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Optimal and sustainable production of tailored fish protein hydrolysates from tuna canning wastes and discarded blue whiting: Effect of protein molecular weight on chemical and bioactive properties

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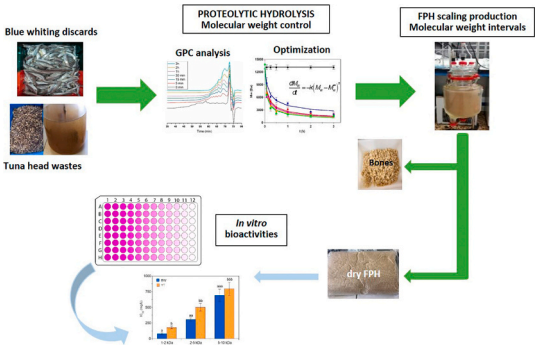
HIGHLIGHTS

- Sustainable valorization of fishing discards and canning wastes.
- Mathematical modeling of protein molecular weight dynamics on enzyme hydrolysis.
- Tailored protein hydrolysates produced at different molecular weight intervals.
- Smaller protein hydrolysates led to higher production yields and bioactives.
- This process may contribute to the sustainability of the fish industry.

GRAPHICAL ABSTRACT



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ABSTRACT

Thousands tons of discards of blue whiting (BW) and tuna heads (YT) by-products are generated each year in Europe. BW is the species most discarded by European fishing fleet and, in some canning factories, YT are processed for the retrieval of oil rich in omega-3, but producing a huge amount of solid remains and effluents disposal as wastes. The development of optimal and sustainable processes for both substrates is mandatory in order to reach clean solutions under the circular economy precepts. This work focused on the mathematical optimization of the production of tailored fish protein hydrolysates (FPH), from blue whiting and tuna residues, in terms of controlling average molecular weights (Mw) of proteins. For the modeling of the protein

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depolymerization time-course, a pseudo-mechanistic model was used, which combined a reaction mechanistic equation affected, in the kinetic parameters, by two non-lineal equations (a first-order kinetic and like-Weibull formulae). In all situations, experimental data were accurately simulated by that model achieving R^2 values higher than 0.96. The validity of the experimental conditions obtained from modeling were confirmed performing productions of FPH at scale of 5 L-reactor, without pH-control in most of cases, at the different ranges of Mw selected (1–2 kDa, 2–5 kDa and 5–10 kDa). The results showed that FPH from BW with lower Mw led to a remarkable yield of production (12 % *w*/w of substrate), largest protein contents (77 % w/w of BW hydrolysate), greatest *in vitro* digestibility (>95 %), highest essential amino acid presence (43 %) and the best antioxidant (DPPH = 62 %) and antihypertensive (IC_{50}-ACE = 80 mg/L) properties. Our results prove that the proposed procedure to produce sustainable FPH, with specific Mw characterisitics, could be extended to other fish waste substrates. Tailored FPH may have the potential to serve as valuable ingredients for functional foods and high-quality aquaculture feed.

1. Introduction

Fish production and consumption has greatly expanded in the last seven decades, growing at around 3 % annual rate and doubling population growth (1.6 %). Wild capture has virtually reached its exploitation limits, peaking at around 90 million tonnes per year, and therefore consumption growth is fundamentally driven by aquaculture (FAO, 2022). Such rise has increased environmental pressure at different levels, including the amount of residues generated. This residual biomass comes from two sources: fish waste, mainly discards, and processing by-products. In the first case, estimates of global fish discards reach 7–16 Mt. (10–12 % of catch) (Gilman et al., 2020). Several countries have implemented legislation to ban discards (Chile, New Zealand, Norway, the European Union) (Borges et al., 2016), resulting in an increase of the landing of fish not suitable for human consumption. In the specific case of Europe, from 2019 the Landing Obligation of the European Commission (EU) Common Fisheries Policy states that all fishing vessels must keep on board and land all the catch of the species subjected to quota, that have a minimum legal size, or are underutilized commercial species (European Commission, 2013).

On the other hand, human consumption accounted for 157 million tonnes (89 %) out of the 178 million tonnes of aquatic animals produced globally in 2020. Although wide geographical variation exist, traditional fish marketing in live, fresh or chilled forms is shifting to value-added products, such as ready-to-eat meals. In 2020, in high-income countries over 50 % of aquatic animals sold for human consumption were frozen, 26 % in prepared and preserved form, and 13 % cured. This trend results in increased amounts of industrial processing by-products, which may account for up to 70 % of processed fish. In contrast, in upper middle income countries, only 20 % of the aquatic food production was frozen, and 11 % was canned; and in low-income countries only 7 % was frozen and 20 % was cured (FAO, 2022). As fish processing expands to middle- and low- income countries, the amount of by-products generated will also foreseeably increase. Considering that high-income countries only consume 20 % of the aquatic food globally produced, by-products in middle- and low-income countries could potentially reach tens of million tonnes.

This scenario urges the development of strategies to deal with this growing biomass. Traditionally, some fish by-products and discards have ended transformed into fishmeal, in the best case, or disposed as waste. These discarded fractions contain however valuable materials suitable for recovery through valorization processes, including polysaccharides (chondroitin sulfate, chitin and chitosan) (López-Álvarez et al., 2020; Namboodiri and Pakshirajan, 2020) protein (collagen and gelatin, fish protein hydrolysates, bioactive peptides, enzymes) (Murthy et al., 2018; Blanco et al., 2020; Vázquez et al., 2020a; Valcarcel et al., 2021), oils (Saini et al., 2018; Karkal and Kudre, 2020), and minerals (López-Álvarez et al., 2017; Terzioğlu et al., 2018). Among these processes, fish protein hydrolysate (FPH) probably represent the most versatile option. On the one hand, because many of these residues contain a significant amount of protein, and on the other, because hydrolysis facilitates the separation of other valuable fractions, such as minerals, cartilage, and fat, which are remained after the solubilisation of protein.

Potential applications of FPH are wide, covering its use as nutritional supplements due to their high essential amino acid content, as food ingredients and additives to modulate emulsification and foaming and as antioxidant, and as nutraceuticals based on its content of bioactive peptides (Gao et al., 2021). Bioactivities tested *in vitro* have shown Ca-binding, immunomodulatory, antidiabetic, antipoliferative, antimicrobial, antioxidant and antihypertensive activities. These depend mainly on chain length, residue composition and sequence, which in turn vary with processing parameters (Halim et al., 2016; Nirmal et al., 2022).

However, these applications still face important challenges, such as the development of commercially viable extraction and purification methods at industrial scale, treatment of off-flavours in certain peptides, knowledge about the structure-function relationship of peptides contained in the FPHs produced, and *in vivo* studies to determine peptide bioactivity (Gao et al., 2021). In this context, one of its most realistic current applications is, for example, as feed ingredients to formulate fishmeal in a more sustainable way. Fishmeals are mainly produced with fresh fish that could be used for human consumption, being the hydrolysates from fish by-products a valuable alternative and more sustainable option (Egerton et al., 2020; Cooney et al., 2023). Inclusion of FPH in aquaculture feed can potentially increase growth, feed efficiency, and immune response in fish, positively impacting sustainability, for instance by reducing the need for chemicals to control pathogens, but these advantages depend largely on the molecular weight of the FPH incorporated (Siddik et al., 2021). Therefore, production of FPH with specific molecular weight distributions would add value to this material as aquaculture feed ingredient and contribute to the economic viability of the valorization process.

Inclusion of low molecular weight FPH in aquaculture diets have resulted in increased growth, feed efficiency, and utilization in a number of species (Aksnes et al., 2006a, 2006b; de Vareilles et al., 2012: Zheng et al., 2013), which may be due to higher palatability, attractant effect, and digestibility of small molecular weight fractions. Evidence also suggest that FPH of different Mw may improve the sanitary conditions of cultured fish: Some studies have also associated FPHs from 0.5 to 3 kDa to stimulation of the immune system (Bøgwald et al., 1996; Gildberg et al., 1996; Leduc et al., 2018), from 3 to 10 kDa with increased antioxidant activity (Sierra et al., 2021), and heightened antibacterial activity for low molecular weight hydrolysates (Pezeshk et al., 2019). The positive effects recorded seem to depend on the rate of inclusion, usually moderate (Siddik et al., 2021). However, the relationship between Mw distributions and physiological benefits remains unclear. In a similar context, various recent works have established the influence of the peptide size of fish hydrolysates in their bioactive *in vitro* properties in the search of attractive and sustainable sources for the manufacture of nutraceutical ingredients (Latorres et al., 2022; Ramakrishnan et al., 2023).

In this study, we propose the use of waste from wild capture and fish processing by-products as potential inputs for aquaculture and food supplements. With this aim, we establish the conditions to produce FPH

of diverse molecular weight by enzymatic digestion from relevant industrial by-products (tuna heads) and discards (eviscerated blue witing individuals). In the first case, tuna species ranked the third most heavily captured finfish group in 2018 globally (Sierra et al., 2021), being for the most part processed for sale as loins, stakes, and canned fish. As a result, tuna processing plants produce a large amount of by-products, mainly composed of heads (13 %) (Pezeshk et al., 2019). In the second case, blue witting appears commonly as by-catch in crustacean bottom otter trawl fisheries (Gamarro et al., 2013; FAO, 2020). In both substrates, we followed at lab scale by gel permeation chromatography (GPC) the kinetics of alcalase and papain hydrolysis at different enzyme to substrate ratios to determine the conditions required for the obtention of the desired molecular weight intervals: 1–2 kDa, 2–5 kDa and 5–10 kDa. We then up-scaled hydrolysates production at the established conditions to 5 L reactors to confirm the validity of the procedure to produce tailored FPH with improved chemical and bioactive properties. Optimizing the molecular weight of FPH may aid in the development of industrial processes, so that hydrolysis conditions can be controlled to break down protein to the extent that bioactive peptides are released, and may represent therefore a first step in studies of structure-activity and bioactivity.

2. Materials and methods

2.1. Fish substrates

Blue whiting (BW, *Micromesistius poutassou*) individuals discarded by Galician fishing fleets were kindly provided by Opromar (Marín, Spain). Those specimens with weights ranging from 100 to 300 g were captured in the North Atlantic Ocean, closed to the Galician coast, and conserved in ice on board (Fig. S1, supplementary material). They were manually eviscerated after landing and the rest of fish, including muscle, bones, skins and heads, were jointly grinded (using a meat mincer), distributed in plastic bags and immediately stored at −18 °C up to use for no >3 months. Industrial remains of yellowfin tuna (YT, *Thunnus albacares*) heads and liquid effluents, generated both wastes from the retrieval of tuna oils by pressing machine working at 60–70 °C and subsequent triple centrifugation, were kindly supplied, frozen, by Valora Marine Ingredients (Jealsa Corporation, Boiro, Spain) (Fig. S1, supplementary material). Tuna heads, still semi-frozen, were also grinded, placed in plastic bags and conserved at −18 °C, as well as the tuna effluents, until processing (in <3 months).

2.2. Optimization of the fish protein hydrolysate (FPH) depolymerization

Initially, a set of experiments were developed by individually testing two proteolytic enzymes to optimize the conditions adequates for the production of FPH with different ranges of molecular weights. The two enzymes, alcalase 2.4 L (Novozymes A/S, Bagsværd, Denmark) and papain (Sigma-Aldrich, Burlington, MA, United States) were selected due to: a) present GRAS characteristics (valid for use in food applications); b) have a moderate cost; c) possess high hydrolytic and mainly endopeptide capacity (Vázquez et al., 2020a); d) have an ample spectre of activity to digest many different marine substrates (Vázquez et al., 2016, 2023). Furthermore, an attempt was made to reduce the amount of ash in <15–17 % (*w*/w) in the final dry FPH. For this, we worked without pH control during the hydrolysis time, in order to avoid the continuous addition of alkalis (inorganic solution that introduces additional ashes) into the reaction system. On an Erlenmeyer flask scale, the experiments were carried out with: a) 50 g of solid substrate (grinded fish material) + 50 mL of tuna effluent or water in the case of BW (solid: liquid ratio = 1:1); b) establishment of an initial pH of the mixture of 8.6 (for alcalase) and 8.0 (for papain) after adding the adequate volume of 5 M NaOH; c) adding different amounts of enzyme (*E*) to each flask (0.01, 0.05, 0.1, 0.25 and 0.5 % *v/w* for alcalase and % w/w for papain); d) incubation of the flasks at 60 °C (alcalase) and 50 °C (papain) under orbital shaking at 250 rpm; e) without maintenance of pH control; f) sampling at different hydrolysis times (*t*).

The range of enzyme concentrations studied was chosen in order to evaluate an extense protease level from a well-known percentage (0.5 %), in which high hydrolysis degrees of FPH was reached (Vázquez et al., 2019), up to 50 times lower concentration. Temperature conditions were the optimal ones validated for the production of BW and YT hydrolysates (Vázquez et al., 2020b, 2022). Finally, each sample was directly heat treated (90 °C/15 min) to deactivate the enzymatic activity, centrifuged (6000 ×*g*/20 min) and the supernatant was collected and stored at −21 °C until analysis.

2.3. Production of each customized FPH at reactor scale

Molecular weight (Mw) intervals were stablished based on: a) the differences in the functional and nutritive properties that FPH with small protein-size content showed in comparison with higher-size Mw hydrolysates (Ramakrishnan et al., 2023); b) the significant effect that the size of the protein material had on the modulation of fish culture growth (Leduc et al., 2018). In both cases, the bioactive hydrolysates were ranged from 0.5 to 10 kDa. In this work, we have decided to set three intervals to study: 1–2 kDa, 2–5 kDa and 5–10 kDa, with the aim to identify the mechanism for their production and to clarify the effect of the hydrolysis process in their chemical and biological features.

After selection of the best time and alcalase concentration to obtain the molecular weights proposed (see R&D section), lab-scale hydrolyses were performed in a 5 L glass-reactor using the same experimental conditions and stages utilized in previous Erlenmeyer experiences. Thus, 1.8 kg of ground fish substrate were mixed in 1.8 L of distilled water (for BW assays) and in 1.8 L of tuna head effluents (for TH experiments) employing the necessary volume of 5 M NaOH to bring the initial pH until a value of 8.6, but without subsequent pH-control. Temperature and agitation were maintained constant at 60 °C and 250 rpm, respectively. At the end of the hydrolysis, bones were removed by filtration (100 μm), the liquid FPH were fast warmed (90 °C for 15 min) for alcalase inactivation, centrifuged (15,000 ×*g*/20 min) and then dried by lyophilisation, vacuum-packed and stored at −18 °C.

2.4. Chemical determinations

Two chemical properties were analysed in the hydrolysates generated in the experiments of optimization: 1) the total soluble protein using Folin–Ciocalteu reagent and spectrophotometric determination at 750 nm (Lowry et al., 1951) and 2) the average molecular weight (Mw) and number average molecular weight (Mn) of peptides by gel permeation chromatography (GPC-HPLC) using Proteema size-exclusion columns (PSS, Mainz, Germany) and a combination of refractive index and dual-angle static light scattering detection (Vázquez et al., 2019). Additional chemical determinations were performed in the liquid FPH produced at reactor scale: 1) amino acids content by ninhydrin reaction (Moore et al., 1958) employing an amino acid analyser (Biochrom 30 series, Biochrom Ltd., Cambridge, UK); 2) *in vitro* digestibility (pepsin method: AOAC Official Method 971.09) according to the modifications suggested by Miller et al. (2002); 3) *in vitro* antihypertensive activity using the Angiotensin I-converting enzyme (ACE) method (Estévez et al., 2012); and 4) antioxidant activities applying Crocin (with Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, as internal control) and 1,1-diphenyl-2-picrylhydrazyl (DPPH, with butylated hydroxytoluene-BHT as internal control) bleaching protocols (Prieto et al., 2015a, 2015b).

Proximal analysis was employed for the characterization of dry FPH as follows: 1) moisture, organic matter (OM) and ash content (AOAC, 1997); 2) total protein as total nitrogen x 6.25 (AOAC, 1997), and 3) total lipids by Soxhlet extraction with diethyl ether and gravimetric quantification (Bligh and Dyer, 1959). Additionally, heavy metals content was determined using inductively coupled plasma mass

spectrometry (ICP-MS) and the ω3/ω6 fatty acid ratio was calculated from oil previously extracted and analysed by gas chromatography–mass spectrometry after chemical methylation (Lepage and Roy, 1986). In all cases, the analyses were done in triplicate or quintupled.

2.5. Mathematical modeling of protein depolymerization

To describe the hydrolysis kinetic profiles (Fig. 2), a mathematical model was developed based on (Valcarcel et al., 2020a): a) a reaction mechanism with $n \leq 3$ to relate the reduction in average molecular weight (M_w) with the proteolysis time (Eq. (1)); b) the Weibull Eq. (2) relating the reaction rate (-*k*-, with units of $kDa^{-3}\ h^{-1}$) to the concentration of enzyme-*E*; c) a first-order kinetics, negative exponential Eq. (3), describing the evolution of the final asymptotic molecular weight of the hydrolysate affected by *E*.

$$\frac{dM_w}{dt} = -k\left(M_w - M_w^{\infty}\right)^n \tag{1}$$

$$k = A\left[1 - exp\left(-ln2\left(\frac{E}{m}\right)^{\alpha}\right)\right] \tag{2}$$

$$M_w^{\infty} = M_w^0 exp(-\mu E) \tag{3}$$

Integrating the differential Eq. (1) we obtain Eq. (4), and subsequently solving for the time variable the Eq. (5) is found:

$$M_w = M_w^{\infty} + \left[\frac{\left(M_w^0 - M_w^{\infty}\right)^{n-1}}{1 + kt(n-1)\left(M_w^0 - M_w^{\infty}\right)^{n-1}}\right]^{\frac{1}{n-1}} \tag{4}$$

$$t = \left(\frac{1}{(n-1)k}\right)\left(\frac{1}{\left(M_w - M_w^{\infty}\right)^{n-1}} - \frac{1}{\left(M_w^0 - M_w^{\infty}\right)^{n-1}}\right) \tag{5}$$

where, M_w is the molecular weight of FPH (average molecular weight, in kDa), n is the kinetic order (dimensionless), k is the rate of decay (in $kDa^{-3}\ h^{-1}$), M_w^{∞} is the molecular weight after 3 h of depolymerization (in kDa), M_w^0 is the initial molecular weight (in kDa) and μ is the (in $\%^{-1}$). There are also three parameters from Weibull equation (A, m and α) that can be estimated from modeling, have the following units (in $kDa^{-3}\ h^{-1}$, % and dimensionless, respectively) but no specific chemical meaning.

2.6. Numerical fittings and statistical analyses

Fitting procedures of experimental data and parametric estimations were calculated by minimizing the sum of quadratic differences between the observed and model predicted values, using the non-linear least-squares (GRG non-linear) method provided by the macro-'Solver' of the Microsoft Excel spreadsheet. Confidence intervals from the parametric estimates (Student's *t*-test) and consistence of mathematical models (Fisher's F test) were evaluated by "SolverAid" macro (Levie's Excellaneous web-site:http://www.bowdoin.edu/~rdelevie/excellaneous). One-way ANOVA test followed by means of Tukey test was applied to know the existence of significant differences between samples. Statistical significance was stablished at $p < 0.05$.

3. Results and discussion

The interest and relevance of this study is given, mainly, by the definition of a proposal that allows: a) the mathematical modeling of the protein depolymerization dynamics (determined by GPC) of BW and YT hydrolysates, b) the validation of the optimal production of FPH with defined Mw at scale of 5 L-reactor, c) the sustainable production of tailored FPH with improved chemical and biological characteristics.

3.1. Optimization of FPH depolymerization conditions

In Fig. 1, a summarized representation of the evolution of molecular weight distributions of protein, analysed by GPC, serves to illustrate the kinetics of depolymerization at various concentrations of alcalase. Elution at higher retention volumes as reaction time increases indicates a reduction in molecular weight (Valcarcel et al., 2020a). Similar behavior was observed in the hydrolysis of cod frames using size exclusion chomatography with Sephadex columns (Himonides et al., 2011). However, no diminution of peaks signal are observed as the hydrolysis progresses, as it was found in the depolymerization of chondroitin sulfate from blue shark cartilage by chondroitinase ABC from *Proteus vulgaris* (Valcarcel et al., 2020a). Nevertheless, the area of the distributions revealed changes that may be associated with the endopeptidase activity of both proteases, which release polypeptide chains within a molecule (breaking peptide bonds of nonterminal amino acids) (Rawlings and Salvesen, 2013).

Although, the use of GPC to analyse the absolute molecular weight of multiple biopolymers including fish protein and peptides has been widely standardized (Bouvier and Koza, 2014; Silva et al., 2015; Vázquez et al., 2019; Alves et al., 2022), its application for the monitoring of protein hydrolysis reaction was still unexplored. Our outcomes demonstrate its validity.

In almost all kinetics, the reduction in protein size was most pronounced in the early stages of hydrolysis processes, until 0.5 h, reaching the stationary phase around 2–3 h (Fig. 2). As expected, with the lowest concentration of both proteases (0.01 %), the rate of proteolysis was slower and the reactions ended earlier. The depolymerization of substrates as complex as YT and BW, made up of multiple and unknown types of proteins and peptides, hinders the possibility of applying purely mechanistic mathematical models based on first principles, since the complexity of the needed equations together with the large set of uncertain parameters makes a reliable estimate impossible from the experimental data. This complexity has been showed, and not fully solved, in the hydrolysis simulation of more simple, and pure, proteins as β-casein and β-lactoglobulin (Vorob'ev, 2022) and polysaccharides as cellulose (Griggs et al., 2012). To our knowledge, this is the first work studying the mathematical prediction and optimization of the enzymatic cleavage of the protein fraction present in fish by-products and discards in terms of the average molecular weight dynamics.

These procedures of modeling were performed using the pseudo-mechanistic model proposed [1–3], which compiling the whole of multiple reaction steps in one simple equation. After estimating the kinetic order (n) from 1 to 3 (maximum limit for a chemical reaction order) for modeling Mw data for both proteases, we decided to establish the value of $n = 3$ due to its best accuracy in the description of experimental dynamics (data not shown). This reaction order was in agreement with that reported for the depolymerization of hyaluronic acid by hyaluronidase (Valcarcel et al., 2020b). We assumed that the concentration of enzyme (E) influenced the reaction rate (k) by means of a non-linear equation as Weibull and the final molecular weight (M_w^{∞}) at the end of hydrolysis time by negative decay exponential formula. Previously, we employed other simpler equations to define those mathematical relationship –as linear, polynomial and Michaelis-Menten–, but produced poorer data fit and were discarded (data not shown). Thus, the goodness of fit (as R^2) for the six series of data were ranged in the interval of 0.963–0.992, revealing the capacity of our models to simulate accurately the proteolysis of BW and YT (Table 1). Additionally, the *p*-values derived from the Fisher F-test were always lower than 0.0001 indicating the robustness and statistical validity of the model.

The differential Eq. (1) can be easily integrated obtaining the explicit function [4]. Once the time of hydrolysis has been isolated from this equation and the relationships [2,3] are replacing in [4], we are able to determine the time required (Eq. (5)) to obtain a given molecular weight for a selected enzyme concentration. As can be observed, a desired range of FPH size can be achieved by different combinations of E and t. Based

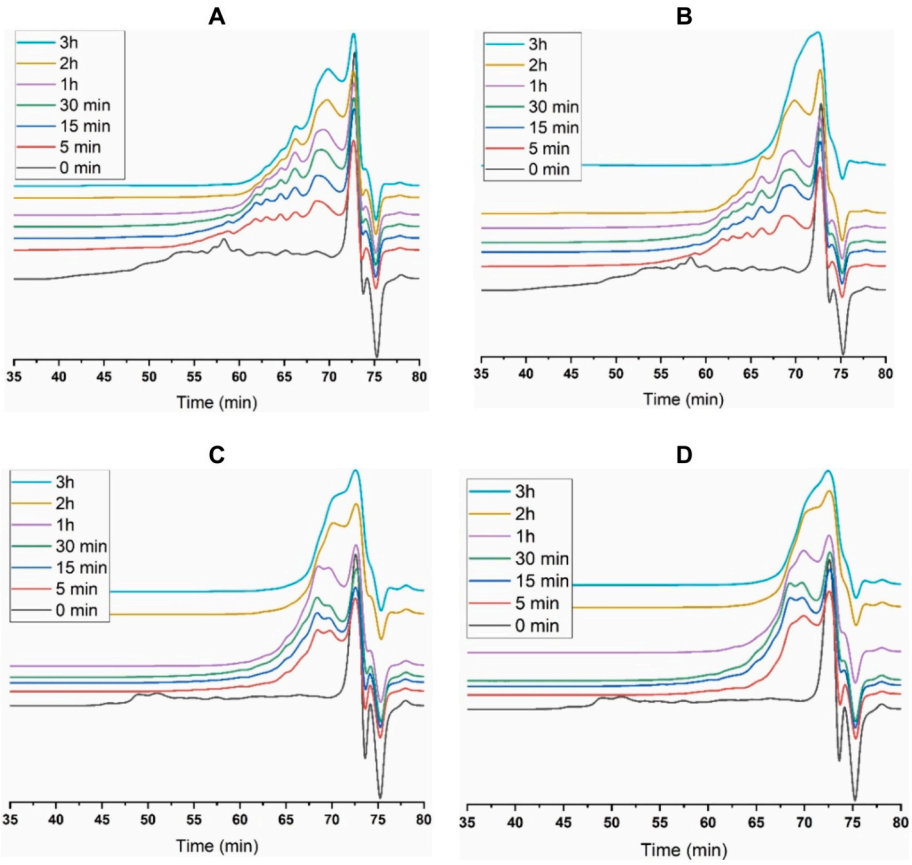

Fig. 1. GPC eluograms (refractive index detector signals) of FPH hydrolysed with alcalase at different reaction times and enzyme concentrations. **A:** YT 0.05 % (*v/w*); **B:** YT 0.1 % (v/w); **C:** BW 0.05 % (v/w); **D:** BW 0.1 % (v/w).

on the numerical parameters from Table 1, the intervals of alcalase amount and reaction time needed to generate the Mw ranges required (1–2 kDa, 2–5 kDa and 5–10 kDa), are summarized in Table 2. Alcalase was finally chosen instead of papain because (Fig. 2 and Table 1): a) it produced less experimental error in the depolymerization dynamics; b) it led to a wider values of Mw; c) it was most cost-effective; and d) it showed better statistical performance in terms of higher R^2 values.

However, the lowest range between 1 and 2 kDa for YT was not able to be released avoiding pH-control during the proteolysis (Fig. 2 and Table 2). It is well-known that the maintenance of pH in the optimal value of maximum enzyme activity produces higher degrees of hydrolysis and lower Mw, but also larger levels of ashes in dry hydrolysates due to the continuous addition of an alkaline solution when working in a pH-stat mode (Vázquez et al., 2022). To solve that problem, we processed the wastes of YT with alcalase at 0.5 % (*v/w*) controlling the pH at the level of 8.6 during 4 h of hydrolysis. Those conditions were enough to obtain FPH with Mw of 1726 Da.

Additionally, the dynamics of net soluble proteins (Pr) were determined in the hydrolysates (Fig. 1B, D, F and H). Such trends were inverse to those shown in protein depolymerization, that is, at higher degree of hydrolysis (not shown), lower Mw is observed and higher concentration of protein is released. This protein concentration was larger in BW samples, mainly using alcalase, and higher with the increase in the added protease. This behavior is expected since with the progress of the catalysis process and when higher concentrations of enzyme are used, an increase of the peptide breakdown occurs, with a consequent increase in the amount of digested biomass, giving rise to a larger amount of protein material solubilized in the liquid fraction and to a decrease in the molecular size of that material (Rawlings and Salvesen, 2013). Furthermore, it is evident that alcalase is more active than papain in hydrolyzing fish substrates, as already happened, for instance, in the proteolysis of ray and small-spotted catshark cartilages (Murado et al., 2010; Blanco et al., 2015).

In conclusion, it was defined the optimal conditions between the time of hydrolysis and the amount of the enzyme from the ranges described in Table 2. These values (BW_{opt} and YT_{opt}) for each range of molecular masses attempted to be the compromise option that minimized, as far as possible, both the process time and the concentration of alcalase (Table 2).

3.2. Production of FPH with tailored molecular weight ranges

Based on the mentioned conditions, the production of hydrolysates was performed at scale of 5 L-reactor in order to: a) validate the optimization proposed; and b) study in a more realistic way the mass balance, chemical characterisitics and bioactive properties of the different FPH produced. The percentages of bones recovered (Y_b) after filtration of the hydrolysate were much larger using YT than BW, and lower with the reduction of molecular weight (Table 3). In concordance, the non digested fraction generated (Y_{nd}) was also higher in the lower size hydrolysates and, as expected because both variables are inversely related, the yield of digestion (Y_{dig}) was superior in the hydrolysate of blue whiting with lowest molecular weight (BW1). Nevertheless, the samples from YT (55–99 g/L) showed higher total soluble protein than those found in BW (37–67 g/L) and, in both cases, at protein size of 1–2 kDa ($p < 0.05$). It must be noted that the production of YT1 was executed using pH-stat mode and in that condition the process of hydrolysis is more exhaustive as it was also found in the production of hydrolysates from seabream and seabass wastes (Valcarcel et al., 2020c).

Attending to the average molecular weight (Mw), we can confirm the validity of optimal conditions proposed, since those values are into the range of protein size initially desired (Table 3). In the case of 1–2 kDa

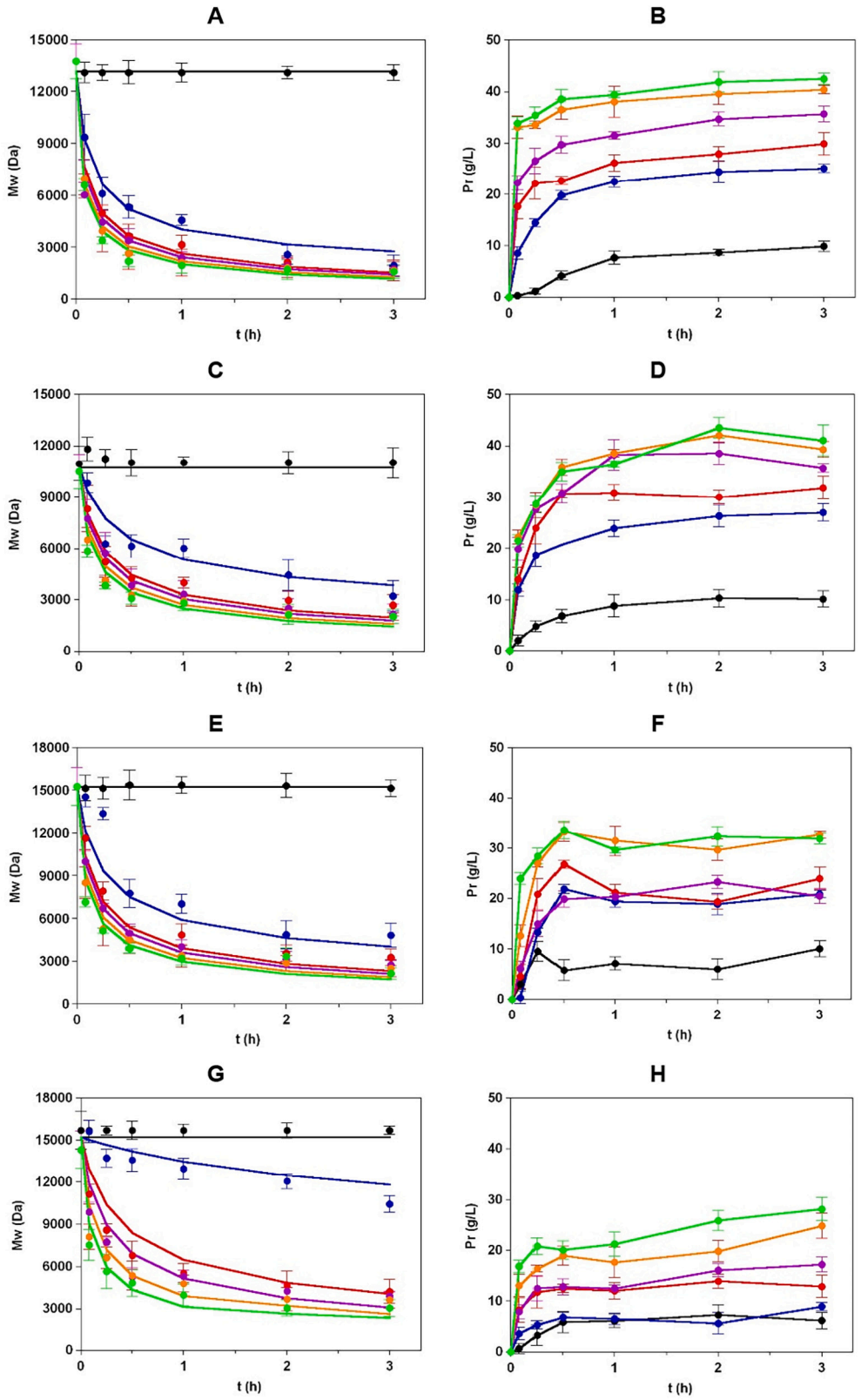

Fig. 2. Kinetics of depolymerization hydrolysis of blue whiting (BW) and yellowfin tuna (YT) substrates by commercial proteases in terms of average molecular weight (Mw) reduction and soluble protein release. Concentrations of enzyme were (in % v/w and % *w*/w for alcalase and papain, respectively): 0 (●), 0.01 (●), 0.05 (●), 0.1 (●), 0.25 (●) and 0.5 (●). Model predicted data are depicted by solid lines, while experimental data are represented by markers. Error bars are the confidence intervals for $n = 2$ and $\alpha = 0.05$. **A:** BW_alcalase (Mw); **B:** BW_alcalase (Pr); **C:** BW_papain (Mw); **D:** BW_papain (Pr); **E:** YT_alcalase (Mw); **F:** YT_alcalase (Pr); **G:** YT_papain (Mw); **H:** YT_papain (Pr).

Table 1
Numerical parameters from Eqs. (1)-(3) obtained from modeling hydrolysis data at the several enzyme concentrations employed. The goodness of fit (R^2) and the robustness of the model are also shown (*p*-value of the F-Fisher test for $\alpha = 0.05$).

Paramater	BW_alcalase	BW_papain	YT_alcalase	YT_papain
M_w^0 (kDa)	13.2 ± 0.5	11.7 ± 0.9	15.2 ± 1.2	15.2 ± 1.6
μ ($\%^{-1}$)	268 ± 23	201 ± 39	255 ± 37	86 ± 29
A (kDa^{-3} h^{-1})	1.53×10^{-6}	1.45×10^{-6}	7.78×10^{-7}	4.45×10^{-6}
m (%)	3204 ± 121	5852 ± 399	2782 ± 198	265 ± 142
α(−)	0.244 ± 0.038	0.272 ± 0.058	0.262 ± 0.053	0.663 ± 0.150
n (−)	3	3	3	3
R^2	0.992	0.976	0.972	0.963
p-value	<0.0001	<0.0001	<0.0001	<0.0001

Table 2
Optimal interval conditions of time and Alcalase concentration, calculated from the Eq. (5), in which is possible to produce the range of FPH molecular weights indicated for each substrate. From these, the final selected value pairs (t, E) for the scale-up productions are also described (BW_{opt} and YT_{opt}).

Alcalase	FPH (1-2 kDa)	FPH (2-5 kDa)	FPH (5-10 kDa)
BW	120–180 min 0.25–0.5 %	10–30 min 0.01–0.5 %	2–10 min 0.01–0.5 %
YT	–	30–180 min 0.05–0.5 %	2–30 min 0.01–0.5 %
BW_{opt}	180 min 0.25 %	12 min 0.01 %	5 min 0.01 %
YT_{opt}	240 min (pH control) 0.5 %	30 min 0.1 %	15 min 0.01 %

Table 3
Chemical characteristics of liquid FPH from blue whiting (BW) and yellowfin tuna (YT) wastes. BW1: FPH from BW in the range of 1–2 kDa. BW2: FPH from BW in the range of 2–5 kDa. BW3: FPH from BW in the range of 5–10 kDa. YT1: FPH from YT in the range of 1–2 kDa. YT2: FPH from YT in the range of 2–5 kDa. YT3: FPH from YT in the range of 5–10 kDa. Y_b: Percentage of wet bones recovered (% w/w of substrate). Y_{db}: Percentage of dry bones recovered (% w/w of substrate). Y_{nd}: Percentage of non-digested substrate (% w/w of substrate). Y_d: Percentage of digested substrate (% w/w of substrate). Pr: Total soluble protein. Mw: Average molecular weight; Mn: Number average molecular weight. Data are shown as average ± confidence intervals ($n = 5$; $\alpha = 0.05$).

FPH	Y_b (%)	Y_{db} (%)	Y_{nd} (%)	Y_{dig} (%)	Pr (g/L)	Mw (kDa)	Mn (kDa)
BW1	4.7 ± 1.2[a]	1.7 ± 0.5[a]	5.9 ± 1.3[a]	85.0 ± 5.2[a]	67.0 ± 6.4[a]	1.41 ± 0.14[a]	1.09 ± 0.14[a]
BW2	6.1 ± 0.9[a]	2.6 ± 0.3[b]	47.9 ± 10.1[b]	45.6 ± 10.7[b]	45.6 ± 5.3[b]	3.08 ± 0.25[b]	1.75 ± 0.12[b]
BW3	12.2 ± 2.2[b]	5.2 ± 0.9[c]	62.1 ± 11.6[b]	26.0 ± 7.6[c]	36.7 ± 10.5[b]	6.73 ± 0.64[c]	3.89 ± 0.59[c]
YT1	23.8 ± 3.0[a]	12.6 ± 1.2[a]	16.5 ± 2.9[a]	61.2 ± 3.5[a]	98.5 ± 2.3[a]	1.73 ± 0.25[a]	1.25 ± 0.18[a]
YT2	27.1 ± 4.5[a]	14.1 ± 1.9[a]	29.5 ± 2.4[b]	52.2 ± 4.7[b]	92.0 ± 7.6[b]	4.03 ± 0.18[b]	2.50 ± 0.18[b]
YT3	42.2 ± 8.6[b]	18.6 ± 2.5[b]	42.5 ± 3.0[c]	15.7 ± 5.1[c]	54.8 ± 6.2[c]	8.80 ± 1.61[c]	4.70 ± 0.81[c]

range, the real data of Mw were 1.41 kDa and 1.73 kDa for BW1 and YT1, respectively. Similar values of Mw, around 1.5 kDa, were also observed in FPH produced from heads of gurnard when alcalase worked during 3 h in control-pH hydrolysis (Vázquez et al., 2023). The next interval (2–5 kDa) was covered by BW2 and YT2 hydrolysates (3.1 kDa and 4 kDa, respectively). The largest range of size (5–10 kDa), reached with the mildest processing conditions, was validated in both cases: 6.7 kDa in BW3 and 8.8 kDa in YT3. The number average molecular weight (Mn), defined as the most repetitive protein size, was also determined by GPC and the data are summarized in Table 3. Those sizes were around 30–90 % smaller than Mw, indicating that the polydispersivity index (PDI) of the hydrolysed protein material is in the interval from 1.29 to 1.87. This size distribution was quite similar when comparing between substrates, being a little larger in tuna, but it was much narrower at lower Mw. It seems clear that we can more accurately obtain protein sizes in the range of 1–2 kDa (PDI = 1.29 and 1.38) than 5–10 kDa (1.73 and 1.87).

As the most widespread commercial format of hydrolysates is in powder form, the liquid hydrolysates were dried by freeze-drying and then extensively analysed (Table 4). The yield of FPH produced from BW (59–122 g/kg of substrate) were lower than obtained from YT (70–173 g/kg of substrate), and the production of smaller Mw led to significant greater amounts of hydrolysates for both type of wastes ($p < 0.05$). Moisture content was quite similar in all cases ranged around 5.6–6.5 % (except in YT2). The objective of reducing ashes below 15 % was only achieved in BW1 and BW2, around 15 % was found in BW3 and YT2, being larger in YT1 and YT3 (22.5–24.5 %). The presence of relevant amount of blood traces in the heads of tuna (rich in iron) and the mineral salts in tuna effluents greatly hinder that aim. The continuous control of pH during the process of proteolysis improves the productive yields, increases the degree of hydrolysis and reduces the Mw of peptides, but also implies an inadequate increase in the level of ash, major complexity of the reaction system (pH-stat reactor) and the consequent rise in the production costs of FPH (Idowu et al., 2019; Vázquez et al., 2019; Kristoffersen et al., 2020).

The organic matter was the predominant component in the dry FPH, presenting a percentage of >5 % in BW samples than those found in YT, meanwhile the amount of total lipids was lower in the smallest FPH: YT1 and BW1 (2.4 % and 3.6 %, respectively), reaching around 7 % in BW3 (Table 4). These results show that by employing a more intense hydrolysis protocol we also improve the concomitant oil recovery after the centrifugation/decantation step of the liquid FPH. Although the substrates studied here are not particularly rich oil sources –BW without viscera and YT generated after mechanical extraction of oil from tuna heads– the strategy of combining enzymatic proteolysis and subsequent separation by centrifugation is an effective procedure for the retrieval of fish oils (Vázquez et al., 2019; Vázquez et al., 2022). The total protein level almost always remained above 70 % (77 % in BW1), except in the tuna with low and high Mw (YT1 and YT3) due to the high presence of ash already mentioned. This result was discrepant with the soluble protein value (larger in YT1 and YT2), indicating that an important amount of protein presented in BW could be mainly particulate. The presence of fish protein aggregates was also observed after heat treatment of hydrolysates (Shaviklo, 2015). In any case, the total protein values of our FPH are highly appreciated by the aquafeed formulating industry, which usually works with fish meals containing a protein level around 60–70 % (Macusi et al., 2023). Fish protein supplements with similar characteristics were also utilized for food fortification (Shaviklo, 2015).

3.3. Chemical and biological properties of tailored FPH

The amino acid profiles from the six FPH are summarized in Tables S1 and S2 (supplementary material). All amino acids –including essential ones for human and cultivated fish species (Ile, Leu, His, Lys, Met, Phe, Tre, Arg and Val)– are present in the hydrolysates produced (Hou et al., 2015; Hou and Wu, 2018), that is, they were not deficient in any of them. Glutamic and aspartic acids (18–19 % and 11–12 %, respectively) were the predominant amino acids in FPH of BW. The presence of lysine, alanine and leucine (larger than 7 %) was also especially remarkable. Hydrolysates performed using megrim, hake and red scorpionfish discards (Vázquez et al., 2020b) and utilizing by-products from seabream and seabass (Valcarcel et al., 2020c) reported quite similar amino acid composition than obtained here for BW. In the hydrolysates of YT, glutamic and glycine (around 11–18 %), followed by alanine and aspartic (8–10 %), were the most abundant protein building blocks. The percentages of proline and hydroxyproline were also

Table 4
Proximal composition of dry FPH with different molecular weights from blue whiting (BW) and yellowfin tuna (YT) wastes. BW1: FPH from BW in the range of 1–2 kDa. BW2: FPH from BW in the range of 2–5 kDa. BW3: FPH from BW in the range of 5–10 kDa. YT1: FPH from YT in the range of 1–2 kDa. YT2: FPH from YT in the range of 2–5 kDa. YT3: FPH from YT in the range of 5–10 kDa. YFPH: Yield of dry hydrolysate produced. Mo: Moisture. Ash: Ash. OM: Organic matter. Lip: Total lipids. Pr-tN: Total protein as total nitrogen x 6.25. Data are shown as average ± confidence intervals (n = 5; α = 0.05).

FPH	Y_{FPH} (%)	Mo (%)	Ash (%)	OM (%)	Lip (%)	Pr-tN (%)
BW1	12.2 ± 1.8[a]	6.39 ± 0.49[a]	12.15 ± 1.12[a]	81.46 ± 1.41[a]	3.60 ± 0.69[a]	76.72 ± 0.78[a]
BW2	7.3 ± 1.2[b]	5.21 ± 0.73[a]	14.12 ± 0.93[b]	80.67 ± 1.34[a,b]	5.14 ± 0.97[a,b]	74.55 ± 1.07[b]
BW3	5.9 ± 0.8[b]	6.52 ± 0.72[a]	15.18 ± 0.73[b]	78.31 ± 1.11[b]	6.81 ± 0.79[b]	71.22 ± 0.89[c]
YT1	17.3 ± 1.2[a]	5.61 ± 0.14[a]	22.51 ± 2.78[a]	71.88 ± 1.96[a]	2.39 ± 0.18[a]	68.13 ± 0.18[a]
YT2	14.7 ± 3.2[a]	7.83 ± 1.12[b]	16.14 ± 2.55[b]	76.03 ± 1.80[b]	4.37 ± 0.58[b]	71.47 ± 1.29[b]
YT3	7.0 ± 1.0[b]	5.81 ± 0.66[a]	24.45 ± 2.33[a]	69.75 ± 2.13[a]	4.61 ± 0.53[b]	64.62 ± 0.69[c]

important, mainly in YT3, and the level of glycine in this FPH increased significantly in relation to the most digested YT1 and YT2. This is indicative of the greater presence of collagen derivate peptides, with the application of non-exhaustive proteolysis conditions, since Pro, Gly and OHPro are the principal amino acids present in that type of protein (Smith and Rennie, 2007). In this context, FPH from wastes generated in the filleting of aquaculture species (salmon and turbot) were also composed with comparable proportions of Glu, Asp, Gly, Pro and OHPro (Vázquez et al., 2019; Vázquez et al., 2020c).

The ratio of essential amino acids per total amino acids (TEAA/TAA) was statistically similar ($p > 0.05$) in the BW hydrolysates (41–43 %), but significant differences were observed in the case of YT ($p < 0.05$). It means that the process of hydrolysis affected the final composition of tuna hydrolysates in high extent than observed in blue whiting, may be due to the great variability and heterogeneity of canning industrial substrates. Nevertheless, the level of essentials in FPH from BW and YT1 are very promising for potential use in human nutritive applications and as ingredient of aquafeeds (FAO/WHO/UNU, 1985; FAO/WHO, 1990).

The fatty acid content of the oil fraction still presents in FPH (Table 5) is also highly valued by feed and nutraceutical formulators, since the ω3/ω6 polyunsaturated ratios in BW and YT were 7–8 and 12–13, respectively, and the joint content of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), the most rewarding omega-3 fatty acids (Gacia-Moreno et al., 2013), ranged from 26 % in BW to 32 % in YT. Heavy metal contamination in FPH was below the limits established by legislation for its use in food and aquaculture feed (Commission Regulation, 2013; Commission Regulation, 2023): <0.12 ppm Cd, <0.25 ppm Hg and < 0.06 ppm Pb.

In order to get further insight on the functional validity of FPH, three types of *in vitro* biological properties were determined: the digestibility (Dig), the antioxidant capacity (using two methods, crocin and DPPH) and the antihypertensive activity (ACE) of FPH. In the first case, the value of Dig increased significantly, in both substrates, with the decrease in the average molecular weight of the hydrolysates, but without differences between BW and YT (Table 5). Such inverse correlation can be explained by the fact that the increase in the degree of hydrolysis generates smaller and more soluble peptides that are much more nutritious and easily digestible (Ramakrishnan et al., 2023). The current values, higher than 91 %, are in line with previously reported results found in other fish hydrolysates (Nisov et al., 2022; Vázquez et al., 2022).

Fig. 3 depicts the antioxidant and antihypertensive activities for FPH. The outcomes from DPPH were evaluated by means of the maximum percentage of activity and by the inhibitory concentration at 50 % (IC_{50}) in relation to the BHT control (Munteanu and Apetrei, 2021; Mendonça et al., 2022). From them, we can verify that in both, tuna and blue whiting hydrolysates, the best antioxidant capacities (higher DPPH% and lower value of mg BHT/g FPH) increased significantly with the reduction in molecular weight ($p < 0.05$). Moreover, the samples from BW revealed higher antioxidant ability than described by YT. Similar trends were observed when FPH samples were analysed by the second *in vitro* antioxidant protocol (Crocin method). In this case, IC_{50} data in terms of Trolox equivalent was significantly lower (larger antioxidant potential) at smaller Mw. These highlights are in agreement with the results reported in hydrolysates of other fish species. FPH from silver catfish (Shaik et al., 2021) and white shrimp (Latorres et al., 2022) processed by ultrafiltration, with the aim of selecting peptide fractions of different Mw, showed an enhanced DPPH sequestering capacity (higher antioxidant activity) when the fraction was below 3 kDa. However, the values of DPPH scavenging activity were moderately promising (percentages inferior to 62 % and $IC_{50} > 70$ mg BHT/g FPH) in comparison with synthetic and antioxidants from terrestrial vegetables and microalgae (Carneiro et al., 2013; Cruz et al., 2019; Aremu et al., 2016), and even regarding other FPH (Amado et al., 2013; Devita et al., 2021).

Finally, the inhibition data of ACE (I_{ACE}) ranged between 16 % and 71 % from large to small hydrolysates of BW, respectively, and in the interval of 21–63 % for the same Mw tendency in YT (Fig. 3). In both substrates, the differences of I_{ACE} between FPH sizes were significant ($p < 0.05$). In concordance with those higher values, FPH from fillets of BW and muscle of red scorpionfish led to 50–75 % of I_{ACE} (Geirsdottir et al., 2011; Aissaoui et al., 2017). However, other hydrolysates from turbot-viscera, grenadier and trout-head, all of them obtained using alcalase and also with Mw smaller than 2 kDa, showed I_{ACE} outcomes around 78–90 % (Vázquez et al., 2019, 2020b, 2020c). Besides, silver catfish protein hydrolysates purified by 3 kDa-ultrafiltration membrane, showed 88 % of I_{ACE} (Shaik et al., 2021). In terms of IC_{50}-ACE, the patterns were complementary to mention for I_{ACE} results: higher activities (80 mg/L in BW and 179 mg/L in YT) were found at smaller size of 1.4 kDa and 1.7 kDa, respectively. These values were much better than those reported for FPH from eels and red scorpionfish-head (490–2130 mg/L) (Baharuddin et al., 2016; Aissaoui et al., 2017), in the same order of magnitude than those found in hydrolysates of gurnard and pouting skins (152–211 mg/L) (Vázquez et al., 2019), but much less active in reducing blood pressure than those defined in the shrimp cooking

Table 5
Omega-3/omega-6 ratio, presence of DHA + EPA, heavy metals content and *in vitro* digestibility for each hydrolysate produced. Data are shown as average ± confidence intervals (n = 3; α = 0.05).

FPH	ω3/ω6	DHA + EPA (%)	Cd (ppm)	Pb (ppm)	Hg (ppm)	Dig (%)
BW1	7–8	28–30	0.018 ± 0.004[a]	0.014 ± 0.008[a]	0.172 ± 0.020[a]	95.8 ± 0.8[a]
BW2	7–8	27–28	0.019 ± 0.005[a]	0.019 ± 0.009[a]	0.248 ± 0.013[b]	94.8 ± 0.7[a]
BW3	7–8	26–29	0.017 ± 0.004[a]	0.055 ± 0.005[b]	0.225 ± 0.013[b]	91.3 ± 0.5[b]
YT1	12.1–13	31–32	0.120 ± 0.016[a]	0.026 ± 0.012[a]	0.155 ± 0.032[a,b]	94.8 ± 1.3[a]
YT2	11.6–13	31–32	0.115 ± 0.010[a]	0.016 ± 0.003[a]	0.135 ± 0.020[a]	94.0 ± 1.1[a]
YT3	11–12	31–32	0.105 ± 0.008[a]	0.012 ± 0.004[a]	0.184 ± 0.022[b]	90.7 ± 1.7[b]

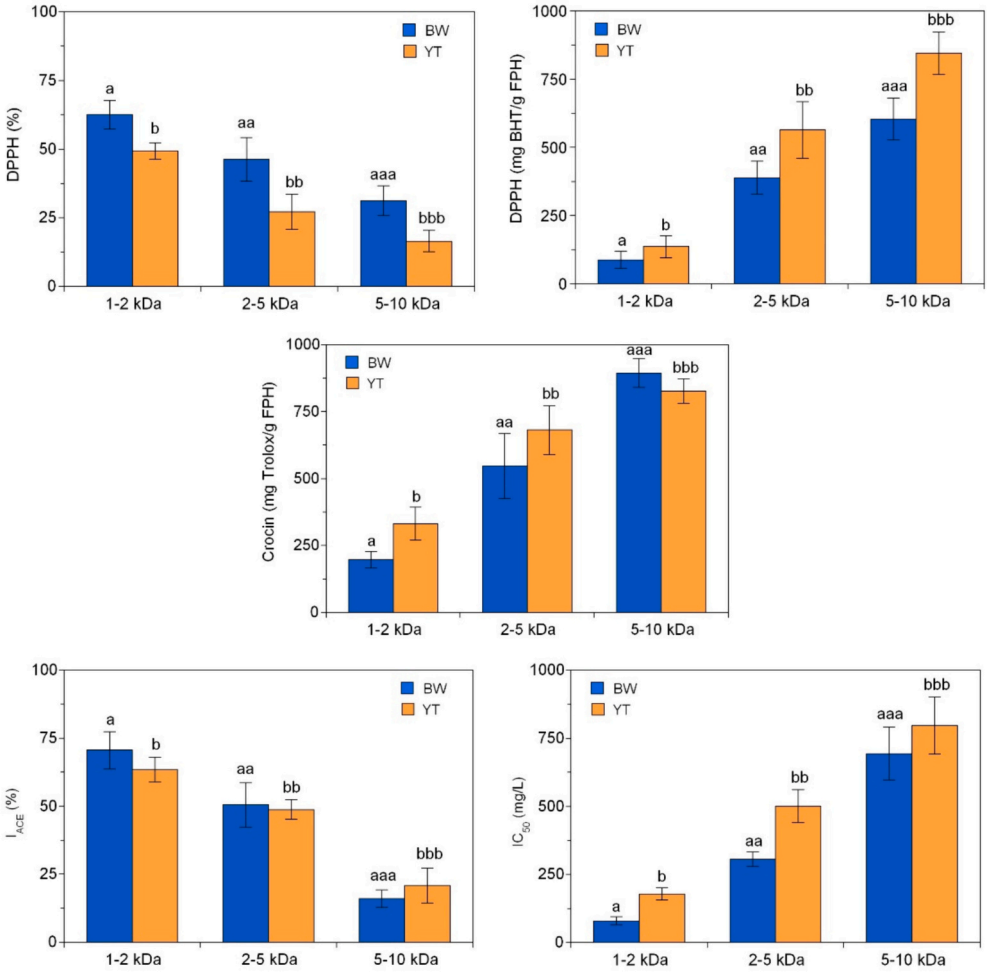

Fig. 3. Histograms showed the bioactivity values (antioxidants and antihypertensive) for the FPH produced at different molecular weights ranges. Different number of letters mean significant differences between hydrolysates of the same substrate (a-letter for BW and b-letter for YT).

wastewaters (2–10 mg/L) processed by a combination of hydrolysis and ultrafiltration (Amado et al., 2016).

Nevertheless and to be honest, the present work has some limitations to be extended to other FPH productions or that hydrolysates can be included in commercial formulations. The main limitations are: 1) Although the mathematical models and the protocol of optimization used can be extrapolated to other proteolytic processes, the numerical values of the parameters are only valid for the type of substrate, enzyme and experimental conditions described here. 2) The *in vitro* bioactive outcomes are preliminary and further studies of purification should be executed to isolate and characterise the peptides responsible of bioactivities. 3) To confirm the real functionality of our hydrolysates in order to be applied in nutraceuticals and fish feed, they must be assessed in fish diets and nutritional intervention trials in humans. 4) Analysis of carbon footprint and life cycle must be further done to validate it from a circular economy viewpoint.

4. Conclusion

This work describes the optimized production of tailored enzymatic protein hydrolysates from blue whiting discards and tuna wastes, using a mathematical model based on the combination of a reaction mechanism and non-linear equations affecting parameters. This resource was adequate to define the conditions needed to obtain customized FPH in terms of controlling average molecular weight of proteins. Scaled-up productions were then performed to obtain hydrolysates of three protein size intervals previously established, confirming the validity of the optimization protocol. FPH with smallest molecular weight led to the largest protein content and the best digestion yield, as well as the highest dry hydrolysate amount and the essential amino acids percentage. Furthermore, the level of heavy metals was below the legislative threshold, and the bioactivities values were also higher at lower protein size of the hydrolysates. The enzyme process here explored supposes a sustainable strategy useful to produce FPH from fish waste substrates with customized average molecular weights and enhancing chemical and biological properties.

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CRediT authorship contribution statement

José Antonio Vázquez: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project

administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Sara Comesaña:** Writing – review & editing, Conceptualization. **José Luis Soengas:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Montse Pérez:** Writing – review & editing, Conceptualization. **Roberto Bermúdez:** Writing – review & editing, Conceptualization. **Josep Rotllant:** Writing – review & editing, Conceptualization. **Jesus Valcarcel:** Writing – review & editing, Methodology, Investigation.

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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Appendix A. Supplementary data

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