare Life Sciences, USA).

2.8.2. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Soluble and insoluble proteins from 600 MPa, 5 min and 20 min of HPP-treated meat and untreated meat were separated in 2D-PAGE, by adopting the method by M. Mansor et al. (2020) (M. Mansor et al., 2020). Briefly, 400 μg/μL of proteins were added in a rehydration buffer and loaded onto immobilised pH gradient (IPG) strips, which were rehydrated overnight at 18 °C. The Ettan IPGphor 2 IEF system (GE Healthcare, Chicago, IL, USA) was used to execute isoelectric focusing (IEF) on a 13 cm pH 4–7 IPG strip (GE Healthcare, Chicago, IL, USA) at 500 V/4 h, 1000 V/1 h, 5000 V/1 h, 8000 V/1 h, and finally 29,000 V/1 h. Equilibrated IPG strips were then laid on polyacrylamide gels, and the Ruby SE 600 electrophoresis equipment was used for electrophoresis (GE Healthcare, Chicago, IL, USA) for 15 min at 20 mA/gel and 3 h at 40 mA/gel. Following the completion of the run, the 2D-PAGE gels were fixed overnight using 40 % methanol and 10 % acetic acid in MilliQ water as fixing solution followed by staining with CBB staining solution.

Three replicates of gels were obtained for each sample. Visualisation of 2D-PAGE gels utilised the calibrated densitometer Biorad GS800 (Biorad Laboratories, Hercules, *CA*, USA) with a 32-bit pixel depth and 600 dpi resolution. Using Progenesis SameSpots software version 3.1v (Nonlinear Dynamic Ltd., Durham, NC, USA), acquired images of 2D-PAGE gels were subjected to the Progenesis SameSpots software for automated stain analysis, where normalised volume was computed and each protein spots were assessed for differential abundance through ANOVA testing.

2.8.3. In-gel digestion

The spots of proteins of interest were excised from the 2D-PAGE gels and went through in-gel protein digestion, following methods by M. Mansor et al. (2020) (M. Mansor et al., 2020). The excised spots were de-stained with 50 % Acetonitrile in 100 mM ammonium bicarbonate as the washing solution. The spots were then reduced with 100 mM DTT in 100 mM ammonium bicarbonate as the reduction solution, at 60 °C for 30 min. Following that, reduced samples were then incubated in darkness with the addition of 55 mM IAA in 100 mM ammonium bicarbonate as the alkylation solution. Samples were then washed and incubated in 100 % ACN for 15 min. ScanSpeed MiniVac Evaporator (Sau, Reutlingen, Germany) was used to dry samples for 1 hour. Trypsin solution (7 μg/mL of trypsin) was added to the dried samples and incubated overnight in a water bath at 30 °C. Digested samples were then mixed with 100 % acetonitrile and dried for 1 hour using ScanSpeed MiniVac Evaporator (Sau, Reutlingen, Germany). Dried peptides were stored at −80 °C until further analysis.

2.8.4. Protein identification by MALDI-TOF/TOF MS

Dried peptides were reconstituted in 0.1 % TFA in 30 % ACN. Zip-Tip C18 (Millipore, Bedford, MA, USA) was then used to desalt the protein samples. The prepared peptides were analysed on an Ultraflextreme MALDI-TOF/TOF mass spectrometer (Bruker, Bremen, Germany) following methods by M. Mansor et al. (2020) (M. Mansor et al., 2020). Spectra of the peptides were then analysed using MASCOT search version 3.5 (Matrix Science, Boston, MA, USA) against Swiss-Prot and National Centre for Biotechnology Information (NCBI) databases for protein identification. The parameters employed in database searches were as follows: Variable modification: Methionine oxidation, Fixed modification: Carbamidomethylation, Mass values: Monoisotopic, Enzyme: Trypsin with one missed cleavage allowed, Peptide mass tolerance: ±300 ppm, Fragment mass tolerance: ±1.0 Da.

2.9. Immunoblotting

Immunoblotting was performed according to M. Mansor et al. (2023) on both 1D SDS-PAGE and 2D SDS-PAGE gel using Biorad Transblot SD Semi-dry Transfer Cell (M. Mansor et al., 2023). After running gel electrophoresis, the gel and activated PVDF membrane were equilibrated in the transfer buffer (25 mM Tris-base, 190 mM glycine, 20 % methanol) for 15 min and the protein transfer was run at 25 V for 35 min.

The membrane containing proteins was then blocked overnight while shaking at 4 °C in a blocking buffer (5 % milk in PBST). After blocking, the membrane was then incubated with 1:1000 dilution of PARV-19 (Sigma-Aldrich, USA) at 4 °C overnight. The membrane was then washed with PBST (0.05 % Tween-20 in PBS) 4 times for 5 min and incubated with goat anti-mouse horse radish peroxidase (HRP)-conjugated IgG solution (Southern Biotech, Birmingham, AL, USA). (1:10,000 dilution) in PBST at RT for 1 hour. The membrane was then rewashed with PBST for 10 min, thrice. After the membrane was washed, it was incubated at RT in Amersham ECL Western blotting reagent GE Healthcare (GE Healthcare, Chicago, IL, USA) for visualisation and imaging was done using Bio-Rad Chemidoc MP imaging system (Bio-Rad Laboratories, Hercules, *CA*, USA).

2.10. Statistical analysis

Statistical analysis was carried out by employing the two-way analysis of variance (ANOVA) with 95 % confidence interval using the statistical software Minitab® version 18 (Minitab Inc., State College, PA, USA). Tukey's multiple comparison test method with $\alpha = 0.05$ was applied to compare significant differences between means of HPP treatments.

3. Results and discussion

3.1. Textural analysis

At present, consumers demand food or food products that are minimally processed, microbiologically safe, more "natural", healthy and nutritious with fewer additives (Tsironi et al., 2019). The textural analysis is utilised to determine the quality of Indian mackerel fillets as it is one of the significant characteristics that impact consumer purchase (Suemitsu & Cristianini, 2019). The effects of HPP pressure level on the texture of Indian mackerel fillets depend on the holding time as seen in Fig. 1. The effect of pressure and holding time on sample hardness followed a direct relationship, *i.e.*, the higher the pressure and holding time used, the higher the fillet hardness obtained. Hardness value is also significantly ($p < 0.05$) higher when treated at 600 MPa compared to treatment of other treatments. Samples treated at 600 MPa for 20 min also showed a significantly ($p < 0.05$) higher hardness than samples treated at 600 MPa for 5 min. An increase in the hardness value of pressure-treated fish fillets has been attributed to aggregation and denaturation of myofibrillar protein (Tsironi et al., 2019). The same was reported by HPP-treated hilsa (Kumar, Rao, Purohit & Kumar 2019) and sea bass (Tsironi et al., 2019).

In terms of springiness, treated Indian mackerel samples showed no significant ($p < 0.05$) changes compared to untreated Indian mackerel samples. For cohesiveness, all pressure-treated samples for both holding times showed a significant ($p < 0.05$) increase in cohesiveness compared to untreated Indian mackerel. For gumminess, Indian mackerel samples treated at 400 MPa and 600 MPa for both holding times (5- and 20-minutes) showed a significant ($p < 0.05$) increase in value than the untreated Indian mackerel. Treatment of 600 MPa treated Indian mackerel samples also showed significantly ($p < 0.05$) higher gumminess compared to treated samples at 400 MPa for both holding times. Samples treated at 600 MPa for 20 min also showed a significantly ($p < 0.05$) higher gumminess than samples treated at 600 MPa for 5 min. The unfolding of actin causes an increase in cohesiveness which conversely increases the gumminess of fish (Puértolas & Lavilla, 2020; Zhao, de Alba, Sun & Tiwari, 2019).

The chewiness of Indian mackerel showed a significant ($p < 0.05$) decrease when treated at 200 MPa for 5 min and then it significantly ($p < 0.05$) increased at treatment of 400 MPa and 600 MPa for 5 min. At 20 min of holding time, chewiness significantly ($p < 0.05$) increased as pressure increased. The same trend can be seen in pressure-treated mackerel fillets whereby the chewiness decreases when treated at a lower pressure level and then starts to increase when treated at a higher pressure level for a lower holding time (de Alba et al., 2019). These changes in tissue texture are the results of muscle protein denaturation and aggregation due to high-pressure levels causing tissue structure shrinkage (Pita-Calvo et al., 2018; Tsai et al., 2022).

Changes in textural attributes of pressure-treated fish fillets can be explained by a pressure-induced degradation of structural proteins such as actin and myosin, which could partially be assisted by proteolytic enzymes liberated from pressure-damaged cells (Cropotova et al., 2020). High pressure in HPP affects the myofibrillar protein resulting in aggregation, agglomeration, unfolding, and network formation of the proteins which affects the textural properties of meat (Nath, Pandiselvam & Sunil, 2023).

3.2. Colorimetric analysis

Colorimetric analysis is an important tool to identify the influence of food processing on the colour parameters of food products as the appearance of food can affect consumer acceptance (Men et al., 2020). In this study, colorimetric analysis was done to investigate the effects of different HPP conditions on Indian mackerel meat. As can be seen from Fig. 2, all HPP treatments caused the Indian mackerel fillets to be whiter and opaquer. The same can be seen in pressure-treated tuna fillets

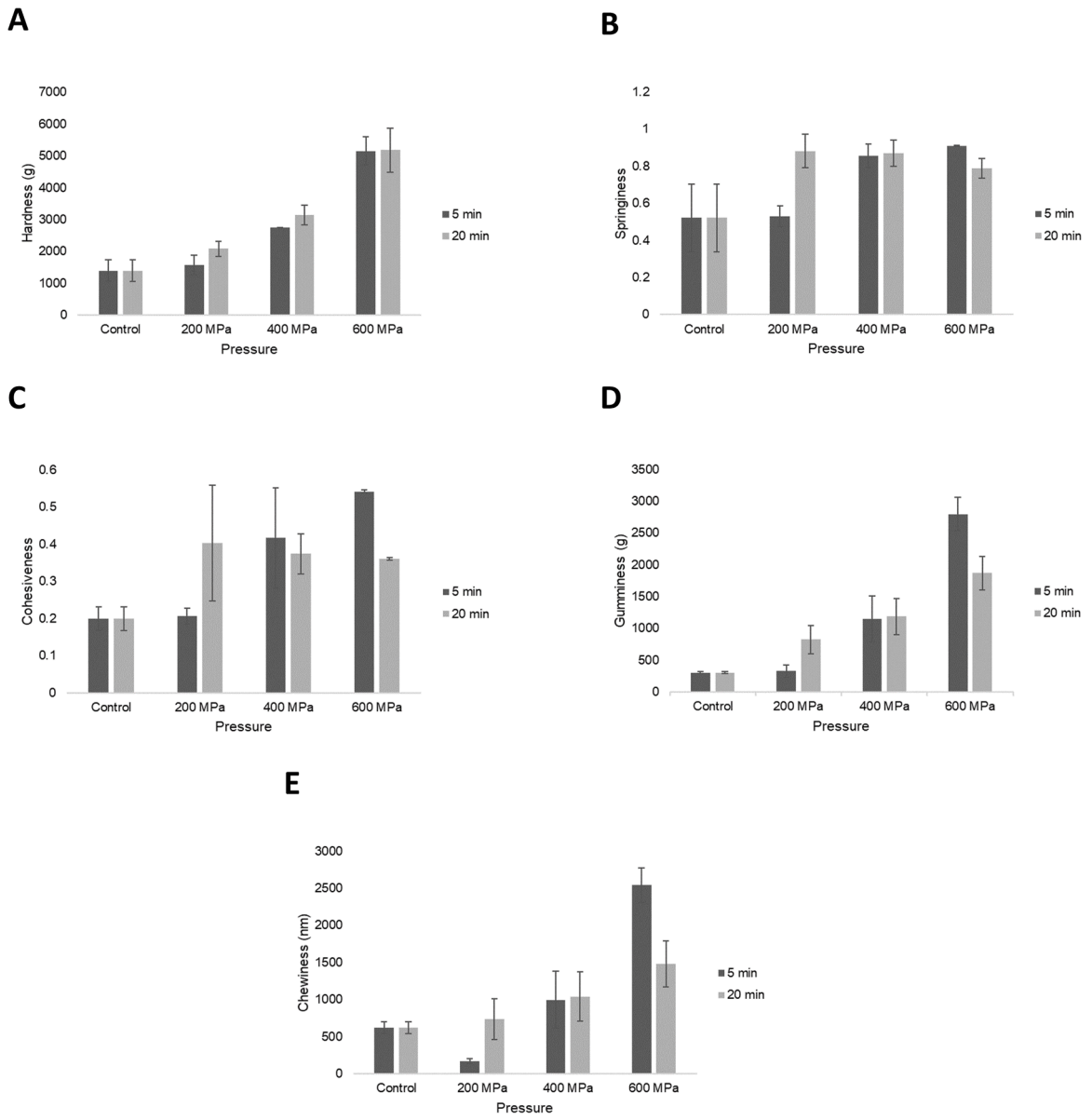

Fig. 1. (A) Hardness (g) of Indian mackerel samples after HPP treatments at 200, 400, and 600 MPa for 5- and 20-min holding times. (B) Springiness of Indian mackerel samples after HPP treatments at 200, 400, and 600 MPa for 5- and 20-min holding times. (C) The cohesiveness of Indian mackerel samples after HPP treatments at 200, 400, and 600 MPa for 5- and 20-min holding times. (D) Gumminess (g) of Indian mackerel samples after HPP treatments at 200, 400, and 600 MPa for 5- and 20-min holding times. (E) Chewiness (nm) of Indian mackerel samples after HPP treatments at 200, 400, and 600 MPa for 5- and 20-min holding times. 0.1 MPa is the untreated sample as the control. Data are means ± *S*.D. ($n = 3$). "*" shows a significant ($p < 0.05$) difference between each treatment to the control. "#" shows a significant ($p < 0.05$) difference between different treatments. Results were obtained from two-way ANOVA followed by Tukey's *post hoc* test.

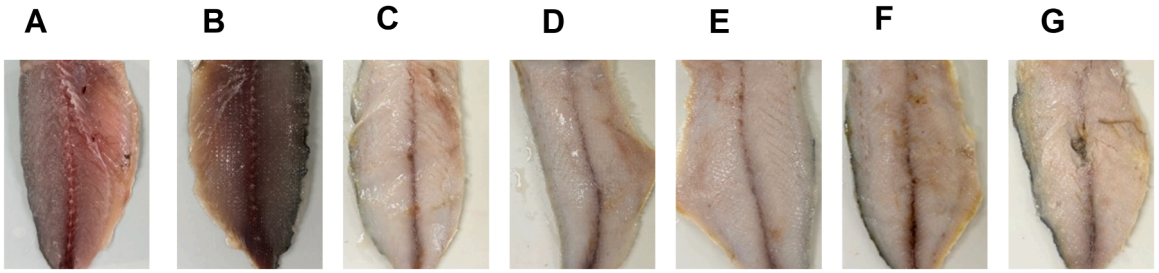

Fig. 2. Indian mackerel fillets after HPP treatments: (A) Control, (B) 200 MPa for 5 min, (C) 200 MPa for 20 min, (D) 400 MPa for 5 min, (E) 400 MPa for 20 min, (F) 600 MPa for 5 min, and (G) 600 MPa for 20 min.

(above 300 MPa for 5 min) as high pressure caused a decrease in pigment activity and caused protein denaturation which changed the characteristics of the sample surface thus increasing the light reflection, producing a whiter appearance (Tsai et al., 2022).

There is a significant ($p < 0.05$) increase in lightness (L*) value for all pressure-treated Indian mackerel samples at both holding times compared to the untreated Indian mackerel (Fig. 3). The L* value is also significantly ($p < 0.05$) increased as pressure and holding time increased. These results were in agreement with the results of HPP on haddock and mackerel minces (Cropotova et al., 2020), European sea bass (Tsironi et al., 2019), mackerel fillet (de Alba et al., 2019) and hilsa fillet (Chouhan, Kaur & Rao, 2015), suggesting that the increase in L* value was caused by both protein coagulation and loss of active pigmentation due to the pressure.

The a* value of Indian mackerel samples treated at 400 and 600 MPa for both holding times showed a significant difference ($p < 0.05$) compared to untreated Indian mackerel. Similar results were observed in hilsa fillet (M. Mansor et al., 2020), European sea bass (Tsironi et al., 2019) and mackerel fillet (de Alba et al., 2019) when subjected to HPP treatment. The decrease in redness value can be attributed to the denatured myoglobin due to the pressure (de Alba et al., 2019; Christensen, Hovda & Rode, 2017). Additionally, HPP at 300 MPa for 5 min of sea bream fillet showed a reduction in a* value compared to fresh fillets (Giannoglou et al., 2020). Regarding the b* and chroma values, pressure treatment at 400 MPa for 20 min showed a significant decrease ($p < 0.05$) compared to untreated samples. Similar effects were observed in pressure-treated mackerel minces, as HPP affects the structural integrity of proteins (Cropotova et al., 2020).

Meanwhile, for the whiteness index (WI), there is an increasing trend with the increase in pressure intensity and holding time. This result is

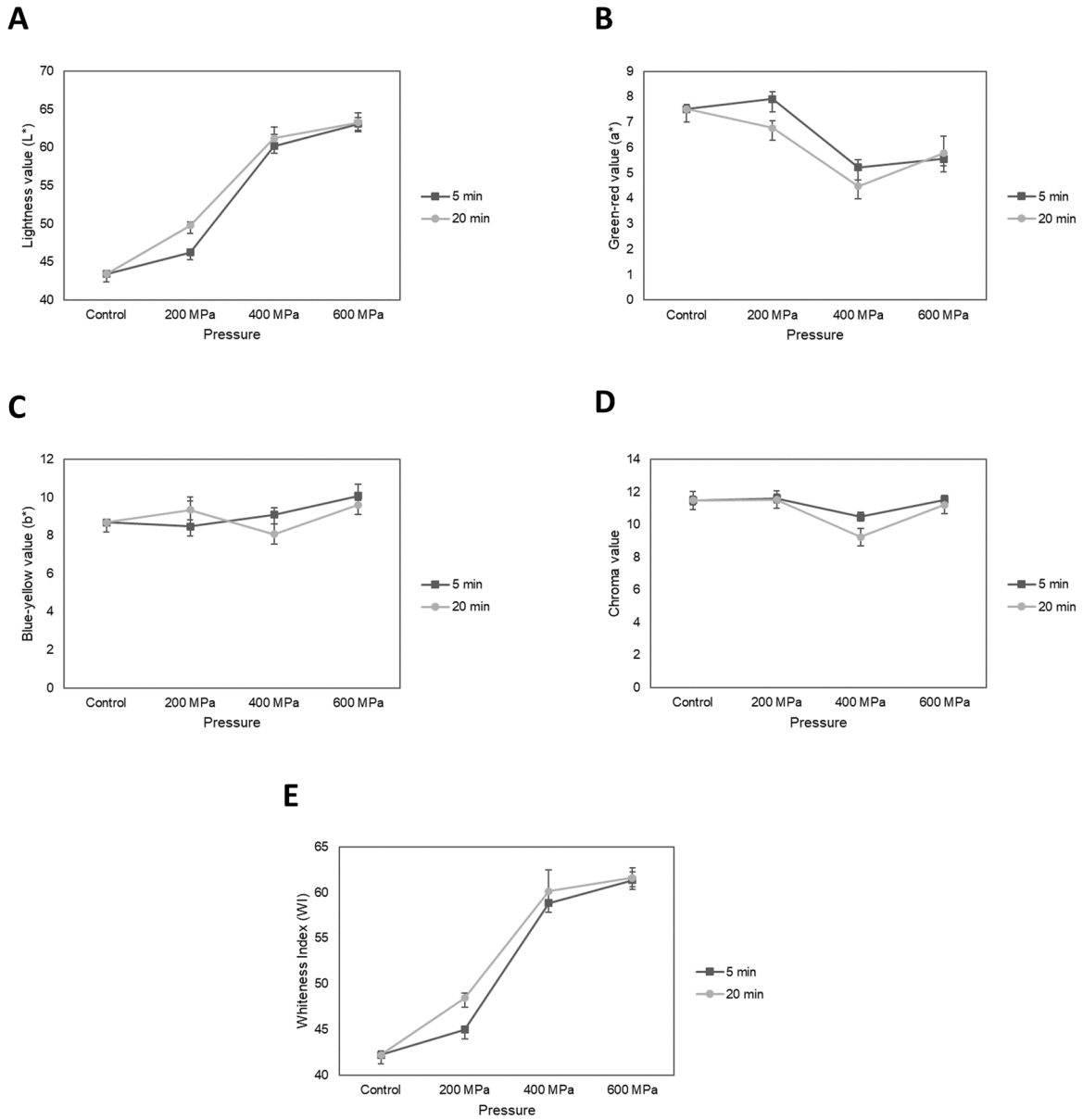

Fig. 3. (A) L* value of Indian mackerel samples after HPP treatments at 200, 400, and 600 MPa for 5- and 20-min holding time. (B) a* value of Indian mackerel samples after HPP treatments at 200, 400, and 600 MPa for 5- and 20-min holding times. (C) b* value of Indian mackerel samples after HPP treatments at 200, 400 and 600 MPa for 5- and 20-min holding times. (D) Chroma value of Indian mackerel samples after HPP treatments at 200, 400, and 600 MPa for 5- and 20-min holding times. (E) WI of Indian mackerel samples after HPP treatments at 200, 400, and 600 MPa for 5- and 20-min holding times. Data are means ± S.D. ($n = 3$). "*" shows a significant ($p < 0.05$) difference between each treatment to the control. "#" shows a significant ($p < 0.05$) difference between different treatments. Results were obtained from two-way ANOVA followed by Tukey's *post hoc* test.

similar to the effect of HPP on sea bass (Tsai et al., 2022), barramundi (Nath, Pandiselvam & Sunil, 2023), and tilapia (Suemitsu & Cristianini, 2019). WI value increase in treated Indian mackerel can be attributed to the unfolding of proteins that make up pigmentations found in the fishes, altering characteristic of sample surface which creates a whiter appearance (Kung et al., 2022).

In fish, the lightness and whiteness of fish fillets are indicators showing the freshness of fish fillets (Tsai et al., 2022). For Indian mackerel fillet, the effect of pressure and holding time on samples' L* value and whiteness index value followed a direct relationship, *i.e.*, the higher the pressure and holding time used, the higher the L* value and whiteness index obtained causing the fillets to have a more "cooked" appearance. The same can be seen in other pressure-treated fish where the increase in pressure level and holding time resulted in a more cooked appearance (Cartagena, Puértolas & Martínez de Marañón, 2020).

3.3. Soluble protein content, protein profile and β-parvalbumin

Protein solubility is an important function that gives rise to food properties such as gelation, emulsification and foaming, which affects food application (Bessada, Barreira & Oliveira, 2019). This study investigated the effects of different HPP treatments on Indian mackerel protein solubility. The effect of HPP pressure level on the soluble protein content of Indian mackerel fillets was dependent on the holding time (Fig. 4). Indian mackerel samples treated at 400 and 600 MPa for both holding times (5- and 20-minutes) showed a significant ($p < 0.05$) decrease in protein content compared to untreated Indian mackerel samples. At a holding time of 5 min, treatment at 600 MPa showed a significant ($p < 0.05$) decrease in protein content compared to treatment at 200 MPa. At a holding time of 20 min, higher pressure caused a significant ($p < 0.05$) decrease in protein content. Increasing the pressure intensity and holding time caused a decreasing trend of soluble protein content in Indian mackerel. The same results can be seen in pressure-treated haddock and mackerel (Cropotova et al., 2020). HPP causes a reduction in protein solubility of these fishes due to the formation of insoluble aggregate (Cropotova et al., 2020; Aubourg, 2018).

SDS-PAGE is a rapid approach that shows information on the changes in protein profiles after food processing (Xue et al., 2020). Thus, this study employed SDS-PAGE to visualise changes in the soluble protein profile of Indian mackerel following different HPP treatments. A protein profile comparison of raw and pressure-treated Indian mackerel in Fig. 4 showed a reduction in protein bands appearing in pressure-treated samples than the raw sample. Furthermore, as the pressure and holding time of HPP increased, the bands in > 37 kDa region also decreased. Similar changes can be seen in European seabass whereby, the protein profile revealed a reduction of protein > 30 kDa following HPP at 600 MPa for 5 min (Prego et al., 2021). The reduction in high molecular weight protein bands was attributed to loss of solubility, aggregation, and denaturation of protein due to HPP treatment (Tsironi et al., 2019). Protein profile studies reveal modifications in the sarcoplasmic proteins of fish which can impact their quality parameters *i.e.* texture and colour (Munekata et al., 2021).

Antigenicity expresses the ability of an antigen to bind to T-cell receptors or antibodies (Jiang & Rao, 2021). Changes in the antigenicity of an allergen indicate modifications in the allergen (Jiang & Rao, 2021). In this study, HPP-treated and untreated Indian mackerel proteins were subjected to immunoblotting using the monoclonal anti-parvalbumin antibody, PARV-19 to investigate the antigenicity of Indian mackerel β-parvalbumin. As can be seen in Fig. 5, β-parvalbumin can be detected in all the samples (treated and raw) regardless of pressure intensity and holding time. Following HPP treatment, β-parvalbumin band intensity ($p < 0.05$) reduced when treated at the pressure level of 600 MPa for 5- and 20-minute holding times. The decrease in β-parvalbumin following HPP treatment can also be seen in fish such as cod (Y. Zhang et al., 2019). HHP treatments affect non-covalent bonds due to their compressibility (Chizoba Ekezie, Cheng & Sun, 2018). The secondary and tertiary structures of proteins are mainly linked by salt bridges, hydrogen bonds, and metal ion bindings which are susceptible to denaturation in HPP, resulting in protein unfolding thus loss of the spatial arrangement of its amino acids known as conformational epitopes that antibodies recognise and bind to. (Khan et al., 2019; Chizoba Ekezie, Cheng & Sun, 2018). As the antigenicity of proteins is dependent on their tertiary structure, high

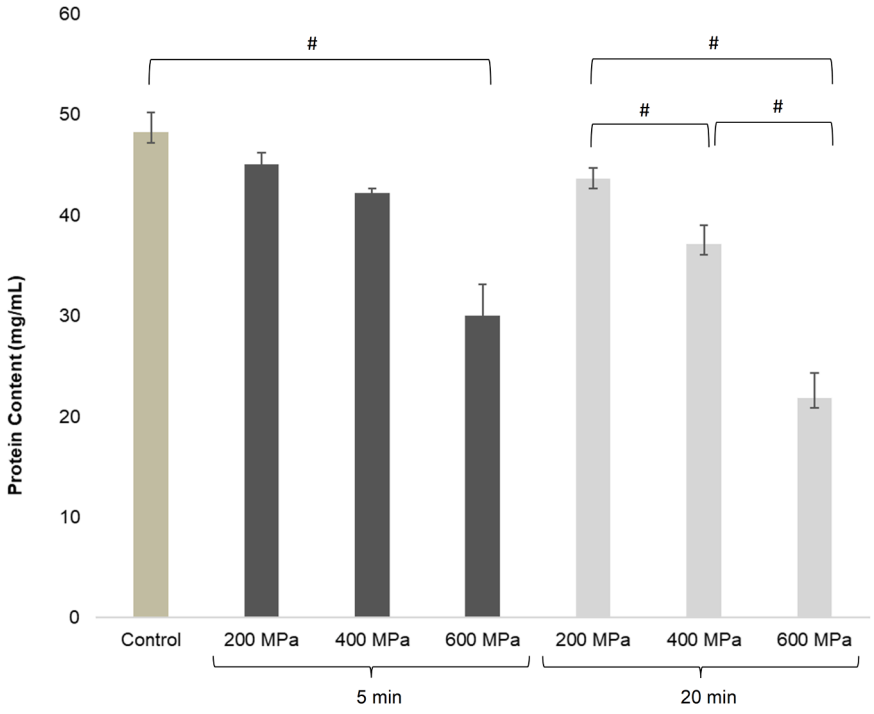

Fig. 4. Protein content from extracted soluble-protein fraction following HPP treatments at 200, 400, and 600 MPa for 5- and 20-min holding time. Data are means ± *S*.D. ($n = 3$). "*" shows a significant difference between each treatment and the control. "#" shows a significant difference between different treatments. Results obtained from two-way ANOVA followed by Tukey's *post hoc* test.

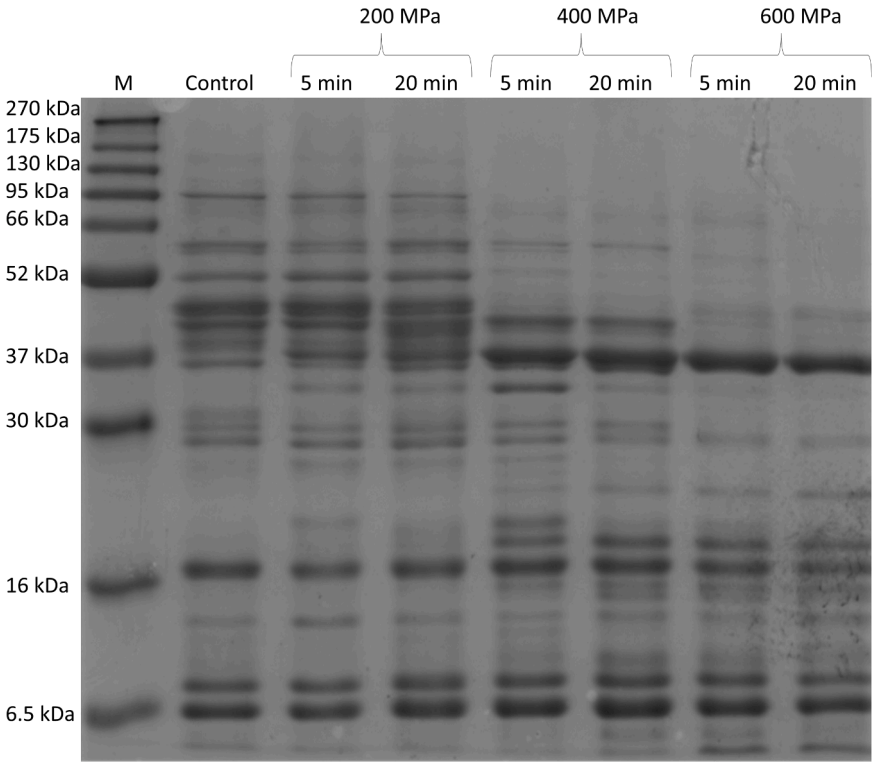

Fig. 5. SDS-PAGE protein profiles of the water-soluble protein fraction of raw Indian mackerel as a control and treated mackerel at 200, 400 and 600 MPa for 5 min and 20 min respectively. Each lane was loaded with 0.5mg/mL of protein and run on 15 % polyacrylamide under denaturing conditions.

pressure in HPP is able to reduce their antibody-binding capacity by destroying conformational epitopes (Lavilla, Puértolas & Orcajo, 2020). Changes in the stability of the secondary structure of β-parvalbumin too have been proven to reduce their antigenicity (Zhang et al., 2020). However, in fish such as horse mackerel and European sea bass, β-parvalbumin abundance increased following HPP treatment as HPP causes lysosome disruption and therefore increases the extractability of proteins (Pazos, Méndez, Vázquez & Aubourg, 2015; W. Fisher & Bianchi, 1984).

3.4. *In vitro digestibility of indian mackerel proteins and β-parvalbumin*

In vitro digestion provides an indication of the digestibility of protein, expressing the bioavailability of protein (Liu, Lin & Sun, 2022). Various food processing methods have been found to alter protein digestibility, but few have investigated the effect of HPP on fish protein and allergen digestibility. This study is the first to investigate the digestibility of Indian mackerel proteins and β-parvalbumin, an allergen, post-HPP treatment. In our study, *in vitro* digestion was performed on untreated (control) and 600 MPa treated fish fillets for both holding times (5- and 20-minutes) as β-parvalbumin showed the most significant change compared to the control, as can be seen in Fig. 6. Furthermore, from previous studies on cod, pressure treatment of 600 MPa resulted in an improvement in the seafood protein digestibility (Y. Zhang et al., 2019). The digestion of Indian mackerel proteins can be seen within 15 min (Fig. 7), as the number of bands drastically reduced throughout the digestion period for both soluble and insoluble protein extracts. There is no obvious distinction in the protein profile between the digestibility of raw and treated samples. A study comparing different food processing methods on cod showed that HPP improved the cod protein digestibility better than other food processing methods (Y. Zhang et al., 2019).

One of the notable characteristics of food allergens are their resistance to gastrointestinal digestibility which allows them to retain their epitopes to bind with antibodies thus triggering allergic response (Pali-Schöll, Untersmayr, Klems & Jensen-Jarolim, 2018). After ingestion, the potential of allergic response in sensitized patients is dependent on sufficiently intact allergens that reach the gut system. The level of sufficient allergen that reaches the gut system is dependent on the abundance of the allergen, its stability to digestive enzymes, and food processing methods (Akkerdaas et al., 2022).

In this study, β-parvalbumin was detected through immunoblotting with monoclonal anti-parvalbumin antibody after digestion. β-parvalbumin can be detected up to 270 min of *in vitro* digestion in the soluble untreated samples (Fig. 8). However, in HPP-treated samples, β-parvalbumin cannot be detected after 15 min of digestion. The same trend can be seen in gilted seabream and European seabass, where β-parvalbumin decreased in its detectability throughout *in vitro* digestion (Schrama et al., 2022). Improvement in β-parvalbumin digestibility after HPP treatment can be attributed to the influence of pressure on the secondary structure of β-parvalbumin (Cepero-Betancourt et al., 2020). While the mechanism of how HPP attenuates the allergenicity of allergens such as β-parvalbumin is not well understood, it is known that HPP treatment can modify hydrophobic and weak hydrogen bonds of the multimeric proteins irreversibly despite not being able to break covalent bonds of the proteins, causes protein denaturation (Pazos et al., 2015). Denaturation of allergens by HPP has been shown to increase the allergens' susceptibility to enzymatic degradation (Lavilla, Puértolas & Orcajo, 2020) and potentially break down linear epitopes of the allergens, effectively reducing antigenicity and immunoreactivity of allergens, as demonstrated previously on HPP-treated cod (Y. Zhang et al., 2019). It is worth noting that antigenicity, however, only describes the capacity of antibodies to bind to a specific allergen (Jiang & Rao, 2021). While it is useful in detecting and measuring the level of target allergens in food, further analysis is needed to determine changes in the allergenicity of β-parvalbumin upon HPP treatment by performing basophil activation test (BAT) assays using IgE antibodies from fish-allergic patients' sera specific for β-parvalbumin. Previous research on the effect of HPP in cod, for example, indicates that disruptions in IgG-binding epitopes often correlate with IgE reactivity changes (Y. Zhang et al., 2019), but this relationship requires further verification for Indian mackerel

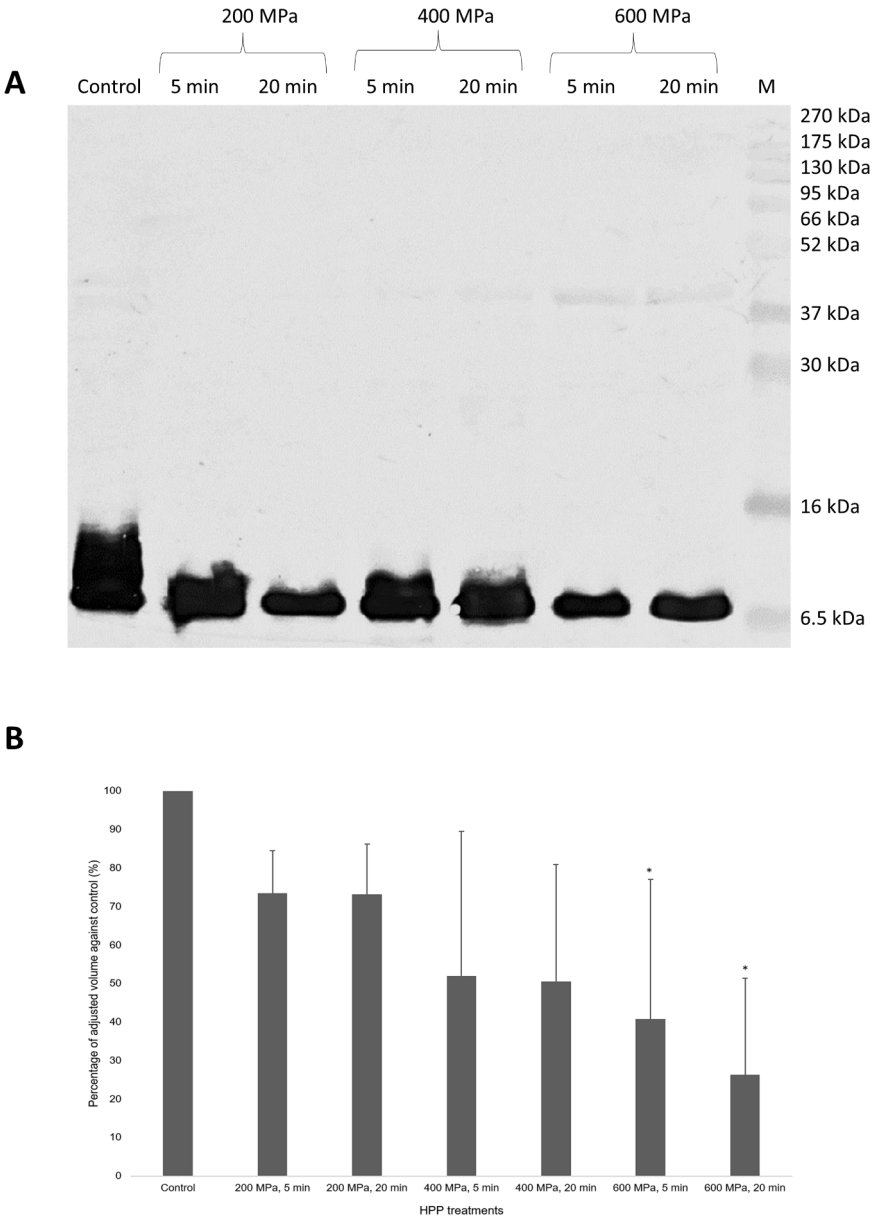

Fig. 6. (A) Representative immunoblot of water-soluble protein fractions detecting the presence of β-parvalbumin from the untreated Indian mackerel samples as a control and treated at 200 MPa for 5 min (Lane 2), 200 MPa for 20 min (Lane 3), 400 MPa for 5 min (Lane 4) 400 MPa for 20 min (Lane 5), 600 MPa for 5 min (Lane 6), 600 MPa for 20 min (Lane 7). (B) Densitometry analysis of the percentage of adjusted band volume against control. Data are means of Western blots ± *S*.D. ($n = 3$). "*" shows a significant ($p < 0.05$) difference between each treatment to the control.

parvalbumin. The weakened IgG-binding to beta-parvalbumin upon HPP treatment observed in Western blots indicates changes in the epitope structure of the parvalbumin, but specific conformational modifications as a result of HPP treatment on Indian mackerel β-parvalbumin should also be investigated in future work to understand the mechanism further by which HPP treatments modify Indian mackerel protein(s). It is also important to note that allergic reactions to fish proteins can also occur through other non-digestive routes, such as oral mucosa contact or inhalation of food particles during preparation (Jiang, Xiang, Huang & Hou, 2024) While this study specifically evaluates the effects of high-pressure processing (HPP) on post-digestion parvalbumin (PV) antigenicity, we acknowledge this as a limitation, as pre-digestive exposure routes remain an important area for future investigation.

3.5. *Effects of HPP on the protein profiles*

Proteomic analysis with SDS-PAGE and mass spectrometric analysis tools is widely utilised to identify changes in protein constituents (Nair & Zhai, 2019). Changes in specific protein biomarkers give rise to different textural and physical characteristics of processed food (Purslow, Gagaoua & Warner, 2021). As of now, no study has been found on the effects of HPP on the insoluble protein fraction of fish meat. Furthermore, this is the first study that investigates the effects of HPP on both soluble and insoluble protein fractions of the Indian mackerel. This study employs proteomic analysis to identify changes in the protein of the HPP-treated Indian mackerel samples (Fig. 9 and Table 1).

The influence of HPP treatments on protein abundance highly depends on the type of proteins. Two of the spots that were identified in MALDI-TOF mass spectrometry analysis as lysyl-endopeptidase in both the soluble and insoluble fractions are from *Pseudomonas aeruginosa. P. aeruginosa* is one of the most common pathogens that can be found in

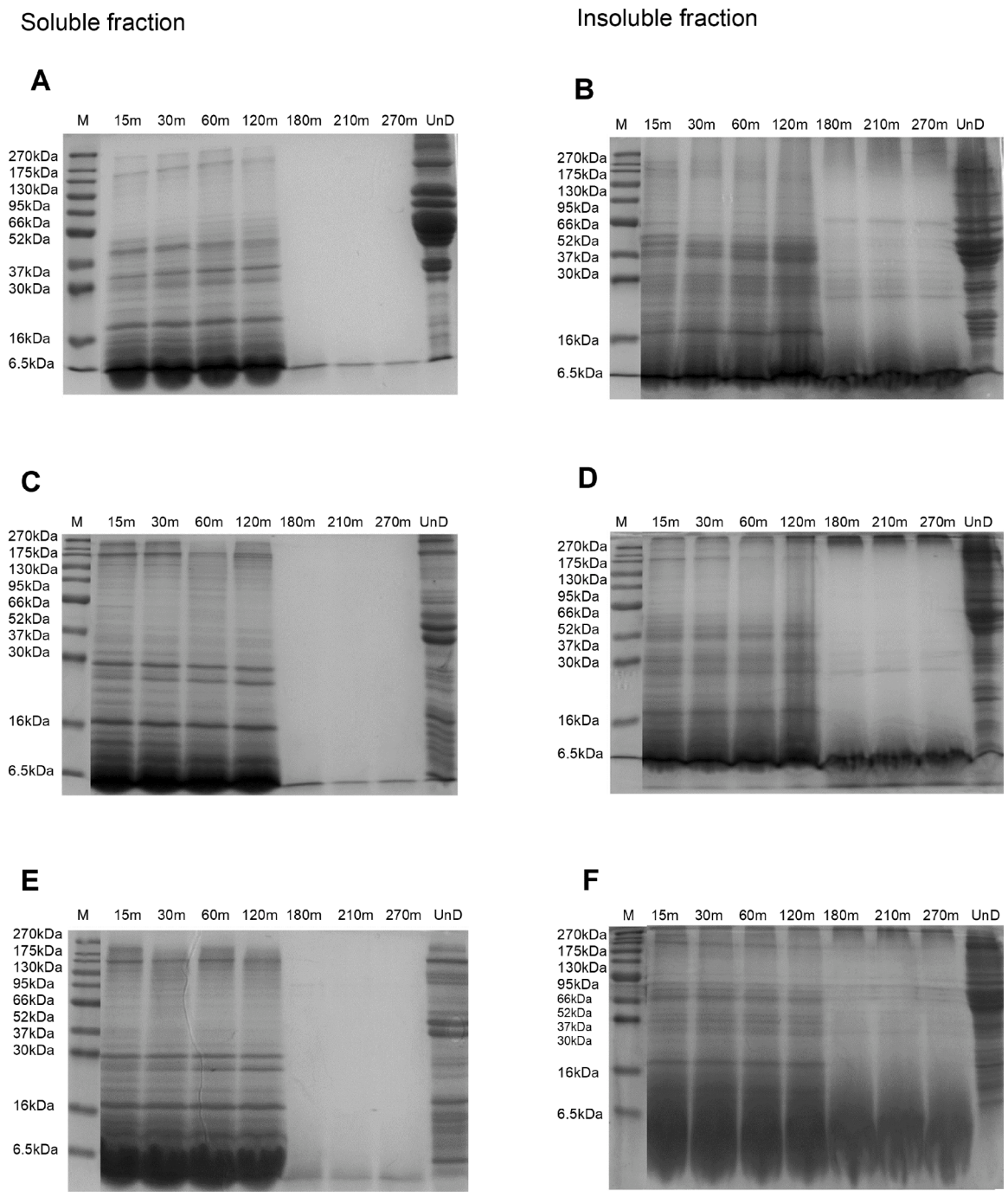

Fig. 7. SDS-PAGE on denaturing 15 % polyacrylamide gel of soluble (A, C, and E) and insoluble (B, D, and F) digested protein fractions from the untreated Indian mackerel and treated samples at 600 MPa for 5 min and 20 min. Untreated samples (A and B), 600 MPa treated for 5 min (C and D), 600 MPa treated for 20 min (E and F). (*M* = marker, UnD = Undigested sample).

fish and is an indication of low water quality (Algammal et al., 2020; Duman et al., 2021). ATP-synthase was identified in both the soluble and insoluble protein fractions in both untreated and HPP-treated samples. Furthermore, ATP-synthase from both the soluble and insoluble fractions showed a significant ($p < 0.05$) reduction in abundance after HPP treatment. No studies have been found investigating the effects of HPP treatments on fish ATP-synthase. However, a study on long-term frozen storage of puffer fish showed that the prolonged freeze treatment caused an abnormality in the mitochondrial function, thus affecting ATP synthesising proteins (Men et al., 2020).

From the soluble fraction, allergenic proteins identified from fish are tropomyosin and β-parvalbumin. Both tropomyosin and β-parvalbumin are major allergens in fish, as the majority of fish-allergic patients are triggered by one or both of these proteins (Ruethers et al., 2021). These allergens showed a significant increase in abundance after HPP treatment at a pressure of 600 MPa for 5 min compared to the untreated samples. In this study, however, the β-parvalbumin from the MALDI-TOF/TOF MS analysis showed an increase in abundance, yet the antigenicity of β-parvalbumin in 1D-immunoblot showed a reduction. The protein spot that had been analysed through MALDI-TOF/TOF is not the main spot of β-parvalbumin, as can be seen in the 2D -immunoblot (Fig. 10). This protein spot is a known isomer of the β-parvalbumin (Ruethers et al., 2018). Therefore, HPP was found to increase the abundance of this specific β-parvalbumin isomer. The effect of HPP on

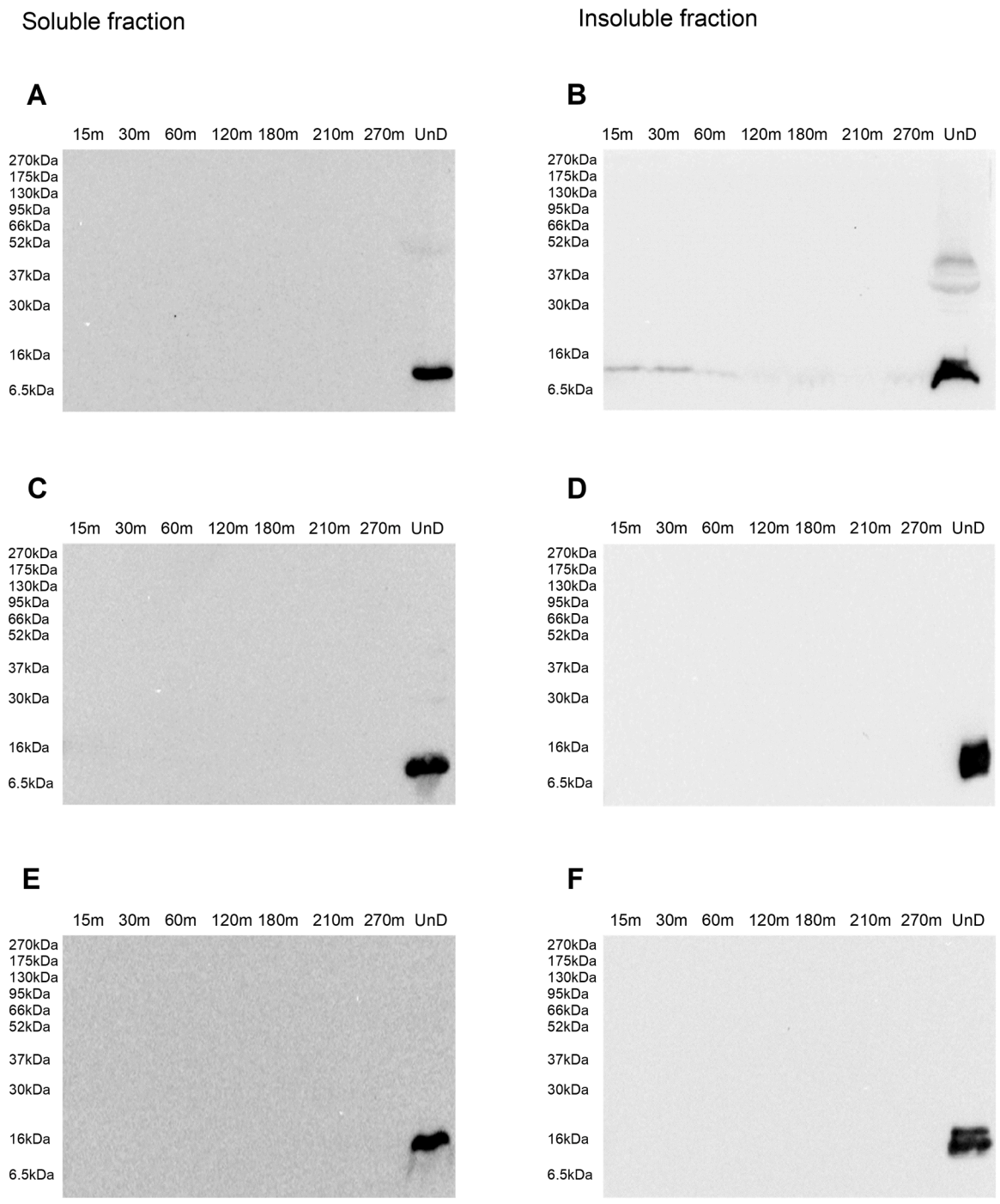

Fig. 8. Immunoblot of soluble (A, C, and E) and insoluble (B, D, and F) digested protein fractions from the untreated Indian mackerel and treated samples at 600 MPa for 5 min and 20 min. Untreated samples (A and B), 600 MPa treated samples for 5 min (C and D), 600 MPa treated samples for 20 min (E and F) (*M* = marker, UnD = Undigested sample) and analysed with PARV-19 against goat anti-mouse horse radish peroxidase (HRP)-conjugated IgG.

β-parvalbumin can cause the protein to increase in extractability which in turn increases the protein abundance (Carrera, Fidalgo, Saraiva & Aubourg, 2018).

From the insoluble fraction, a protein identified from the fish sample as actin showed a decrease in abundance when pressure treated at 600 MPa for 5 min compared to the untreated sample. The same effect can be observed in mud carp, where treatment at a pressure of 300 MPa for 15 min and above caused a significant reduction in actin (Liu et al., 2022). Actin is one of the major myofibrillar proteins that contribute to the structural integrity of meat (Liu et al., 2022). HPP treatment causes the unfolding of fish actin, which leads to changes in the textural attributes of fish samples and affects the quality of the fish fillets (Liu et al., 2022).

The successful identification of proteins using MALDI-TOF/TOF MS depends on having a database containing the spectra of known proteins (Stahl & Schröder, 2017). In this study, seven proteins out of 78 protein spots were significantly identified by MALDI-TOF/TOF MS. Currently, there is a lack of proteome database for Indian mackerel, which hinders the comprehensive identification of proteins in this study (Surachat et al., 2022).

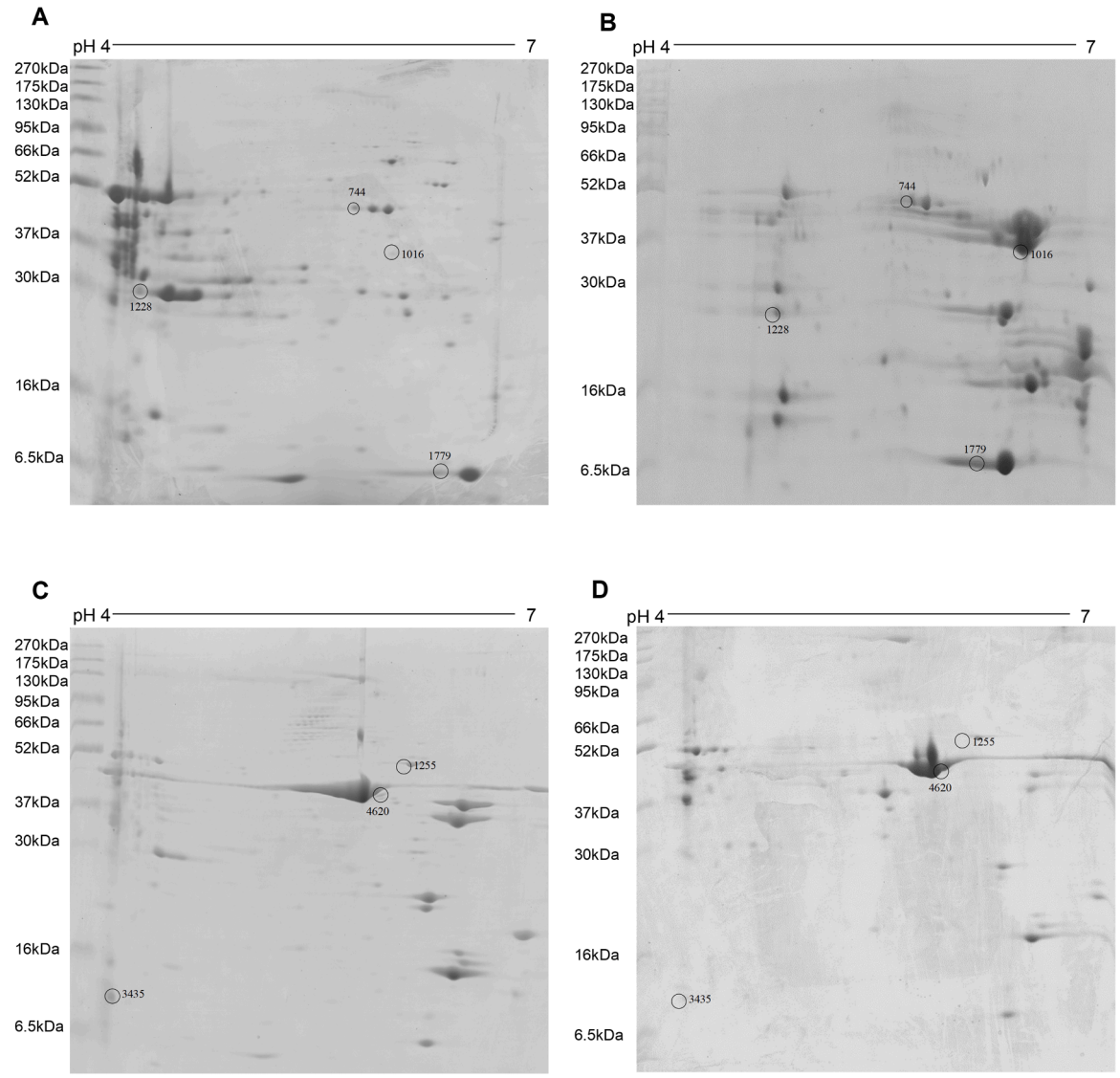

Fig. 9. Representative 2-DE gels of soluble protein fraction obtained from untreated Indian mackerel muscle sample. Protein extracts (400 μg) were resolved on a 12 % bis-acrylamide gel after being run on a 13 cm pH 4–7 IPG strip. Untreated samples (A and C), samples treated at 600 MPa for 5 min (B and D). (A) and (B) are soluble protein fractions whereas (C) and (D) are insoluble protein fractions. Protein spots identified through MALDI-TOF/TOF MS are marked and numbered.

4. Conclusion

As the pressure and holding time of HPP increased, Indian mackerel developed a more cooked appearance and firmer texture, attributed to structural changes in the proteins. HPP also led to a reduction in soluble protein content with increasing pressure and holding time. Despite these changes, β-parvalbumin remained detected in all samples, both treated and raw, regardless of the HPP conditions, albeit significantly lower β-parvalbumin band intensity ($p < 0.05$) observed when treated at the pressure level of 600 MPa for 5- and 20-minute holding times. Our findings also suggest that HPP enhances the digestibility of β-parvalbumin, as demonstrated by a reduction in antigenicity during *in vitro* digestion in both the SGF and SIF phases, particularly in the insoluble protein fractions from treatments at 600 MPa for 5 and 20 min, compared to the raw samples. From the MALDI-TOF/TOF analysis, seven protein spots were successfully identified ($p < 0.05$, 2-fold increase), including two major fish allergens, β-parvalbumin and tropomyosin, as well as actin, which is associated with the textural properties of the meat. Additionally, lysyl-endopeptidase from *Pseudomonas aeruginosa* and ATP-synthase from fish mitochondria were identified. The potential of HPP to produce hypoallergenic fish products warrants further exploration, particularly on other allergens such as β-enolase, collagen, tropomyosin, and aldolase A, which were not addressed in this study while also looking into the sensorial effects that may affect the quality of the fish.

Ethical statement

This study does not involve the use of live animal or human subjects. Therefore, ethics approval is not required.

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CRediT authorship contribution statement

Marcella Meia Gary Enchangan: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Muhammad Syamil Seman:** Methodology, Investigation, Formal analysis, Data curation. **Jameel R. Al-Obaidi:** Writing

Table 1
Identified proteins contained in the soluble and insoluble fraction that were significantly ($p \leq 0.05$) altered with max fold change of ≥ 2 due to HPP at 600 MPa for 5 min.

Spot ID	Protein name	Species	Accession number	Score	Theoretical pI	Mass (Da)	Mw theoretical (kDa)	Matches	Average normalised volumes	
									Untreated sample	**Treated sample (600 MPa, 5 m)**
	Soluble Fraction									
744	ATP synthase subunit beta, mitochondrial	*Cyprinus carpio*	Q9PTY0	87	6.05	55,327	56	3	7.867e+005	3.778e+005
1016	Tropomyosin alpha-1 chain	*Liza aurata*	P13104	55	6.41	32,760	39	3	2.870e+006	2.097e+007
1228	Lysyl-endopeptidase	*Pseudomonas aeruginosa*	Q9HWK6	72	4.63	48,547	29	2	5.31E+06	3.846e+005
1779	β-parvalbumin	*Scomber japonicus*	P59747	79	6.08	11,652	7	7	4.30E+06	8.69E+06
	Insoluble Fraction									
1255	ATP synthase subunit beta, mitochondrial	*Cyprinus carpio*	Q9PTY0	84	6.1	55,327	50	4	9.879e+005	2.904e+005
4620	Lysyl-endopeptidase	*Pseudomonas aeruginosa*	Q9HWK6	63	6.01	48,547	42	2	7.644e+004	2.684e+004
3435	Actin, alpha cardiac muscle	*Cyprinus carpio*	P53480	51	4.11	42,290	14	2	5.279e+005	1.971e+005

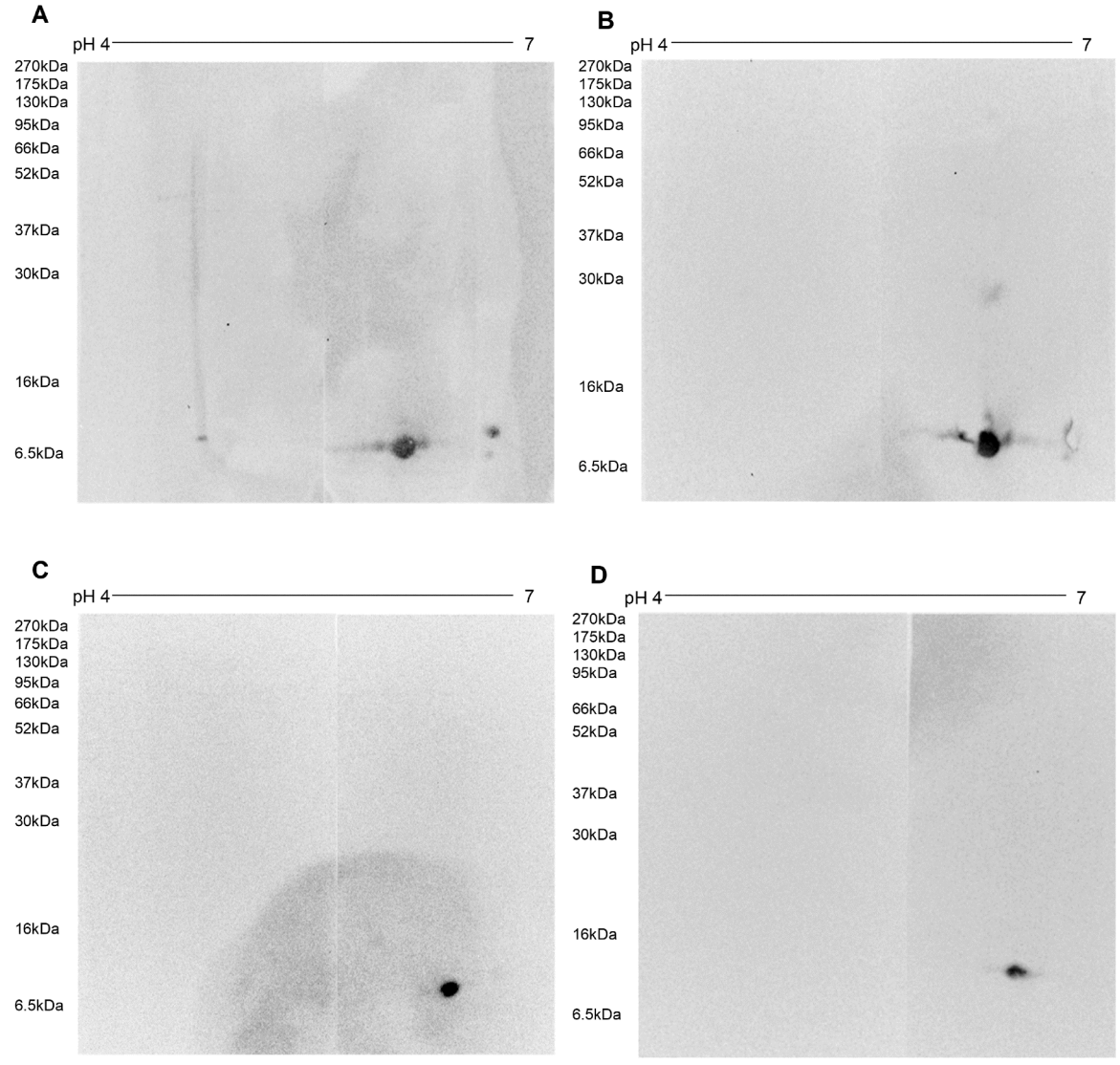

Fig. 10. 2D-Immunoblot of soluble and insoluble protein fraction obtained from Indian mackerel muscle: untreated (A and C) and treated at 600 MPa, 5 min (B and D). (A) Soluble untreated, (B) Soluble 600 MPa treated for 5 min, (C) Insoluble untreated (c), and (D) 600 MPa treated for 5 min.

– review & editing, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Atiqah Farah Zakaria:** Supervision, Resources. **Ahmad Faizal Abdull Razis:** Resources. **Nazamid Saari:** Supervision, Resources. **Hanis Hazeera Harith:** Resources. **Nuzul Noorahya Jambari:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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