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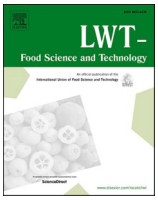

Impact of raw material and enzyme type on the physico-chemical and functional properties of fish by-products hydrolysates

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ABSTRACT

To examine the impact of raw material and enzyme type on the physico-chemical and functional properties of fish by-products hydrolysates, mackerel (*Scomber scombrus*) and sardine (*Sardina pilchardus*) by-products were the raw material, and papain, pepsin and Protamex™ were the protease enzymes. Hydrolysates' physico-chemical and functional properties were affected by the fish species used as raw material and the employed enzyme type. Mackerel produced hydrolysates with higher foaming properties and solubility at pH 10 than sardines. Hydrolysis, oil retention capacity, and turbidity were higher for the hydrolysates obtained from the sardine by-products. The Papain and Protamex™ hydrolysates exhibited similar functional properties, while significant differences were observed between these and the pepsin hydrolysates. Papain and Protamex™ displayed the highest degree of hydrolysis and relevant emulsifying properties. The Pepsin hydrolysates had good foaming and oil retention capacities. These results underline the need to consider fish species and hydrolysis enzymes when protein hydrolysates and peptides with specific functions are desired.

1. Introduction

The world fish and seafood production in 2020 was around 200 million tonnes, and this quantity is expected to continue to grow in forthcoming years (FAO, 2022). Small pelagic species, including Atlantic mackerel (*Scomber scombrus*), and sardine (*Sardina pilchardus*), constitute 30% of the EU's fishery and aquaculture products (FAO, 2022). Both species are well appreciated for their high nutritional value, and after tuna, sardine, sardine-type, and mackerel are the main canned fish species consumed worldwide (Ferraro et al., 2013). Fish production leaves vast quantities of fish loss and waste in different value chain parts, such as harvesting, processing, distribution, or consumer households (Kruijssen et al., 2020). It is estimated that volumes as high as 130 million tonnes are annually wasted as a result of poor seafood resources management, which generates a value loss of up to 43 billion euros and poses serious environmental problems (Racioppo et al., 2021). While processing fish for human consumption, large amounts of these by-products are produced and come in the form of heads, viscera, frames, skins, tails, fins, mince, and blood. Depending on the species, fish by-products may constitute up to 70% of the total fish weight (European Commission, 2020). When not discarded, fish by-products are transformed into low-market-value products, such as fish meal, fish silage, or fertilizers (Coppola et al., 2021; FAO, 2021). Instead, they can be employed to obtain high-value products with nutritional and functional properties, such as collagen, chitin, enzymes, or protein hydrolysates (Coppola et al., 2021; Espinales et al., 2023).

Protein hydrolysates are small fractions of peptides and amino acids that have drawn considerable attention as a source of bioactive and functional compounds for food, pharmaceutical, agricultural or cosmetic applications (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012). Different studies have reported important technical-functional properties of fish protein hydrolysates, such as solubility, oil-binding capacity, emulsifying, film-forming, gel-forming, and foam capabilities (Kvangarsnes et al., 2023; Noman et al., 2018). Due to its high protein content, fish waste has been successfully used to obtain peptides with these and other functional capacities (Henriques et al., 2021; Naghdi, Rezaei, Tabarsa, & Abdollahi, 2023a; Zamorano-Apodaca et al., 2020; Zhang, Li, Hong & Luo, 2020). Functionally active peptides can be produced by different methods, such as solvent extraction, microbial fermentation, and enzymatic hydrolysis (Najafian & Babji, 2012). The extraction method largely influences the functionality of protein hydrolysates, which is determined by the degree of hydrolysis and the molecular weight of the obtained peptides (Espinales et al., 2023). Enzymatic hydrolysis is the preferred method for producing functional peptides because solvent or toxic residue is absent in the

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Abbreviations

DH	Degree of hydrolysis
DLS	Dynamic light scattering
EAI	Emulsifying activity index
ESI	Emulsion stability index
FC	Foaming capacity
FS	Foam stability
MA	Mackerel by-products hydrolysed by papain
ME	Mackerel by-products hydrolysed by pepsin
MR	Mackerel by-products hydrolysed by Protamex
ORC	Oil retention capacity
PCA	Principal components analysis
SA	Sardine by-products hydrolysed by papain
SE	Sardine by-products hydrolysed by pepsin
SDS	Sodium dodecyl sulfate
SR	Sardine by-products hydrolysed by Protamex
TNBS	2,4,6-trinitrobenzene sulphonic acid
ZP	Zeta Potential

final product (Najafian & Babji, 2012). This method consists of using proteolytic enzymes to break down proteins into amino acids and polypeptides. The final product's characteristics depend on different factors, including the employed enzyme and the protein precursor type (Akbarian, Khani, Eghbalpour, & Uversky, 2022; Naghdi, Rezaei, Tabarsa, & Abdollahi, 2023b).

Enzyme origin plays a crucial role in determining the specificity of proteolytic enzymes during the hydrolysis process and influencing the properties of the hydrolysate (Siddik, Howieson, Fotedar, & Partridge, 2021). Enzymes derived from animal sources tend to be more specific in their site of action than plant enzymes, while microbial proteases show a higher heterogeneity. For instance, pepsin, the main protease from human gastric juice, cleaves at the phenylalanine or leucine bond, whereas papain is less specific, cleaving bonds at the hydrophobic regions including phenylalanine, arginine, and lysine, and Protamex™, a commercial blend of microbial endo-proteases, usually displays a wide specificity (Bing et al., 2024; Wang et al., 2021). Therefore, enzyme-substrate specificity is a crucial factor in determining the functional properties of protein hydrolysates. However, there is little information comparing the impact of specific proteases on the physicochemical and functional properties of different raw materials, such as sardine and mackerel by-products.

In this context, this study aimed to determine the influence of fish species and the enzyme used during the hydrolysis process on the physico-chemical and functional properties of fish by-products hydrolysates. For this purpose, Atlantic mackerel (*Scomber scombrus*) and sardine (*Sardina pilchardus*) by-products were employed as raw material, and three protease enzymes of different origins were used for the hydrolysis process: papain (vegetal), Protamex™ (bacterial) and pepsin (animal). The results of this work will help to elucidate the optimal enzyme and raw material to obtain peptides with relevant functional properties using by-products as a substrate.

2. Material and methods

2.1. Chemicals

All the chemicals used in the experiments were of analytical grade. Pepsin of porcine origin (EC.3.4.23.1), L-leucine, 2,4,6-trinitrobenzene sulfonic acid (TNBS), and sodium dodecyl sulfate (SDS) were acquired from Sigma-Aldrich Co. Ltd. (USA). Papain (EC.3.4.22.21) was obtained from CYGYC Biocon (Spain). Protamex™ (EC.3.4.21.62) was purchased from Novozymes (Denmark). Finally, HCl and NaOH were supplied by Scharlab (Spain).

2.2. Raw material

Atlantic mackerel (*Scomber scombrus*) and sardine (*Sardina pilchardus*) were purchased from a local market (Spain). Heads, bones, skin, fins, and viscera were separated, washed twice with water, and then stored inside sealed plastic bags (250 g) at −40 °C until used. Before the hydrolysis process, the bags containing the fish by-products were thawed overnight in a refrigerator at 4 °C.

2.3. Proximate composition of fish by-products

The fish by-products' moisture, lipid, protein, and ash contents were determined according to AOAC Methods 950.46, 991.36, 928.08, and 920.153, respectively (AOAC, 1997). The results were expressed as g per 100 g of sample.

2.4. Preparation of fish by-products hydrolysates

Fish by-products were minced and homogenized with distilled water at a 1:1 (w/v) ratio in a food processor (Thermomix, Germany) to produce uniform suspensions. The mixture was heated in a boiling water bath for 20 min to inactivate endogenous enzymes. Next samples were pre-incubated under the optimal pH and temperature conditions for each enzyme: papain (pH 6, 70 °C), pepsin (pH 2.5, 37 °C), and Protamex™ (pH 7, 50 °C). Then, protein substrates were enzymatically hydrolysed for 6 h by adding papain, pepsin, or Protamex™ (1.5 g per 100 g of sample) with stirring (600 rpm). During the reaction, the mixture's pH was periodically adjusted by adding NaOH. At the end of the reaction time, enzymes were inactivated by heating samples at 95 °C for 20 min. Hydrolysates were centrifuged at 8,000*g* and 4 °C for 20 min and supernatants were paper-filtered to remove impurities. Finally, the hydrolysate solutions were stored at −40 °C overnight and then freeze-dried (LyoQuest-55, Telstar, Spain) for 48 h. Six hydrolysates were obtained: mackerel by-products hydrolysed by the action of papain (MA), pepsin (ME), or Protamex™ (MR); sardine by-products hydrolysed by papain (SA), pepsin (SE) or Protamex™ (SR). The freeze-dried powders were stored at −40 °C for further analysis.

2.5. Physico-chemical analysis of fish by-products hydrolysates

2.5.1. Turbidity, pH, and zeta potential

For the turbidity and pH determinations, 0.5 g of the hydrolysate sample was diluted in 5 mL of distilled water. The turbidity of solutions was measured by reading absorbance at a wavelength of 600 nm in a UV–visible spectrophotometer (Helios Zeta, Thermo Scientific, UK). pH was measured at 25 °C in a pH-meter Crison (Crison Instruments, Spain).

The zeta potential (ZP) was determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Before measurements, samples were dispersed in distilled water, sonicated for 2 min, and then measured at 25 °C. The ZP values were calculated from the electrophoretic mobility measurements using the Smoluchowski model.

2.5.2. Degree of hydrolysis

The degree of hydrolysis (DH) was quantified by the determination of the free amino groups released during the hydrolysis following the TNBS method. The applied TNBS method was described by Gallego, Arnal, Barat, and Talens (2020). Briefly, 40 μL of a sample, 320 μL of sodium phosphate buffer (0.2 M, pH 8.2), and 320 μL of TNBS solution were mixed and heated at 50 °C for 60 min. Next 640 μL of HC1 (0.1 M) was added and the mixture was incubated at room temperature for 30 min. Finally, absorbance was read at 340 nm. Samples' complete hydrolysis was carried out by dissolving hydrolysates with 6 M HCl at 110 °C for 24 h. The DH values were calculated according to Eq. (1) and

the results were expressed as mg L-leucine equivalents per g sample:

$$DH = \frac{H_t\text{-}H_0}{H_c\text{-}H_0} \quad (1)$$

where H_t is the concentration of the free amino groups in hydrolysates, H_0 is the concentration of the free amino groups before hydrolysis and H_c is the content of the free amino groups after samples' complete hydrolysis.

2.6. Functional properties of fish by-products hydrolysates

2.6.1. Solubility

Hydrolysates' solubility was determined at different pH values. Briefly, 0.5 g of hydrolysate sample was dispersed in 5 mL of deionized water and the pH of the solution was adjusted to 4, 7, and 10 with 6 M HCl or 6 M NaOH. The mixture was stirred at room temperature for 30 min and then centrifuged at 7,500*g* for 15 min. Finally, the supernatant was collected and protein content was quantified by the Kjeldahl method. Protein solubility was calculated using Eq. (2) and expressed as a percentage (%) of water-soluble protein in 100 g protein.

$$\text{Solubility (\%)} = \frac{\text{Protein content in the supernatant}}{\text{Total protein content in the sample}} \times 100 \quad (2)$$

2.6.2. Oil retention capacity

The oil retention capacity (ORC) analysis was carried out according to the method described by Fernández-López et al. (2009). In a falcon tube, 1 g of sample was mixed with 30 mL of sunflower oil, vortexed for 1 min, and stored at room temperature for 24 h. After centrifuging samples (3,000*g*, 20 min), the supernatant was discarded and the residue was weighed. ORC was expressed as g of oil retained in a g of sample.

2.6.3. Emulsifying properties

Hydrolysates' emulsifying capacity was quantified by two different parameters: the emulsifying activity index (EAI) and the emulsion stability index (ESI). The EAI refers to the volume of oil (mL) that can be emulsified per gram of protein hydrolysate, while the ESI measures the emulsion's capacity to resist changes in its properties over time. Both parameters were determined following the method described by Noman et al. (2018) with modifications. Approximately 0.2 g of sample was mixed with 45 mL of deionized water and 15 mL of sunflower oil. The mixture was homogenized using an Ultra-Turrax T25 (IKA Labortechnik, Germany) at maximum speed for 1 min. Next 50 μL from the bottom of the formed emulsion were collected and mixed with 2 mL of SDS solution. The mixture's absorbance was immediately measured at 500 nm (A_0) or 10 min after emulsion formation (A_{10}). Both the EAI (m^2/g) and ESI (min) were calculated according to Eq. (3) and Eq. (4), respectively.

$$EAI = \frac{2 \times 2.303 \times A_0}{0.25 \times \text{protein weight}} \quad (3)$$

$$ESI = A_0 \times \frac{A_{10}}{A_0\text{-}A_{10}} \quad (4)$$

2.6.4. Foaming properties

Foaming capacity (FC) and foam stability (FS) were measured according to the methods described by Noman et al. (2018) with some modifications. Approximately 0.5 g of hydrolysate was mixed with 30 mL of deionized water (V_0) in a 100 mL volumetric cylinder. The mixture was homogenized using an Ultra Turrax at maximum speed for 1 min. Then the foam volume was immediately measured (V_1) 30 min after the foam formed (V_2). FC and FS were calculated according to Eq. (5) and Eq. (6), respectively.

$$FC\ (\%) = \frac{(V_1\text{-}V_0)}{V_0} \times 100 \quad (5)$$

$$FS\ (\%) = \frac{(V_2\text{-}V_0)}{V_0} \times 100 \quad (6)$$

2.6.5. Statistical analysis

All the analyses were performed in triplicate. A statistical analysis was performed using Statgraphics Centurion XVIII (Statpoint Technologies, Inc., USA). A one-way analysis of variance (ANOVA) was carried out to test any significant differences in raw material's proximate composition. The effect of raw material and the enzyme on hydrolysates' physico-chemical and functional properties was determined by a multifactor ANOVA. The Tukey HSD (Tukey's Honest Significant Difference) procedure was used to test for differences between means at the 5% significance level. To study the relation between hydrolysates' physico-chemical and functional properties with raw material and enzyme type, a Principal Component Analysis (PCA) was run with the MATLAB® PLS Tool-box, 6.3 (Eigenvector Research Inc., USA).

3. Results and discussion

3.1. Proximate composition of fish by-products

Table 1 shows the proximate composition of the Atlantic mackerel and sardine by-products. Moisture content was significantly higher for the Atlantic mackerel than for the sardine by-products, and fat content was significantly higher for the latter than for the former. No significant differences were observed in the protein and ash content of both species by-products. The protein content was 16.3 g and 14.9 g for 100 g of the Atlantic mackerel and the sardine by-products, respectively. The high protein content found in both species' waste demonstrated the potential use of these by-products as a source of protein hydrolysates and functional peptides. The composition of fish by-products could be substantially affected by different factors, such as different amounts of lipids, blood, proteins, undigested feed in their stomach and intestines, or the percentage of the different fractions (heads, viscera, frames, etc.) (Kandyliari et al., 2020; Nikoo, Regenstein & Yasemi, 2023). Every by-product category has specific nutritional features: skin is considered the most significant protein source; trimmings and bones are high in calcium; head, intestines, and bones are a good source of lipids. In contrast, other authors have also reported similar results in these fish species' by-products composition (Dumay, Donnay-Moreno, Barnathan, Jaouen, & Bergé, 2006).

3.2. Physico-chemical analysis composition of fish by-products hydrolysates

Hydrolysate turbidity has been proposed as an indirect measurement of peptide aggregate formation (Groleau, Gauthier, & Pouliot, 2003), and a relevant parameter to consider hydrolysates to be an ingredient in food products (Barlow, 1993). The turbidity values of the hydrolysate samples are shown in Table 2. Turbidity was significantly affected by raw material and enzyme type. The sardine hydrolysate samples generally obtained higher absorbance values than the mackerel hydrolysates. For each raw material, papain produced hydrolysates with the highest turbidity. Conversely, pepsin resulted in hydrolysates with the

Table 1
Proximate composition of the fish by-products used as raw material for hydrolysates preparation.

Composition	Atlantic mackerel	Sardine
Moisture (%)	76.0 (0.6)[a]	72.4 (0.8)[b]
Protein (%)	16.3 (0.2)	14.9 (0.9)
Fat (%)	3.4 (0.5)[a]	5.8 (0.4)[b]
Ash (%)	4.1 (1.6)	5.5 (0.6)

Values are expressed as mean (SD) (n = 3). Different letters in the same row indicate significant differences ($p \leq 0.05$).

Table 2
Turbidity, zeta potential (ZP) and pH of fish by-product hydrolysates.

Raw material	Enzyme	Turbidity	ZP (mV)	pH
Mackerel	Papain	0.11 (0.02)^{aA}	−23.23 (1.87)^{aA}	6.21 (0.01)^{aA}
	Pepsin	0.03 (0.01)^{aB}	−4.11 (0.30)^{aB}	3.89 (0.01)^{aB}
	Protamex™	0.06 (0.00)^{aC}	−15.01 (0.71)^{aC}	6.77 (0.01)^{aC}
Sardine	Papain	0.16 (0.01)^{bA}	−18.10 (2.54)^{bA}	6.24 (0.01)^{bA}
	Pepsin	0.10 (0.01)^{bB}	−2.86 (0.26)^{bB}	3.63 (0.00)^{bB}
	Protamex™	0.13 (0.03)^{bC}	−12.52 (0.75)^{bC}	6.66 (0.04)^{bC}

Values are expressed as mean (SD) (n = 3). Different lower-case and upper-case letters, in the same column, indicate significant differences among hydrolysates for raw material and enzyme factors, respectively ($p < 0.05$).

lowest turbidity values from each by-product. Noman, Ali, AL-Bukhaiti, Mahdi, and Xia (2020) compared the physico-chemical characteristics of lyophilized Chinese sturgeon protein hydrolysates prepared with papain and alcalase 2.4L. These authors found that by increasing the concentration of protein, the turbidity of the papain hydrolysate significantly increased compared to that of alcalase hydrolysate. Moreover, the particle size distribution analysis of samples showed that alcalase 2.4L hydrolysate exhibited a smaller particle size (822.047 ± 61.26 nm) than papain hydrolysate (1425.39 ± 44.82 nm). These authors attributed the higher turbidity values obtained for papain hydrolysates to larger particle sizes.

ZP is an endpoint related to peptide size and aggregate formation. This parameter expresses the electrochemical equilibrium between molecules and the dispersing liquid medium (Lunardi, Gomes, Rocha, De Tommaso, & Patience, 2021), and it is often used as an indicator of dispersions and emulsions stability (Bhattacharjee, 2016). ZP values within or close to the instability range (from +30 to −30 mV) promote sedimentation and agglomerate formation due to van der Waals interparticle attraction, whereas extremely positive or negative ZP values produce large repulsive forces, and particles remain dispersed and suspended (Gupta & Trivedi, 2018). In this regard, enhanced turbidity and low ZP values have been related to the formation of protein aggregates due to van der Waals interparticle attraction (Bengoechea, Peinado & McClements, 2011; Ravindran, Williams, Ward, & Gillies, 2018). As Table 2 indicates, the ZP values of all the samples in the distilled water solution had a net negative charge and fell within the instability range. However, significant differences were observed in the ZP values depending on the raw material and enzyme used during the hydrolysis process of each by-products type. The mackerel and sardine by-products hydrolysed with papain obtained the lowest ZP values, followed by Protamex™ and pepsin. As previously mentioned, papain and Protamex™ show broad specificity for hydrophobic amino acids, while pepsin displays a higher specificity degree and only cleaves proteins at the phenylalanine and leucine bonds. Surface hydrophobicity is positively correlated with an absolute value of zeta potential and negatively correlated with particle size (Gupta & Trivedi, 2018). In our work, and independently of the employed raw material, the pepsin hydrolysates exhibited the lowest turbidity and absolute ZP values. In contrast, the papain samples resulted in higher turbidity and absolute ZP values. In this regard, the higher number of hydrophobic amino groups as a consequence of papain and Protamex™ cleavage, may be responsible for increased peptide aggregation, resulting in the higher turbidity values observed. These results suggest that larger particle sizes or peptide aggregation formations are related to the higher turbidity values observed for papain and Protamex™ han for pepsin samples. Regarding the raw material, the higher turbidity values found for the sardine than the mackerel hydrolysates may indicate that factors, such as fish species, or contaminant substances, may also play a role (Alfaro, Balbinot, Weber, Tonial, & Machado-Lunkes, 2015).

Hydrolysates' pH was also affected by the employed raw material and enzyme (Table 2). The mackerel by-products hydrolysed with Protamex™ obtained the highest pH values, followed by papain and pepsin. A similar trend was observed for the sardine hydrolysate samples, with the highest pH value for Protamex™, followed by papain, and finally by pepsin. The medium's pH is the most important factor in determining ZP values, and it becomes more positive and negative in magnitude with an acidic and a basic pH, respectively (Bhattacharjee, 2016). As observed in Table 2, the pepsin samples, which had less negative ZP values, also had the most acidic pH values for both the mackerel and sardine hydrolysates. The more negative ZP values samples presented more basic pH values.

Protein hydrolysis process efficiency was estimated by assessing the DH. This parameter is defined as the free amino group content quantified by the TNBS method. The DH obtained by the different samples ranged from 34.74% to 55.61% (Fig. 1). The higher DH values were for the sardine by-products hydrolysed with papain and Protamex™ with respective DH percentages of 55.39% and 55.61%. The lower DH values were for both the samples hydrolysed by pepsin as follows: ME (34.45%) and SE (34.74%).

Various authors have used different methods to compare the DH of fish by-products hydrolysates produced with various protease enzymes and have obtained varying results. Similarly to this work, Hou, Li, Zhao, Zhang, and Li (2011) did not find any differences between papain and Protamex™ when analyzing the DH of Alaska pollock frames hydrolyzates by the ninhydrin colorimetric method. In contrast, Tan, Chang, and Meng (2019), found that the proteases deriving from plant sources (papain, ficin, and bromelain) gave higher DH values than the proteases of bacterial origin (alcase, Protamex™, novo-pro D, and thermolysin) when studying the hydrolysis process of Channel catfish (*Ictalurus punctatus*) by-products with the TNBS method. Je, Qian, Byun, and Kim (2007) hydrolysed tuna backbone protein by different proteases, including papain and pepsin, and estimated the DH by measuring the nitrogen content soluble in 10% trichloroacetic acid. The proteolytic process with papain was conducted at pH 8 and 37 °C and gave around 50% DH. Pepsin hydrolysates were obtained at pH 2 and 37 °C as the proteolysis conditions, which resulted in 74.5% DH. However, our results were higher than those found by Liaset, Lied, and Espe (2000), who hydrolysed fish frames without heads from Atlantic cod and Atlantic salmon using pepsin at pH 2.5 and 37 °C for 120 min. No significant differences were found between both fish species, which yielded 15.6% DH and 14.5% DH, respectively, according to the TNBS method. All together, this information suggests that both the type of raw material and enzyme employed during the hydrolysis process can determine the physico-chemical properties and the DH of fish by-products

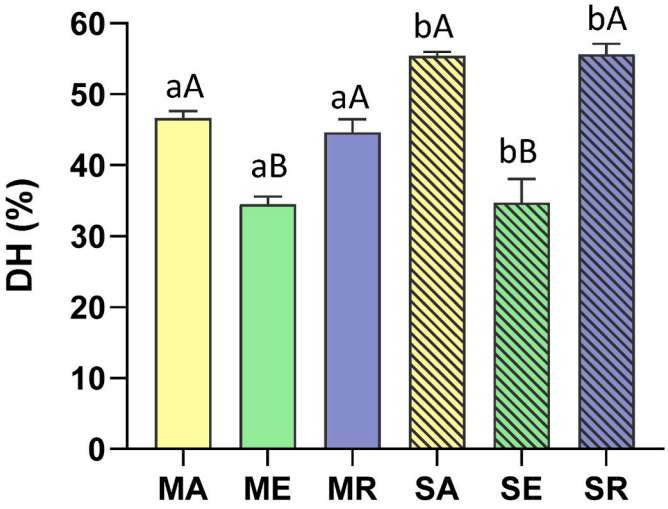

Fig. 1. Degree of hydrolysis (DH) of the mackerel and sardine by-products samples hydrolysed by Papain, Pepsin and Protamex™. Values are means ± SD (n = 3). Different lower-case and upper-case letters indicate significant differences among hydrolysates for raw material and enzyme factors, respectively ($p < 0.05$).

hydrolysates.

3.3. Functional properties of fish by-products hydrolysates

Protein functionality includes the ability of both peptides and proteins to dissolve under different pH conditions, absorb fat, form foam, or emulsify. All these properties are relevant for different applications in diverse food industry products, including soups, creams, beverages, and meat, or extruded, bakery and confectionary products (Aryee, Agyei, & Udenigwe, 2017). Of the different functionalities of protein hydrolysates, solubility is considered one of the most relevant because it influences a large number of other functional and bioactive properties, and determines their potential industrial applications (Kristinsson & Rasco, 2000). High solubility over a wide pH range is a useful characteristic for many food applications. Hydrolysates' increased solubility is due to the generation of low-molecular-weight peptides and the number of polar groups subsequently grows during the enzymatic hydrolysis process (Pacheco-Aguilar, Mazorra-Manzano & Ramíerz-Suárez, 2008). In this work, fish by-products hydrolysates' solubility was measured at pH 4, 7, and 10 (Table 3). Samples' solubility ranged from 65.22% to 86.05% at different pH values. However, differences were observed at the three studied pH levels. The lowest solubility for all the samples was observed at pH 10. The higher solubility of the samples hydrolysed by pepsin was observed at pH 4, while the papain and Protamex™ hydrolysates presented higher solubility values at pH 7. This may be attributed to differences in the isoelectric point of the peptides that constituted samples. As previously indicated, the pepsin hydrolysates obtained higher ZP values and a more acidic pH than the papain and Protamex™ samples. Surface hydrophobicity, which promotes aggregate formation, and net charge, which increases as pH moves away from the isoelectric point, may both be responsible for determining hydrolysates' solubility (González-Serrano et al., 2022).

The influence of raw material and enzyme type on ORC and the emulsifying and foaming properties of hydrolysates is shown in Table 4. ORC consists of the amount of oil absorbed per protein hydrolysate weight, which is a relevant property of the ingredients used in the meat and confectionary industries for fat and flavor retention and texture (Aryee et al., 2017). Different protein hydrolysates' characteristics, such as protein source, processing method, bulk protein density, hydrolyzing enzyme specificity, the DH, the size and concentration of peptides, the number of non-polar amino acids, and protein-lipid interactions, have been described to affect hydrolysates' ability to bind fat (Aryee et al., 2017; Pires & Batista, 2013). In this work, the highest ORC was for mackerel hydrolysed with pepsin, while the lowest values were for both the samples hydrolysed by Protamex™. Our results showed that both the employed raw material and enzyme affected ORC. Papain and pepsin are better capable of retaining oils than Protamex™, while better raw material performance depends on the employed enzyme. The mackerel samples gave better yields for retaining oil when the hydrolysis process was performed with pepsin and the sardine samples when hydrolysed with papain. The ORC of the protein hydrolysates correlated with surface hydrophobicity because a high content of non-polar or hydrophobic amino acids is essential for binding to the hydrocarbon chains of fats (Kristinsson & Rasco, 2000). The pepsin hydrolysates, which had the highest ORC, were also the closest ones to zero ZP values, a scenario that is indicative of more hydrophobic surfaces. The solubility and the DH of the protein hydrolysates affect ORC due to a smaller molecular size, which leads to reduced oil absorption (Noman et al., 2018).

Table 3
Protein solubility of fish by-product hydrolysates at different pH values.

Sample	Solubility (%)		
	pH 4	pH 7	pH 10
MA	72.85 (0.70)^{aA}	78.35 (1.31)^{aA}	70.21 (0.03)^{aA}
ME	86.05 (0.85)^{aB}	69.54 (1.05)^{aB}	65.22 (0.11)^{aB}
MR	76.44 (4.23)^{aA}	81.32 (0.24)^{aC}	69.39 (0.01)^{aA}
SA	79.19 (0.43)^{aA}	78.55 (0.46)^{aA}	67.47 (0.59)^{bA}
SE	81.95 (2.10)^{aB}	66.62 (0.16)^{aB}	65.13 (0.15)^{bB}
SR	77.70 (1.30)^{aA}	83.29 (1.42)^{aC}	66.62 (0.17)^{bA}

Values are expressed as mean (SD) (n = 3). Different lower-case and upper-case letters indicate significant differences among hydrolysates for raw material and enzyme factors, respectively ($p < 0.05$).

Table 4
Oil retention capacity (ORC), emulsifying activity index (EAI), emulsion stability index (ESI), foaming capacity (FC), and foam stability (FS) of fish by-product hydrolysates.

Sample	ORC (g_{oil}/g)	EAI (m^2/g)	ESI (mg/g)	FC (%)	FS (%)
MA	1.96 (0.00)^{aA}	2.12 (0.41)^{aA}	1.56 (0.31)^{aA}	5.00 (2.36)^{aA}	3.33 (4.71)^{aA}
ME	3.17 (0.04)^{aB}	0.22 (0.01)^{aB}	0.08 (0.05)^{aB}	20.00 (9.43)^{aB}	8.33 (2.36)^{aB}
MR	1.36 (0.12)^{aC}	1.15 (0.02)^{aC}	0.52 (0.13)^{aC}	23.33 (4.71)^{aC}	10.00 (0.00)^{aC}
SA	2.52 (0.11)^{bA}	1.20 (0.04)^{bA}	0.82 (0.02)^{aA}	3.33 (0.00)^{aA}	3.33 (0.00)^{bA}
SE	2.10 (0.13)^{bB}	0.23 (0.04)^{bB}	0.10 (0.00) ^{aB}	35.00 (2.36)^{aB}	3.33 (0.00)^{bB}
SR	1.29 (0.05)^{bC}	1.07 (0.10)^{bC}	0.77 (0.47) ^{aC}	5.83 (1.18)^{aC}	5.83 (1.18)^{bC}

Values are expressed as mean (SD) (n = 3). Different lower-case and upper-case letters, in the same column, indicate significant differences among hydrolysates for raw material and enzyme factors, respectively ($p < 0.05$).

The protein hydrolysates' capability to form stable emulsions and foams is also a relevant feature for them to be applied in a variety of foods, such as ice cream, dressings, mousses, pâtés margarines or beverages (Aryee et al., 2017). Proteins are surface-active molecules with hydrophobic and hydrophilic amino acids that, as with ORC, are responsible for their emulsifying and foaming properties (Haque et al., 2016). Hydrolysates' emulsifying capacity was quantified by both the EAI and ESI. As shown in Table 4, greater emulsifying activity and emulsion stability were found for both the samples hydrolysed by papain (MA and SA). Inversely, the samples hydrolysed by pepsin (ME and SE) showed lesser emulsifying properties. The lower values compared to those reported by other authors (Klompong, Benjakul, Kantachote, & Shahidi, 2007; Noman et al., 2018) might be related to the high DH achieved during the hydrolysis process in this work. The influence of both the DH and enzyme type employed for protein hydrolysis on the emulsifying properties has been reported by different authors (Siddik et al., 2021). Alavi, Jamshidian, and Rezaei (2019) observed an inverse relation between the DH and the emulsifying properties of kilka fish protein hydrolysates. Similar results have been reported by Vásquez, Sepúlveda, and Zapata (2022), who observed that the hydrolysates with the highest emulsifying properties had the lowest tested DH (DH 5%) and the highest proportion in high-molecular-weight peptides. Hence the fish protein hydrolysates with a lower DH presented a larger amount of high-molecular-weight peptides and higher surface hydrophobicity, which contribute to better flexibility and orientation at the oil-water interface. On the contrary, as the DH increases, a larger number of smaller peptides form, which may result in a drastic loss of emulsifying properties. Therefore, enzyme selection plays an important role in emulsifying properties because it strongly influences the molecular size and hydrophobicity of the resulting peptides. Vieira, Pinho, and Ferreira (2017) found that the sardine protein hydrolysates produced by alcalase yielded high-molecular-weight peptides and better emulsifying stability and activity compared to protease. Similar results have been obtained by Klompong et al. (2007) when comparing alcalase and Flavourzyme for the hydrolyzation of Yellow stripe trevally. Therefore, a low DH and a careful choice of enzymes are key issues if enhanced emulsifying properties are desired.

Foaming properties were evaluated by quantifying protein

hydrolysates' ability to form and stabilize foam seeing that both are key parameters for the texture of a variety of food products. The greater ability to form foam (FC) was for SE (35%), followed by MR (23.33%) and ME (20%) (Table 4). The values for FS were low for all the samples, ranging from 3.33% to 10%, with a higher capacity to stabilize foam for the Protamex™ samples. Moreover, mackerel hydrolysates exhibited a higher ability to stabilize foam than sardines' hydrolysates. Similarly, several authors have also established that further hydrolysis could reduce foaming stability because more microscopic peptides do not have the necessary strength to maintain foam stability (Alavi et al., 2019; Klompong et al., 2007).

In general, DH and solubility have been proposed as relevant factors to affect emulsifying and foaming properties (Pires & Batista, 2013). In our work, the DH correlated positively with the emulsifying properties and negatively with the foaming properties of hydrolysates. According to Pires and Batista (2013), emulsifying properties usually decrease as the DH increases. However, we found that papain and Protamex™ exhibited better emulsifying attributes and higher DH values than the pepsin hydrolysates. These results agree with those found in the hydrolysates obtained from surimi processing by-products and common carp collagen by-products (González-Serrano et al., 2022; Liu et al., 2014). In our study, the high solubility at pH 7 observed for papain and Protamex™ is probably related to its emulsifying properties because solubility facilitates rapid migration and adsorption at the oil-water interface to form a film (Mune Mune, 2015).

The fish by-product hydrolysates' foaming properties displayed a different pattern. For lower DH values, hydrolysates displayed higher foaming capacity and more foam stability. In line with this, González-Serrano et al. (2022) studied the functional properties of different fractions of collagen hydrolysed from common carp by-products with alcalase. These authors found that molecular weights of more than 30 kDa possessed stronger FC and FS than those with lower molecular weights. Similarly, Zamorano-Apodaca et al. (2020) noted that FC was conditioned by molecular weight. According to these authors, low-molecular peptides and proteins do not present the necessary strength to form foam because of their inability to reorganize their structure at the air-water interface (Zamorano-Apodaca et al., 2020). Foam stability is due to the orientation of peptides at the interface, with the polar head located in the aqueous phase and the hydrophobic chain in the apolar phase (Thiansilakul, Benjakul & Shahidi, 2007). Accordingly, Naghdi et al. (2023b) suggested that more air can be combined into a solution of low molecular weight peptides, and as a consequence, these small peptides with more air cells have less capacity to keep durable foams. However, different results can be found in the literature about the relation between DH and foaming properties, and higher foaming properties have also been related to higher DH values in sardinella (Ben Khaled et al., 2014) or rainbow trout roe (Rajabzadeh, Pourashouri, Shabanpour, & Alishahi, 2018) protein hydrolysates. Liu et al. (2014) observed that the hydrolysates with 20% DH and 30% DH prepared by Alcalase exhibited superior foam properties to those of the samples with the same DH prepared by Protamex™. Other factors along with the DH, such as surface hydrophobicity of unfolded proteins, solubility, size, and charge of peptides, may be responsible for the differences noted in hydrolysates' foam properties (Liu et al., 2014; Pacheco-Aguilar et al., 2008; Pires & Batista, 2013).

3.4. Effect of raw material and enzyme on hydrolysates' parameters/ multivariate analysis

The multifactor ANOVA was carried out to determine the impact of the two factors (raw material and enzyme type), as well as their interaction, on each physico-chemical and functional parameter. Table 5 shows the F-ratio and the significance level of the factors and their interactions for each parameter. The F-ratio value is directly proportional to the statistical effect of each factor on the response variables. Both the raw material and enzyme type used during the hydrolysis process, in addition to their interaction, strongly influenced the evaluated parameters. In particular, the enzyme factor significantly affected all the variables, while the raw material factor affected all the variables, except for solubility at pH 4 and pH 7, the ESI, and FC ($p < 0.05$). The interactions between raw material and enzyme affected all the studied variables, except for the ZP. Moreover, as evidenced by higher F-ratio values, a stronger effect of enzyme than raw material was found for all the considered variables, except for turbidity, and solubility at pH 10 and FS. Similarly, the effect of the interaction was more marked than the raw material factor considered individually for all the measured parameters, except for the DH, solubility at pH 10, the EAI, and FS.

Table 5
F-ratio values and significance levels were obtained in the multifactor ANOVA for the physico-chemical parameters and functional properties according to factors: fish by-product employed as raw material (F), enzyme (E), and their interaction (F x E).

	F	E	F x E
Turbidity	35521.60[c]	12801.10[c]	202.90[c]
pH	92.07[c]	23942.05[c]	51.40[c]
Zeta potential (ZP)	28.47[c]	395.98[c]	3.18
Degree of hydrolysis (DH)	63.33[c]	161.40[c]	15.09[c]
Solubility at pH 4	2.88	53.33[c]	19.31[c]
Solubility at pH 7	0.65	758.56[c]	21.54[c]
Solubility at pH 10	326.58[c]	231.14[c]	52.22[c]
Oil retention capacity (ORC)	36.58[c]	684.92[c]	250.76[c]
Emulsifying activity index (EAI)	112.13[c]	708.84[c]	89.27[c]
Emulsion stability index (ESI)	0.35	68.20[c]	23.66[c]
Foaming capacity (FC)	0.04	98.67[c]	38.01[c]
Foam stability (FS)	172.9**	13.00[b]	4.43[a]

ns: non-significant.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.

The PCA was used to visualize the relation between hydrolysates' physico-chemical and functional properties. The results revealed that 78.8% variability was explained by two principal components (PCs). The first PC (PC1) explained 61.7% of the total variance. As Fig. 2 depicts, PC1 included the variance generated by the enzyme factor type. The most important variables for PC1 were DH (loading value: 0.307), ESI (0.329), EAI (0.338), pH (0.342), and solubility at pH 7 (0.329) with a positive weight, and FC (−0.298), the ZP (−0.341), solubility at pH 4 (−0.320) and ORC (−0.218) with a negative weight. PC1 explained the variability between the pepsin hydrolysates and the papain and Protamex™ hydrolysates. The papain and Protamex™ hydrolysates both exhibited high values for pH, DH, and emulsifying properties, but low values for the ZP, ORC, and FC. Conversely, the pepsin samples showed low DH, pH, and emulsifying properties, but high ORC and ZP values, and good FS performance. Enzymatic hydrolysis results in amino acids and peptides of varied sizes depending on the targeting specific peptide cleavage bonds of the employed enzyme (Fernández-Lucas, Castañeda & Hormigo, 2017). Papain and Protamex™ are both cysteine proteases that show broad specificity for hydrophobic amino acids, resulting in high DH values and a high number of hydrophobic amino groups exposed. The high hydrophobic nature of the Papain and Protamex™ hydrolysates may be responsible for the physicochemical and functional properties observed. Richness in hydrophobic peptides has been related to improved emulsifying properties due to the greater hydrophobicity that allows them to form more stable emulsions (Liu et al., 2014). Instead, low ORC has been related to hydrophilic polar side chains and high DH values (Noman et al., 2018). Thus, the high DH of Papain and Protamex™ samples produces a large number of small peptides that lead to a decrease in the absorption of oil (Noman et al., 2018), generating low ORC values. Besides that, the decrease in FC and FS values has been attributed to the aggregation of proteins that interfere with the interactions between proteins and the water needed for the formation of foam (Noman et al., 2018). This agrees with the low ZP and FC values

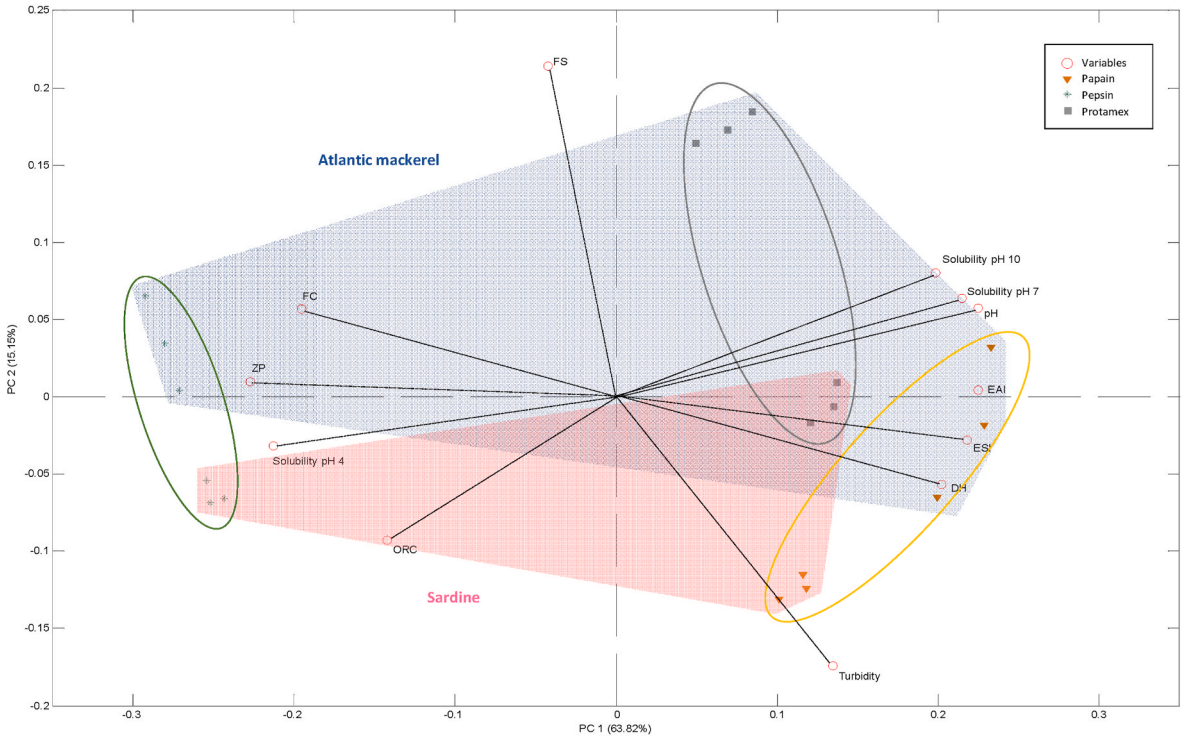

Fig. 2. PCA biplot of the physico-chemical and functional properties of hydrolysates. The drawn ellipses and colour stains highlight the natural samples clustering for enzyme type and fish species, respectively, but do not represent any statistical significance. Abbreviations: DH, degree of hydrolysis; EAI, Emulsifying activity index; ESI, Emulsion stability index; FC, Foaming capacity; FS, Foam stability; ORC, Oil retention capacity; ZP, Zeta Potential. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

observed for these samples. By contrast to papain and Protamex™, pepsin exhibits a higher specificity degree in the amino acid sequences and, as a consequence, produces protein hydrolysates with lower DH. The formation of larger peptides in pepsin hydrolysates may be responsible for the observed low emulsifying properties, but the high ORC and FS. High molecular weight peptides are generally positively related to the emulsifying properties, ORC, and foam stability of protein hydrolysates (Liu et al., 2014; Noman et al., 2018). Moreover, it has been reported that pH affects emulsifying properties by changing protein surface hydrophobicity (Halim et al., 2016). Studies have shown that the highest EAI is produced within a pH range of 6–10, with the lowest EAI occurring at pH 4 (Taheri, Anvar, Ahari, & Fogliano, 2013). In our work, pepsin samples showed the most acidic pH values for the mackerel and sardine hydrolysates, below pH 4. The acidic pH of the pepsin hydrolysates difficult the efficient exposure of hydrophilic and hydrophobic thus preventing significant interactions at the oil-water interface (Taheri et al., 2013).

Regarding the raw material, sardine, and mackerel hydrolysates were grouped according to PC2 (15.1%). The mackerel hydrolysates were clustered together in the upper half of the plot and were characterized by high performance in FS and, to a lesser extent, solubility at pH 10. Instead, the sardine hydrolysates were clustered in the lower part of the plot and had high values for turbidity, DH, and ORC. The key variables for PC2 were FS, turbidity, and ORC. These functional properties of protein hydrolysates are determined by their molecular size, hydrophobicity, the number of reacting amino acid residues, distribution of electrostatic charges, etc. But they are also affected by the physico-chemical properties of the parent protein used as the substrate, the specificity of the protease, or the hydrolysis conditions (temperature, pressure, pH, ionic strength, water activity, and solvent polarity) (Pires & Batista, 2013). Fish by-products employed as a raw material were mainly formed by heads, viscera, skin, scales, and bones. These materials from small pelagic species contain important quantities of proteins and polyunsaturated fatty acids. The amino acid profile of sardine and mackerel by-products was compared by Khan et al. (2003) showing no differences in the amino acid pattern and peptide molecular weight between both fish species. However, when these authors evaluated the functional properties of fish by-products hydrolysates they found significant differences. Because these fish by-products presented similar amino acid composition, the differences observed were attributed to other components, such as non-protein nitrogenous substances, and ash or lipid contents. Similarly, Ferraro et al. (2013) reviewed the extraction and the valorization of bioactive compounds from sardine, sardine-type fish, and mackerel canning by-products, not establishing differences depending on the fish species employed as a raw material. However, these authors highlighted the importance of gaining information about the effect of different factors, such as processing conditions, storage, and seasonal variability of the raw material characteristics on the hydrolysates' properties.

All together, this information confirms that the proteolytic enzymes and to a lesser extent the selected raw material determined the physico-chemical properties and the functionality of fish by-products hydrolysates.

4. Conclusions

Enzymatic hydrolysis of fish by-products is a favorable tool for reducing the pollution generated by the tonnes of fish waste produced annually during fish processing and their revalorization. In short, the results of this study highlight that the raw material and enzyme type used during the hydrolysis process, as well as their interaction, affect hydrolysates' physico-chemical and functional properties. Protein hydrolysates with physico-chemical and functional properties of interest for several applications, including food industry purposes, are obtained from both raw materials. The hydrolysates obtained from the mackerel by-products showed better foaming properties, while the sardine hydrolysates displayed better ORC. Fish by-products hydrolysates with better oil retention and foaming properties can be obtained by using

pepsin, while employing the papain and Protamex™ enzymes seems more appropriate when hydrolysates with a higher DH and better emulsifying properties are required. These results underline the need to consider both fish species and the hydrolysis enzyme when protein hydrolysates and peptides with specific functions are desired.

CRediT authorship contribution statement

Cristina Fuentes: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Samuel Verdú:** Formal analysis. **Raúl Grau:** Supervision. **José Manuel Barat:** Resources, Funding acquisition. **Ana Fuentes:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Formal analysis, 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.

Data availability

Data will be made available on request.

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References

AOAC. (1997). *Official methods of analysis of AOAC International (16th Edition)*. Arlington, Va: Association of Official Analytical Chemists.

Akbarian, M., Khani, A., Eghbalpour, S., & Uversky, V. N. (2022). Bioactive peptides: Synthesis, sources, applications, and proposed mechanisms of action. *International Journal of Molecular Sciences, 23*(3), 1445. https://doi.org/10.3390/ijms23031445

Alavi, F., Jamshidian, M., & Rezaei, K. (2019). Applying native proteases from melon to hydrolyze kilka fish proteins (*Clupeonella cultriventris caspia*) compared to commercial enzyme Alcalase. *Food Chemistry, 277*, 314–322. https://doi.org/10.1016/j.foodchem.2018.10.122

Alfaro, A. da T., Balbinot, E., Weber, C. I., Tonial, I. B., & Machado-Lunkes, A. (2015). Fish gelatin: Characteristics, functional properties, applications and future potentials. *Food Engineering Reviews, 7*, 33–44. https://doi.org/10.1007/s12393-014-9096-5

Aryee, A. N. A., Agyei, D., & Udenigwe, C. C. (2017). Impact of processing on the chemistry and functionality of food proteins. *Proteins in Food Processing*, 27–45. https://doi.org/10.1016/B978-0-08-100722-8.00003-6

Barlow, P. J. (1993). Encyclopedia of food science and technology. *Food Chemistry, 46*(4). https://doi.org/10.1016/0308-8146(93)90019-c

Ben Khaled, H., Ktari, N., Ghorbel-Bellaaj, O., Jridi, M., Lassoued, I., & Nasri, M. (2014). Composition, functional properties and in vitro antioxidant activity of protein hydrolysates prepared from sardinelle (*Sardinella aurita*) muscle. *Journal of Food Science and Technology, 51*(4), 622–633. https://doi.org/10.1007/s13197-011-0544-4

Bengoechea, C., Peinado, I., & McClements, D. J. (2011). Formation of protein nanoparticles by controlled heat treatment of lactoferrin: Factors affecting particle characteristics. *Food Hydrocolloids, 25*(5), 1354–1360. https://doi.org/10.1016/j.foodhyd.2010.12.014

Bhattacharjee, S. (2016). DLS and zeta potential–what they are and what they are not? *Journal of Controlled Release, 235*, 337–351. https://doi.org/10.1016/j.jconrel.2016.06.017

Bing, S. J., Chen, X. S., Zhong, X., Li, Y. Q., Sun, G. J., Wang, C. Y., … Mo, H. Z. (2024). Structural, functional and antioxidant properties of Lentinus edodes protein hydrolysates prepared by five enzymes. *Food Chemistry, 437*, Article 137805. https://doi.org/10.1016/j.foodchem.2023.137805

Chalamaiah, M., Dinesh Kumar, B., Hemalatha, R., & Jyothirmayi, T. (2012). Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A review. *Food Chemistry, 135*(4), 3020–3038. https://doi.org/10.1016/J.FOODCHEM.2012.06.100

Coppola, D., Lauritano, C., Esposito, F. P., Riccio, G., Rizzo, C., & de Pascale, D. (2021). Fish waste: From problem to valuable resource. *Marine Drugs, 19*(2), 116. https://doi.org/10.3390/MD19020116

Dumay, J., Donnay-Moreno, C., Barnathan, G., Jaouen, P., & Bergé, J. P. (2006). Improvement of lipid and phospholipid recoveries from sardine (*Sardina pilchardus*) viscera using industrial proteases. *Process Biochemistry, 41*(11), 2327–2332. https://doi.org/10.1016/j.procbio.2006.04.005

Espinales, C., Romero-Peña, M., Calderón, G., Vergara, K., Cáceres, P. J., & Castillo, P. (2023). Collagen, protein hydrolysates and chitin from by-products of fish and shellfish: An overview. *Heliyon, 9*(4), Article e14937. https://doi.org/10.1016/j.heliyon.2023.e14937. Article.

European Commission. (2020). Blue bioeconomy report. In *EUMOFA - European market observatory for fisheries and aquaculture products* (Issue December).

FAO. (2021). Fish waste management. *Fish Waste Management*. https://doi.org/10.4060/CB3694EN

FAO. (2022). *The state of world fisheries and aquaculture 2022*. The State of World Fisheries and Aquaculture. https://doi.org/10.4060/cc0461en, 2022.

Fernández-López, J., Sendra-Nadal, E., Navarro, C., Sayas, E., Viuda-Martos, M., & Alvarez, J. A. P. (2009). Storage stability of a high dietary fibre powder from orange by-products. *International Journal of Food Science and Technology, 44*(4), 748–756. https://doi.org/10.1111/j.1365-2621.2008.01892.x

Fernández-Lucas, J., Castañeda, D., & Hormigo, D. (2017). New trends for a classical enzyme: Papain, a biotechnological success story in the food industry. *Trends in Food Science and Technology, 68*, 91–101. https://doi.org/10.1016/j.tifs.2017.08.017

Ferraro, V., Carvalho, A. P., Piccirillo, C., Santos, M. M., Castro, P. M., & Pintado, M. E. (2013). Extraction of high added value biological compounds from sardine, sardine-type fish and mackerel canning residues—a review. *Materials Science and Engineering: C, 33*(6), 3111–3120. https://doi.org/10.1016/j.msec.2013.04.003

Gallego, M., Arnal, M., Barat, J. M., & Talens, P. (2020). Effect of cooking on protein digestion and antioxidant activity of different legume pastes. *Foods, 10*(1), 47. https://doi.org/10.3390/FOODS10010047

González-Serrano, D. J., Hadidi, M., Varcheh, M., Jelyani, A. Z., Moreno, A., & Lorenzo, J. M. (2022). Bioactive peptide fractions from collagen hydrolysate of common carp fish byproduct: Antioxidant and functional properties. *Antioxidants, 11*(3), 509. https://doi.org/10.3390/antiox11030509

Groleau, P. E., Gauthier, S. F., & Pouliot, Y. (2003). Effect of residual chymotryptic activity in a trypsin preparation on peptide aggregation in a β-lactoglobulin hydrolysate. *International Dairy Journal, 13*(11), 887–895. https://doi.org/10.1016/S0958-6946(03)00112-2

Gupta, V., & Trivedi, P. (2018). *In vitro* and *in vivo* characterization of pharmaceutical topical nanocarriers containing anticancer drugs for skin cancer treatment. *Lipid Nanocarriers for Drug Targeting*, 563–627. https://doi.org/10.1016/B978-0-12-813687-4.00015-3

Halim, N. R. A., Yusof, H. M., & Sarbon, N. M. (2016). Functional and bioactive properties of fish protein hydolysates and peptides: A comprehensive review. *Trends in Food Science & Technology, 51*, 24–33. https://doi.org/10.1016/j.tifs.2016.02.007

Haque, M. A., Timilsena, Y. P., & Adhikari, B. (2016). Food proteins, structure, and function. *Reference Module in Food Science, 5*(3), 163–166. https://doi.org/10.1016/b978-0-08-100596-5.03057-2

Henriques, A., Vázquez, J. A., Valcarcel, J., Mendes, R., Bandarra, N. M., & Pires, C. (2021). Characterization of protein hydrolysates from fish discards and by-products from the north-west Spain fishing fleet as potential sources of bioactive peptides. *Marine Drugs, 19*(6), 338. https://doi.org/10.3390/md19060338

Hou, H., Li, B., Zhao, X., Zhang, Z., & Li, P. (2011). Optimization of enzymatic hydrolysis of Alaska pollock frame for preparing protein hydrolysates with low-bitterness. *WT-Food Science and Technology, 44*(2), 421–428. https://doi.org/10.1016/j.lwt.2010.09.009

Je, J. Y., Qian, Z. J., Byun, H. G., & Kim, S. K. (2007). Purification and characterization of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis. *Process Biochemistry, 42*(5), 840–846. https://doi.org/10.1016/j.procbio.2007.02.006

Kandyliari, A., Mallouchos, A., Papandroulakis, N., Golla, J. P., Lam, T. K. T., Sakellari, A., et al. (2020). Nutrient composition and fatty acid and protein profiles of selected fish by-products. *Foods, 9*(2), 190. https://doi.org/10.3390/foods9020190

Khan, M. A. A., Hossain, M. A., Hara, K., Osatomi, K., Ishihara, T., & Nozaki, Y. (2003). Effect of enzymatic fish-scrap protein hydrolysate on gel-forming ability and denaturation of lizard fish Saurida wanieso surimi during frozen storage. *Fisheries Science, 69*(6), 1271–1280. https://doi.org/10.1111/j.0919-9268.2003.00755.x

Klompong, V., Benjakul, S., Kantachote, D., & Shahidi, F. (2007). Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry, 102*(4), 1317–1327. https://doi.org/10.1016/j.foodchem.2006.07.016

Kristinsson, H. G., & Rasco, B. A. (2000). Fish protein hydrolysates: Production, biochemical, and functional properties. *Critical Reviews in Food Science and Nutrition, 40*(1), 43–81. https://doi.org/10.1080/10408690091189266

Kruijssen, F., Tedesco, I., Ward, A., Pincus, L., Love, D., & Thorne-Lyman, A. L. (2020). Loss and waste in fish value chains: A review of the evidence from low and middle-income countries. *Global Food Security, 26*, Article 100434. https://doi.org/10.1016/j.gfs.2020.100434

Kvangarsnes, K., Dauksas, E., Tolstorebrov, I., Rustad, T., Bartolomei, M., Xu, R., et al. (2023). Physicochemical and functional properties of rainbow trout (Oncorhynchus mykiss) hydrolysate. *Heliyon, 9*(7), Article e17979. https://doi.org/10.1016/j.heliyon.2023.e17979

Liaset, B., Lied, E., & Espe, M. (2000). Enzymatic hydrolysis of by-products from the fish-filleting industry; chemical characterisation and nutritional evaluation. *Journal of the Science of Food and Agriculture, 80*(5), 581–589. https://doi.org/10.1002/(SICI)1097-0010(200004)80:5<581::AID-JSFA578>3.0.CO;2-I

Liu, Y., Li, X., Chen, Z., Yu, J., Wang, F., & Wang, J. (2014). Characterization of structural and functional properties of fish protein hydrolysates from surimi processing by-products. *Food Chemistry, 151*, 459–465. https://doi.org/10.1016/J.FOODCHEM.2013.11.089

Lunardi, C. N., Gomes, A. J., Rocha, F. S., De Tommaso, J., & Patience, G. S. (2021). Experimental methods in chemical engineering: Zeta potential. *Canadian Journal of Chemical Engineering, 99*(3), 627–639. https://doi.org/10.1002/cjce.23914

Mune Mune, M. A. (2015). Influence of degree of hydrolysis on the functional properties of cowpea protein hydrolysates. *Journal of Food Processing and Preservation, 39*(6), 2386–2392. https://doi.org/10.1111/jfpp.12488

Naghdi, S., Rezaei, M., Tabarsa, M., & Abdollahi, M. (2023a). Fish protein hydrolysate from sulfated polysaccharides extraction residue of tuna processing by-products with bioactive and functional properties. *Global Challenges, 7*(4), Article 2200214. https://doi.org/10.1002/gch2.202200214

Naghdi, S., Rezaei, M., Tabarsa, M., & Abdollahi, M. (2023b). Parallel extraction of sulfated polysaccharides and protein hydrolysate from skipjack tuna head and their bioactive and functional properties. *Food and Bioprocess Technology, 16*(6), 1258–1279. https://doi.org/10.1007/s11947-022-02988-w

Najafian, L., & Babji, A. S. (2012). A review of fish-derived antioxidant and antimicrobial peptides: Their production, assessment, and applications. *Peptides, 33*(1), 178–185. https://doi.org/10.1016/j.peptides.2011.11.013

Nikoo, M., Regenstein, J. M., & Yasemi, M. (2023). Protein hydrolysates from fishery processing by-products: Production, characteristics, food applications, and challenges. *Foods, 12*(24), 4470. https://doi.org/10.3390/foods12244470

Noman, A., Ali, A. H., Al-Bukhaiti, W. Q., Mahdi, A. A., & Xia, W. (2020). Structural and physicochemical characteristics of lyophilized Chinese sturgeon protein hydrolysates prepared by using two different enzymes. *Journal of Food Science, 85*(10), 3313–3322. https://doi.org/10.1111/1750-3841.15345

Noman, A., Xu, Y., Al-Bukhaiti, W. Q., Abed, S. M., Ali, A. H., Ramadhan, A. H., et al. (2018). Influence of enzymatic hydrolysis conditions on the degree of hydrolysis and functional properties of protein hydrolysate obtained from Chinese sturgeon (*Acipenser* sinensis) by using papain enzyme. *Process Biochemistry, 67*, 19–28. https://doi.org/10.1016/J.PROCBIO.2018.01.009

Pacheco-Aguilar, R., Mazorra-Manzano, M. A., & Ramírez-Suárez, J. C. (2008). Functional properties of fish protein hydrolysates from Pacific whiting(Merluccius productus) muscle produced by a commercial protease. *Food Chemistry, 109*(4), 782–789. https://doi.org/10.1016/j.foodchem.2008.01.047

Pires, C., & Batista, I. (2013). Functional properties of fish protein hydrolysates. *Utilization of Fish Waste*, 59–75.

Racioppo, A., Speranza, B., Campaniello, D., Sinigaglia, M., Corbo, M. R., & Bevilacqua, A. (2021). Fish loss/waste and low-value fish challenges: State of art, advances, and perspectives. *Foods, 10*(11), 2725. https://doi.org/10.3390/foods10112725

Rajabzadeh, M., Pourashouri, P., Shabanpour, B., & Alishahi, A. (2018). Amino acid composition, antioxidant and functional properties of protein hydrolysates from the roe of rainbow trout (*Oncorhynchus mykiss*). *International Journal of Food Science and Technology, 53*(2), 313–319. https://doi.org/10.1111/ijfs.13587

Ravindran, S., Williams, M. A. K., Ward, R. L., & Gillies, G. (2018). Understanding how the properties of whey protein stabilized emulsions depend on pH, ionic strength and calcium concentration, by mapping environmental conditions to zeta potential. *Food Hydrocolloids, 79*, 572–578. https://doi.org/10.1016/j.foodhyd.2017.12.003

Siddik, M. A. B., Howieson, J., Fotedar, R., & Partridge, G. J. (2021). Enzymatic fish protein hydrolysates in finfish aquaculture: A review. *Reviews in Aquaculture, 13*(1), 406–430. https://doi.org/10.1111/raq.12481

Taheri, A., Anvar, S. A. A., Ahari, H., & Fogliano, V. (2013). Comparison the functional properties of protein hydrolysates from poultry by-products and rainbow trout (*Onchorhynchus mykiss*) viscera. *Iranian Journal of Fisheries Sciences, 12*, 154–169.

Tan, Y., Chang, S. K. C., & Meng, S. (2019). Comparing the kinetics of the hydrolysis of by-product from channel catfish (*Ictalurus punctatus*) fillet processing by eight proteases. *Lebensmittel-Wissenschaft & Technologie, 111*, 809–820. https://doi.org/10.1016/J.LWT.2019.05.053

Thiansilakul, Y., Benjakul, S., & Shahidi, F. (2007). Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus maruadsi*). *Food Chemistry, 103*(4), 1385–1394. https://doi.org/10.1016/J.FOODCHEM.2006.10.055

Vásquez, P., Sepúlveda, C. T., & Zapata, J. E. (2022). Functional properties of rainbow trout (*Oncorhynchus mykiss*) viscera protein hydrolysates. *Biocatalysis and Agricultural Biotechnology, 39*, Article 102268. https://doi.org/10.1016/j.bcab.2021.102268

Vieira, E. F., Pinho, O., & Ferreira, I. M. (2017). Bio-functional properties of sardine protein hydrolysates obtained by brewer's spent yeast and commercial proteases. *Journal of the Science of Food and Agriculture, 97*(15). https://doi.org/10.1002/jsfa.8432

Wang, Y. Y., Wang, C. Y., Wang, S. T., Li, Y. Q., Mo, H. Z., & He, J. X. (2021). Physicochemical properties and antioxidant activities of tree peony (*Paeonia suffruticosa Andr.*) seed protein hydrolysates obtained with different proteases. *Food Chemistry, 345*, Article 128765. https://doi.org/10.1016/j.foodchem.2020.128765

Zamorano-Apodaca, J. C., García-Sifuentes, C. O., Carvajal-Millán, E., Vallejo-Galland, B., Scheuren-Acevedo, S. M., & Lugo-Sánchez, M. E. (2020). Biological and functional properties of peptide fractions obtained from collagen hydrolysate derived from mixed by-products of different fish species. *Food Chemistry, 331*, Article 127350. https://doi.org/10.1016/J.FOODCHEM.2020.127350

Zhang, L., Li, Q., Hong, H., & Luo, Y. (2020). Prevention of protein oxidation and enhancement of gel properties of silver carp (*Hypophthalmichthys molitrix*) surimi by addition of protein hydrolysates derived from surimi processing by-products. *Food Chemistry, 316*. https://doi.org/10.1016/j.foodchem.2020.126343