

FUNCTIONAL, BIOACTIVE AND ANTIGENICITY PROPERTIES OF BLUE WHITING PROTEIN HYDROLYSATES: EFFECT OF ENZYMATIC TREATMENT AND DEGREE OF HYDROLYSIS

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ABSTRACT

BACKGROUND: Fish discards represent an important underutilization of marine resources. This study evaluated the up-grading of the protein fraction of blue whiting (*Micromesistius poutassou*) discards by the production of fish protein hydrolysates (FPH) exhibiting functional, antioxidant, angiotensin-I converting enzyme (ACE)-inhibitory and antigenicity properties.

RESULTS: FPH with low DH (4%) showed better emulsifying, foaming and oil binding capacities, particularly those obtained using only trypsin. FPH with DH 4% exhibited also the stronger antioxidant activity, especially the one obtained using only subtilisin ($IC_{50}=1.36$ mg protein mL^{-1}). The presence of hydrophobic residues at the C-terminal of the FPH produced using subtilisin also led to the stronger ACE-inhibitory activity. However, FPH with high DH (12%), which implies a higher proportion of short peptides, was required to enhance ACE-inhibition ($IC_{50}=172$ μg protein mL^{-1}). The antigenic levels of the FPH were also reduced with DH independently of the enzymatic treatment. Nevertheless, the highest degradation of fish allergens (e.g. parvalbumin) was also obtained when using only subtilisin.

CONCLUSION: These results suggest that added-value products for food applications can be produced from the protein fraction of discards.

Keywords: discards, fish protein hydrolysates, functional properties, antioxidant activity, ACE-inhibitory activity, antigenicity

INTRODUCTION

Fish industry generates a large amount of by-products, being discards the most significant waste of fish resources.¹ Discards represent not only an irresponsible underutilization of marine stocks, which affects negatively future fishing production, but also they have a negative impact on marine ecosystem. Indeed, most of the individuals which are returned to the sea are died or dying, which alters the structure of trophic chains since it favors the presence of depredator species.² Thus, the European Commission is currently undertaking a depth reform in common fisheries policy, adopting a set of measures towards the complete elimination of discards in EU fisheries, the so-called zero-discard policy.³ Nevertheless, discards bans must be accompanied with the production of added-value products from this non-commercial material which must be totally landed. In the West Mediterranean Sea, trawling is one the main fishing gears with a global discard rate of 23%. Among the discarded species, specimens of blue whiting (*Micromesistius poutassou*) represent the majority of the total discards within this fishing gear. Although blue whiting is a target species, it is discarded due to minimum landing size requirements (15 cm), fishing quota and high grading practices.⁴

Blue whiting discards can be up-graded based on their protein content, 180 kg⁻¹ along the year given in a wet basis (not published results). In this sense, enzymatic hydrolysis of proteins is an effective process for the recovery of protein material from fish by-products since the reaction is carried out at mild conditions, side reactions are avoided and the products obtained have a high nutritional value.⁵ Fish protein hydrolysates (FPH), containing modified proteins, peptides and amino acids, have numerous applications in the food industry due to their functional properties such as improved solubility, emulsifying and foaming properties and water holding and oil binding capacities.⁶⁻⁷ Furthermore, the production of FPH exhibiting bioactive properties has gained an increasing interest in the last decade due to the added value of these products.⁸ In this regard, several studies have reported protein hydrolysates with antioxidant activity obtained from discarded fish species such as mackerel, sardine, horse mackerel, bogue, axillary seabream and small-spotted

catshark.⁹⁻¹⁰ FPH exhibiting angiotensin I-converting enzyme (ACE)-inhibitory activity have been also produced as alternative antihypertensive agents from different fish species.¹¹⁻¹² In addition, enzymatic hydrolysis of fish protein is expected to degrade fish allergens (e.g. parvalbumin) reducing the immunoreactivity of the native protein. This is of special importance when developing food products containing fish protein since parvalbumin retains its allergenicity even after heat treatment or exposure to extreme pH.¹³ It should be also noted that the functional and biological properties of FPH will depend, apart from the characteristics of the substrate hydrolysed, on the enzymatic treatment employed as well as on the extent of the hydrolysis reaction. Subtilisin and trypsin are enzymes widely used to produce FPH exhibiting technological^{14,15} and bioactive properties;¹⁶⁻¹⁷ however, the combination of both enzymes has not been previously evaluated on blue whiting proteins.

In light of the above, this work aimed to study the potential of blue whiting for the production of FPH exhibiting: i) functional properties (solubility, emulsifying and foaming capacities and water and fat absorption capacities), and ii) biological activities (antioxidant, ACE-inhibitory and antigenicity). Particularly, the influence of both enzymatic treatment (subtilisin, trypsin and combined treatment of subtilisin and trypsin) and degree of hydrolysis (DH: 4, 8 and 12%) on FPH properties was evaluated. Contrarily to Geirsdottir et al.¹⁸, who employed as raw material a protein isolate from fillets of blue whiting, we used a dewatered and defatted protein cake obtained by hydraulic pressing of whole fish.

MATERIALS AND METHODS

Raw material

Blue whiting (*Micromesistius poutassou*) was provided by the fishing harbour of Motril (Spain). Fish were kept in ice during transportation. In the same day, whole fish, including skin, bones and internal organs, were preheated at 40 °C for 30 min (Digiterm 100, Selecta, Barcelona, Spain) and hydraulic pressed according to García-Moreno et al.¹⁰ in order to obtain a dewatered and defatted

protein cake. The proximal composition of the protein cake was determined according to the AOAC official methods,¹⁹ and it was as follows: 184 g kg⁻¹ of proteins, 17 g kg⁻¹ of lipids, 40 g kg⁻¹ of mineral matter and 770 g kg⁻¹ of moisture.

Enzymatic hydrolysis

Two serine endoprotease enzymes were employed: one of bacterial origin (subtilisin, EC 3.4.21.62) and other from an animal source (pancreatic trypsin, EC 3.4.21.4). Both were provided by Novozymes (Denmark) as Alcalase 2.4L and PTN 6.0S, respectively. Three different enzymatic treatments were assayed: i) enzymatic hydrolysis with subtilisin (S), ii) hydrolysis with trypsin (T), and iii) hydrolysis employing a mixture 1:1 (by weight) of both enzymes (ST). For each enzymatic treatment, three protein hydrolysates were produced at different DH, namely 4%, 8% and 12%.

A given mass of grinded press cake was homogenised with demineralised water until reaching a final volume of 200 mL. This suspension, having a protein concentration of 25 g L⁻¹, was then transferred into a jacketed reactor of volume capacity 250 mL. The experiments were conducted at the optimum conditions for each enzymatic treatment: S, pH 8 and 50 °C; T, pH 8 and 40 °C; ST, pH 8 and 45 °C. In order to have hydrolysis time larger than 20 min, the enzyme protein ratio for each treatment assayed was varied from 0.1 to 6% (by weight) depending on the desired DH. The DH was calculated according to the pH-stat method as described by García-Moreno et al.¹⁰ Reactions were stopped by heating the reaction mixture at 100 °C for 15 min. Then, hydrolysates were cooled down to room temperature and freeze-dried in a Labconco freeze drying system (Kansas City, MO, USA). The lyophilised obtained was employed for the subsequent analysis.

Protein determination

The protein content of the powdered hydrolysates was determined by the Kjeldahl method, assuming a nitrogen-to-protein conversion factor of 6.25.¹⁹

Molecular mass distribution

The molecular weight distribution of the hydrolysates was determined by size exclusion chromatography (SEC). Lyophilized hydrolysates were dissolved with ultrapure water at protein concentration of 5 mg mL⁻¹. In order to obtain the molecular size distribution, 100 µL of the above solutions were injected in a fast protein liquid chromatography system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) mounted with Superdex Peptide 10/300GL column (GE Healthcare, Uppsala, Sweden). The samples were eluted at 0.5 mL min⁻¹ with 70:30 water/acetonitrile with 1 mL L⁻¹ of TFA. The fractionation was monitored by measuring the absorbance at 280 nm. The column was calibrated with compounds of known molecular weight: Gly (75 Da), Ala (89 Da), Phe-Gly-Gly (279 Da), (Gly)₆ (360 Da), vitamin B12 (1355 Da), insulin (5733 Da), aprotinin (6511 Da) and ribonuclease (13700 Da).

Functional properties

Solubility

Solubility of the powdered hydrolysates was determined according to method proposed by Amiri-Rigi et al.²⁰ To this end, 100 mg of powdered hydrolysate were mixed with 30 mL of ultrapure water and stirred at 500 rpm and 20 °C for 5 min. The resulting suspension was centrifuged at 3,000×g for 5 min. Supernatant, 15 mL, was recovered and dried in an oven at 110 °C for 4 h. The solubility of the sample was expressed as the percentage of total solids, related to the initial mass, recovered in the supernatant.

Emulsion capacity and stability

The emulsifying property of the protein hydrolysates was evaluated by means of the emulsifying capacity (EC) and the emulsion stability (ES). Both indices were determined according to Klompong et al.²¹ with some modifications. A mixture containing 300 mg of powdered hydrolysate, 30 mL of deionized water and 10 mL of sunflower oil was homogenized at 13,000 rpm for two min

by means of an Ultra-Turrax (T25 basic, IKA, Staufen, Germany). At that point, 50 μL were drawn from the bottom of the homogenizer, and stabilized with 5 mL of dodecyl sulfate sodium (10 mL L^{-1}). This step was repeated after 10 min, to evaluate the emulsion stability. The emulsifying property was calculated from the absorbance of both samples at 500 nm, according to the formulas below:

$$EC \text{ (m}^2 \text{ g}^{-1}\text{)} = \frac{2 \cdot 2.303 \cdot A_0}{0.25 \cdot m_p} \quad [1]$$

$$ES = \frac{A_0 \cdot \Delta t}{A_0 - A_{10}} \quad [2]$$

where A_0 and A_{10} stand for the absorbance at 500 nm for the first and second aliquots, m_p is the mass of protein (g) and Δt is the time interval (min) between both aliquots were drawn (i.e. 10 min).

Foaming properties

The foaming properties of the samples were measured according to Elavarasan et al.²² with some modifications. A suspension containing 250 mg of powdered hydrolysate in 25 mL of deionized water was homogenized at 13,500 rpm (Ultra-Turrax T25 basic, IKA, Staufen, Germany) for 3 min at room temperature. This operation incorporates air to the sample. After this treatment, the sample was transferred into a 50-mL cylindrical cylinder. The volume occupied by the foam was measured at initial time and after 60 min, to evaluate the stability of the foam. The foaming capacity (FC) was calculated as the initial volume of foam related to that of the suspension before whipping (25 mL). Similarly, the foaming stability (FS) of the sample was calculated from the volume of foam after 60 min.

Water Holding Capacity

The water holding capacity of the hydrolysates was measured against hake (*Merluccius merluccius*) muscle, following the method of Slizyte et al.²³ A mixture of 300 mg of powdered hydrolysate and 10 g of hake muscle mince was suspended in 10 mL of deionized water. After homogenization, the samples were centrifuged for 10 min at $500\times g$ and room temperature. The supernatant (i.e. water unbound to the protein matrix) was recovered, expressing the water holding capacity (WHC) of

each sample as the volume of water retained in the centrifuge slurry, related to the mass of water initially added (10 mL).

Oil Binding Capacity

Oil binding capacity (OBC) determination was adapted from Sathivel et al.²⁴ To this end, 250 mg of the powdered hydrolysate were suspended in 5 mL of standard olive oil and transferred to a centrifuge tube. After homogenization, the tubes were centrifuged for 25 min at 2,560×g and room temperature. The supernatant (i.e. oil unbound to the hydrolysates) was recovered by allowing the centrifuge tube to drain at 45° inclination for 10 min. OBC was expressed as the volume of oil retained in the centrifuge slurry, related to the amount of powdered hydrolysate.

Bioactive properties

Antioxidant activity

The antioxidant activity of the hydrolysates was determined by measuring their 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity according to Picot et al.²⁵ Lyophilized hydrolysates were dissolved in distilled water, obtaining different solutions (0.5-5 mg mL⁻¹). In short, a volume of 1 mL of each protein hydrolysate solution was added to 1 mL of 0.1 mM DPPH in methanol. The mixture was then kept at room temperature in the dark for 30 min, and the absorbance was measured at 515 nm. A blank was run in the same way by using distilled water instead of sample, and sample control was also made for each sample by adding methanol instead of DPPH solution. Triplicate measurements were carried out for each sample and DPPH scavenging activity was calculated as follows:

$$\text{DPPH inhibition (\%)} = \left(1 - \frac{A_{\text{sample}} - A_{\text{sample, control}}}{A_{\text{blank}}}\right) \times 100 \quad [3]$$

The IC₅₀ value, which is defined as the concentration of hydrolysate (mg protein mL⁻¹) needed to inhibit DPPH activity by 50%, was calculated for each hydrolysate.

ACE-inhibitory activity

The ACE inhibitory activity of the hydrolysates was determined *in vitro* by the methodology described by Shalaby et al.²⁶ This method is based in the hydrolysis of the tripeptide N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG, Sigma F7131) with the Angiotensin converting enzyme (ACE) from rabbit lung (Sigma A6778). The assay was carried out in 96-well microplate at 37 °C. Each well contained 10 µL of enzyme solution (0.25 U mL⁻¹), 10 µL of sample, and 150 µL of 0.88 mM of FAPGG in buffer Tris-HCl 50 mM, pH 7.5 and 0.3 M of NaCl. The wavelength was set at 340 nm and the absorbance was monitored during 30 minutes by means of a Multiskan FC microplate photometer (Thermo Scientific, Vantaa, Finland). Each sample was analysed in triplicate.

The absorbance decreases linearly with time as ACE hydrolyses the substrate FAPGG. The slope of this descent is commonly used as a measurement of the enzyme activity. Indeed, the numerical value of inhibitory activity of each hydrolysate can be calculated by Eq. 4:

$$\text{ACE inhibition (\%)} = \left(1 - \frac{\rho_i}{\rho_o}\right) \times 100 \quad [4]$$

where ρ_i was the slope in the presence of inhibitor (hydrolysate) and ρ_o the slope obtained in the absence of inhibitor (pure water). These slopes were calculated from the values obtained within the interval of 10 to 25 min, where a better linearity was observed.

The IC₅₀ value, which is defined as the concentration of hydrolysate (µg protein mL⁻¹) needed to inhibit 50% of ACE activity, was also determined for each hydrolysate.

Antigenicity

An inhibition ELISA test was employed to quantify the level of antigenicity of the protein hydrolysates. Polystyrene microtiter plates (Fisher Scientific, Waltham, MA, USA) were coated with 200 µL per well of a solution containing 50 mg mL⁻¹ of blue whiting native protein in a coating buffer Na₂CO₃/NaHCO₃ at pH 9.6. After overnight incubation, the microplates were washed and filled with 20 mg mL⁻¹ of bovine serum albumin in phosphate saline buffer with tween (PBST, pH

7.4), followed by incubation at 37°C for 30 min. The reaction antigen-antibody was carried out separately, by incubating (37°C, 1 h) a mixture containing 1 part of serial 10-fold PBST solutions of the powdered hydrolysates with 9 parts of a 1:2000 solution of monoclonal anti-Parvalbumin (PV 235, Swant, Marly, Switzerland) in PBST. After washing the plates 3 times with PBST, 200 µL of the incubated solution were transferred to each well and incubated again at 37°C for 1 h. At that point, the microplate was again washed three times and 200 µL of a PBST solution 1:1000 of peroxidase-conjugated radish anti-mouse IgG. Binding was visualized using 5 mL L⁻¹ of 3,3',5,5'-tetramethylbenzidine (Sigma Aldrich, St. Louis, MO, USA) and 0.25 mL L⁻¹ of hydrogen peroxide in citrate buffer (25 mM citric acid, 50 mM Na₂HPO₄). After the color was developed, the reaction was stopped with 2.5 N sulfuric acid and the absorbance was measured at 450 nm. Percentage inhibition was then calculated as follows:

$$\text{Inhibition (\%)} = \left(\frac{A_{\max} - A_{\text{sample}}}{A_{\max} - A_0} \right) \times 100 \quad [5]$$

where A_{sample} is the absorbance of a given sample, $A_{\max}$ is the maximum value of absorbance, corresponding to a dilution 1:2000 of anti-Parvalbumin in PBST and A_0 is the absorbance of the blank (i.e. PBST solution).

Statistical analysis

Data were subjected to analysis of variance (ANOVA) by means of a statistical program (Statgraphics 5.1.). The Tukey's multiple range test was used to determine whether significant differences existed among enzymatic treatments and degree of hydrolysis. Differences among mean values were considered significant at a level of confidence of 95% ($p < 0.05$).

RESULTS AND DISCUSSION

Characterization of hydrolysates

Protein content

The protein content of the powdered hydrolysates presented no significant differences among the enzymatic treatments, but did with DH. Effectively, it decreased from an average $828 \pm 7 \text{ mg kg}^{-1}$ for DH 4% down to $744 \pm 24 \text{ mg kg}^{-1}$ for DH 12%. These differences in protein content are due to increasing levels of salts in the final hydrolysate.²⁷ This is attributable to the pH-stat method, which compensates the pH drop by adding titration agent (NaOH 0.5 N) to the reactor vessel.

Molecular weight distribution

Fig. 1 shows the size exclusion chromatograms obtained for the hydrolysates. It was observed that increasing the extent of hydrolysis reaction led to significant changes in the molecular size profile of the hydrolysates produced. For evaluating the influence of enzymatic treatment and DH on molecular weight profile, four clearly different fractions observed in the hydrolysates were used: fraction A $>903 \text{ Da}$, fraction B ranged between $903 - 337 \text{ Da}$, fraction C varied between $337 - 92 \text{ Da}$ and fraction D which contained compounds below 92 Da . In addition, fraction D could also contain small peptides larger than 92 Da which interact with the stationary phase.

As expected, the proportion of large peptides (fraction A) decreased when increasing DH (Table 1). Moreover, independently of DH, the combination of both enzymes led to hydrolysates with lower proportion of large peptides. Fraction B ($903 - 337$) was generally the smallest fraction, presenting a maximum proportion of 15.6%. The proportion of this fraction seems to increase when DH change from 4 to 8%. However, after that increment, the proportion of peptides did not change when raising the DH to 12%. In the case of fraction C ($337-92 \text{ Da}$), there are differences between treatments assayed. While trypsin hydrolysates increased their proportion of fraction C with DH, the two hydrolysates employing subtilisin presented a significant increment in fraction C only at

high DH (12%). Fraction D (<92 Da), composed by amino acids and peptides which interact with the stationary phase of the column, was the most abundant fraction for all the hydrolysates. Hydrolysates produced with the combination of subtilisin and trypsin presented the highest proportion of fraction D (Table 1).

In general, the hydrolysates produced had considerable high proportion of short peptides (smaller than 900 Da), which ranged from 75 to 90 %. In this line, Chabeaud et al.²⁸ found that peptides lower than 1000 Da in subtilisin-hydrolysate of saithe (DH 10.96) represented the 56% of the total peptide content.

Functional properties

Solubility

The percentage solubility of the nine hydrolysates is presented in Fig. 2, where the samples were arranged in three groups corresponding to the enzymatic treatments. As a general trend, it can be observed that DH affected positively the solubility ($p < 0.05$). Indeed, the average solubility of subtilisin hydrolysates increased from $78.90 \pm 1.03\%$ (DH 4%) to $85.00 \pm 0.70\%$ (DH 12%). Similarly, the solubility of the hydrolysates produced by the combined treatment improved from $78.90 \pm 1.70\%$ to $86.70 \pm 1.40\%$. As for the trypsin hydrolysates, they presented a maximal solubility ($84.70 \pm 2.40\%$) at DH 8% which was maintained at DH 12% ($83.90 \pm 0.20\%$). These results were expected since increasing DH leads to smaller average peptide size and improves the exposure of polar residues to the medium.^{6,21,29} Effectively, the size exclusion chromatograms (Table 1) confirmed that the extent of the hydrolysis reaction increased the percentage of short peptides below 900 Da (i.e. fractions B, C and D). According to the multiple comparison test, there were no significant differences among the three enzymatic treatments studied. In this line, Chamalaiah et al.³⁰ reported similar levels of protein solubility for subtilisin and trypsin hydrolysates from carp roe, being superior to pepsin or papain hydrolysates. Subtilisin is a wide spectrum protease, commonly reported to improve protein solubility. In contrast, trypsin cleaves preferably peptide

bonds with Arg or Lys residues, releasing positively charged (and therefore hydrophilic) peptides to the medium.³¹

Emulsifying properties

The emulsifying properties of FPH are related to the adsorption and stabilization of the protein on oil-water interfaces. They depend on the surface properties of the protein, mainly the presence of hydrophobic groups which facilitate the adsorption of protein to oil-water interface.¹⁴ Therefore, the emulsifying properties are highly influenced by the degree of hydrolysis. As a general trend, emulsifying properties of FPH worsen with increasing DH due to the release of small peptides which exhibit less surface activity and hydrophobicity.^{6,32} The average values of emulsifying capacity (EC) and emulsion stability (ES) are represented in Fig. 3a and Fig. 3b, respectively. It can be observed that EC was negatively correlated to DH for the three enzymatic treatments assayed. Furthermore, the values of EC for the hydrolysates at DH 4% were markedly superior to the rest of samples. Maximal values of EC were attained at DH 4% for the trypsin hydrolysates ($75.3 \text{ m}^2 \text{ g}^{-1}$), followed by the subtilisin-trypsin hydrolysates ($51.1 \text{ m}^2 \text{ g}^{-1}$). Regarding the emulsion stability, subtilisin hydrolysates at DH 4% presented the maximal ES (92.7 min) in the experimental series. This value remained constant at 40 min for the rest of subtilisin hydrolysates, and was on average 58 min for the trypsin hydrolysates. The poor emulsion properties exhibited by subtilisin hydrolysates are in line with the results reported on Alcalase hydrolysates from several fish species such as Cape hake,¹⁴ blue whiting¹⁸ or fresh water carp.²² Moreover, the superior emulsifying properties exhibited by trypsin hydrolysates, compared to those produced by Alcalase, were also found by Blanco et al.¹⁵ on boardfish FPH.

Foaming properties

Fish protein hydrolysates are able to form stable foams which are responsible for the texture of some food products such as fish based pâtés or soufflés.¹⁴ Since foaming properties are dependent

on the surface activity of the proteins, they present a similar trend with DH as previously reported for the emulsifying properties.²¹

The blue whiting hydrolysates were evaluated for their foaming capacity (FC) and foaming stability (FS) indices, as shown in Fig. 4. As a general trend, it was observed that both indices decreased significantly with the DH for the three enzymatic treatments assayed. The highest values of FC were evaluated for trypsin hydrolysates at DH 4% (91.5 %) followed by subtilisin-trypsin hydrolysates (67.1%), as it occurred with the emulsifying capacity. These values dropped significantly for the rest of hydrolysates. The levels of FC obtained for subtilisin hydrolysates are inferior to those reported for other FPH employing the same enzyme, such as Cape hake,¹⁴ yellow stripe trevally²¹ or fresh water carp.²²

With regard to foam stability, the highest values were observed at DH 4% for the subtilisin-trypsin hydrolysates (29.1%) followed by subtilisin hydrolysates (18.9%). These values are comparable to those reported for Cape hake hydrolysates with alcalase,³³ which presented FS values between 10 – 30% after 60 min. Trypsin hydrolysates presented similar levels of FS at DH 4% and 8% (15.8% on average). In our case, no significant difference was found between trypsin and subtilisin hydrolysates at DH 4% and 8%. This is in contrast with the results found by Blanco et al.¹⁵ for boarfish hydrolysates, where FS for trypsin hydrolysates (35% after 30 min) was clearly superior to that of Alcalase hydrolysates (7%).

Water Holding Capacity

Water Holding Capacity (WHC) is of great interest in food industry given its influence on the economic feasibility of food processing, as well as the sensory properties of the final product.¹⁴ Kristinsson and Rasco⁶ reported that the addition of variable levels of fish protein hydrolysates to protein matrices improved their ability to bind and hold water molecules. Enzymatic hydrolysis increases the amount of polar carboxyl and amino groups released to the medium; and therefore, increases the hygroscopicity of the fish protein hydrolysate when compared to the native protein.

Accordingly, Wassswa et al.³⁴ and Balti et al.³⁵ observed an increase of WHC of protein matrices with the degree of hydrolysis of the FPH added.

In contrast, this work did not find significant differences in WHC among enzymatic treatments and DH, except for the subtilisin hydrolysate at DH 4% (Fig. 5a). This hydrolysate presented $45.9 \pm 0.8\%$ WHC, while the average of the rest of hydrolysates was 35.4%. The addition of blue whiting hydrolysates improved the WHC of the hake muscle in 95% for the subtilisin hydrolysate at DH 4% and 50% for the rest of samples. These results are in line with previous works on blue whiting hydrolysates¹⁸ or cobia frame hydrolysates.³⁶ Geirsdottir et al.¹⁸ evaluated WHC on a cod mince supplemented with blue whiting hydrolysates produced at increasing DH. The samples presented an average WHC of 97%, unaffected by DH. In this case, the addition of hydrolysates only increased in 18% the initial WHC of the cod mince. Šližytė et al.²³ reported a similar trend between WHC and DH for cod backbones hydrolysates. Indeed, they found that the addition of FPH with low degree of hydrolysis (10 min hydrolysis) gave the highest WHC in cod mince. This value decreased and remained constant for the rest of hydrolysates, produced at longer times of reaction. The authors concluded that the high WHC of partially hydrolyzed proteins may be explained by their high ratio of surface area to mass. Moreover, the unfolding of quaternary structure exposed previously buried hydrophilic groups, increasing the affinity of protein for water molecules.

Oil Binding Capacity

Oil Binding Capacity (OBC) is a technological property of interest in the meat and confectionery industries,¹⁴ where the lipids are commonly present in protein-fat emulsion systems. Previous studies have related certain properties of fish protein hydrolysates, such as enzyme treatment and DH, with their ability to bind fats.^{6,32}

The average values of OBC, reported as the volume of oil (mL) bound per gram of FPH, are shown in Fig. 5b. It can be observed that both the enzymatic treatment and the degree of hydrolysis affected significantly the levels of fat absorption. Both subtilisin and subtilisin-trypsin hydrolysates presented a negative correlation between OBC and DH. The average values of OBC for subtilisin

hydrolysates dropped with DH from an average 3.74 ml g⁻¹ (DH 4%) to 1.85 ml g⁻¹ (DH 12%). These levels are similar to those reported by Geirsdottir et al.¹⁸ for the blue whiting hydrolysates produced with Alcalase, where OBC decreased from 3.1 ml g⁻¹ (DH 2.5%) to 2.2 ml g⁻¹ (DH 15%). The combined treatment employing subtilisin plus trypsin led to the higher levels of fat binding, ranging from 5.39 ml g⁻¹ (DH 4%) to 2.03 ml g⁻¹ (DH 12%). A similar trend has been reported for blue whiting,¹⁸ salmon,³⁷ and herring³⁸ protein hydrolysates, among other species. As said above for WHC, the extent of the hydrolysis releases polar groups to the medium. This fact reduces the hydrophobicity of the hydrolysates, and therefore their ability to bind fats. As for the trypsin hydrolysates, DH did not affect significantly their values of OBC, binding on average 3.61 ml g⁻¹.

Bioactive properties

Antioxidant activity

Lipid oxidation reactions in food reduce the nutritional value and quality of the product. Besides, oxidation and physiological level generates free radicals (e.g. OH·) which initiate or propagate chain reactions, resulting in the damage of lipid membranes, structural proteins, enzymes and DNA structure.³⁹ Therefore, antioxidants compounds which can delay or interrupt chain oxidation reactions by scavenging free radicals or acting as chain terminators are required.

In this study, the DPPH radical scavenging activity of the FPH was measured as a function of the protein concentration in order to determine their IC₅₀ values (Fig. 6a). The IC₅₀ values obtained ranged from 1.36 to 2.46 mg protein mL⁻¹. These results are in line with previous studies producing FPH from different species such as sardine, horse mackerel, axillary seabream and bogue,¹⁰ 0.91-2.91 mg protein mL⁻¹, and rohu, 1.5 mg hydrolysate mL⁻¹.⁴⁰

Independently of the enzymatic treatment, FPH with the lowest DH assayed (4%) presented the highest antioxidant activity, having lower IC₅₀ values (Fig. 6a). This may be explained by the different size of the peptides in the hydrolysates with different DH. Hydrolysates with DH 4%

presented a higher proportion of peptides larger than 903 Da (Table 1). In this sense, Wu et al.⁴¹ reported that mackerel hydrolysate, with molecular weight of approximately 1400 Da, exhibited a stronger antioxidant activity when compared to mackerel hydrolysates with peptides of 900 and 200 Da. In the same line, Klompong et al.²¹ also found a stronger DPPH scavenging activity for hydrolysates of yellow strip trevally with DH 5% when compared to 15 and 25%. The same effect of DH was observed by Theodore et al.⁴² for channel catfish hydrolysates produced with Protamex. Nevertheless, contradictory results on the influence of DH of FPH on DPPH scavenging activity have also been published. For instance, Raghavan and Kristinsson⁴³ reported an increase in the antioxidant activity for tilapia hydrolysates with DH. These authors found the same trend when producing the tilapia hydrolysates using different enzymes (e.g. Flavourzyme, Neutrase, Cryoting F, Protease A Amano-2 and Protease N Amano).

Apart from peptide size, the amino acid composition and sequence have a great importance on the antioxidant activity of FPH. This is mainly determined by the specificity of the enzyme employed.⁴⁴ Fig. 6a shows that for all the DH values assayed, hydrolysates produced with subtilisin exhibited a higher antioxidant activity, except for DH 8% when compared to trypsin. This is because subtilizing favors the cleavage at the C-terminal of hydrophobic residues (e.g. Val, Gly and Pro) which contribute to the DPPH scavenging activity.⁴⁵ This explains the highest antioxidant activity of the hydrolysate with DH 4% and produced with subtilisin ($IC_{50}=1.36$ mg protein mL⁻¹).

ACE-inhibitory activity

One of the most common methods to reduce blood pressure is the inhibition of ACE, since this enzyme plays a crucial role on the regulation of blood pressure since.⁴⁶ In the renin-angiotensin system, ACE transforms the inactive decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) into the potent vasoconstrictor octapeptide, angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). Furthermore, in the kallikrein-kinin system, ACE catalyzes the degradation of bradykinin, a vasodilator nonapeptide.⁴⁷

All the hydrolysates produced exhibited ACE-inhibitory activity, with IC_{50} values ranging from 172 to 764 $\mu\text{g protein mL}^{-1}$ (Fig. 6b). These results are in the line of those reported by García-Moreno et al.¹² for other discarded species such as sardine, horse mackerel, bogue, axillary seabream and small-spotted casthark, IC_{50} between 279 and 768 $\mu\text{g mL}^{-1}$; but are considerably lower than the IC_{50} values obtained for sardinelle hydrolysates,¹⁷ 1.2-7.4 mg hydrolysate mL^{-1} , and for blue whiting hydrolysates, 1.34-2.41 mg protein mL^{-1} .¹⁸

Fig. 6b shows a different effect of DH on ACE-inhibitory activity depending on the enzymatic treatment. Hydrolysates with DH 4%, independently of the enzymatic treatment, exhibited an important ACE inhibition. This is in agreement with previous works indicating a sharply increase in the ACE-inhibitory activity of the hydrolysates at the beginning of the hydrolysis reaction.^{9,12} Then, the ACE-inhibitory activity of the hydrolysates slightly (subtilisin treatment) or considerably (trypsin and combined subtilisin and trypsin treatments) decreased for the hydrolysates of DH 8%. This indicates that ACE-inhibitory peptides previously generated were then cleaved at sites that do not facilitate ACE inhibition. As the reaction advanced (DH 12%), the ACE-inhibitory activity remained constant in the case of the hydrolysate obtained by the combined enzymatic treatment. This trend was also reported by Bougatef et al.¹⁷ for sardinelle hydrolysates for digestion above DH 6% using crude enzyme extract of sardine viscera. On the contrary, hydrolysates with DH 12% produced using subtilisin and trypsin as single enzymes exhibited a stronger ACE inhibition when compared to hydrolysates with lower DH. It may be attributed to the higher proportion of short peptides (337-92 Da) presented in these hydrolysates (Table 1). This is in the line of previous studies reporting a higher ACE-inhibitory activity when increasing DH.^{18,48}

The ACE-inhibitory peptides are normally constituted by 2-12 amino acids and they normally contain hydrophobic residues (e.g. Phe, Trp, Tyr, Pro) in the C-terminal tripeptide sequence. This enhances the interaction of the peptide with the three hydrophobic zones placed at the active site of the enzyme.⁴⁷ Therefore, the highest ACE-inhibitory activity of the hydrolysate with DH 12% obtained using subtilisin ($IC_{50}=172 \mu\text{g protein mL}^{-1}$) may be related to a higher content of short

peptides containing hydrophobic residues, whose formation is favored by the specificity of this enzyme.

Antigenicity

The antigenicity of fish proteins is caused by the presence of specific sequences in the allergen proteins (i.e. parvalbumins) called epitopes, which may be recognized by the immune system, triggering an allergenic response. Enzymatic hydrolysis is expected to degrade allergenic epitopes and therefore reduce the immunoreactivity of the native protein. This assumption was confirmed by the values of percentage inhibition obtained by the ELISA assays. Effectively, the antigenicity of the hydrolysates was decreased by both the enzymatic treatment and the degree of hydrolysis. As an example, Fig. 7 shows the inhibition curves for the native protein extract and the DH 4% hydrolysates for the three enzymatic treatments assayed. Native blue whiting protein presented 100% inhibition against anti-Parvalbumin at $10^3 \mu\text{g mL}^{-1}$, estimating an IC_{30} value (i.e. concentration of protein corresponding to 30% antigenicity) of $1.26 \mu\text{g mL}^{-1}$. At the same concentration and DH, trypsin hydrolysates reduced 6% the antigenicity of the native protein, increasing the IC_{30} value up to $25.1 \mu\text{g mL}^{-1}$. For these hydrolysates, the levels of antigenicity decreased significantly at increasing DH, dropping down to 14% inhibition at DH 12% and $10^3 \mu\text{g mL}^{-1}$ of protein. The combined treatment subtilisin plus trypsin improved slightly the levels of antigenicity observed for the tryptic hydrolysates. Indeed, as shown in Fig. 7, DH 4% hydrolysates presented 84% inhibition at $10^3 \mu\text{g mL}^{-1}$ of protein, corresponding to an IC_{30} value of $31.6 \mu\text{g mL}^{-1}$. Finally, subtilisin hydrolysates presented the lowest antigenic levels among the three treatments studied. Compared to the native protein, the values of IC_{30} were increased 4000 times at DH 4%, while the hydrolysates at DH 8% and DH 12% did not show inhibition against anti-Parvalbumin within the range of concentrations assayed in this work.

The effect of enzymatic hydrolysis on the reduction of protein immunoreactivity has been widely studied in cow milk proteins, while studies on the control of allergenic reactivity of parvalbumins in fish are still limited.⁴⁹ The efficiency of subtilisin to reduce the antigenicity of whey protein

hydrolysates have been confirmed by previous studies.^{50,51} Tryptic hydrolysates have been tested in combination with exo-proteases to reduce the antigenicity of cheese whey.⁵² The mixture trypsin-papain (1:1) led to the lowest inhibition against anti α -lactalbumin and anti β -lactoglobulin (40% at $10^3 \mu\text{g mL}^{-1}$ of protein), improving largely the results obtained by single enzymes.

CONCLUSIONS

The protein fraction of blue whiting discarded specimens can be up-graded through the production of FPH since they presented: a) acceptable functional properties, b) significant DPPH scavenging and ACE-inhibitory activities, and c) reduced antigenicity. Independently of the enzymatic treatment, FPH with low DH (4%) are preferred in order to obtain better emulsifying, foaming and oil binding capacities and higher antioxidant activity. Although tryptic hydrolysates can better unfold at the interface producing emulsions and foams, FPH obtained using subtilisin led to the highest DPPH scavenging activity ($\text{IC}_{50}=1.36 \text{ mg protein mL}^{-1}$). The employment of only subtilisin, which favors the cleavage at the C-terminal of hydrophobic residues, was also preferred for the production of FPH with stronger ACE-inhibitory activity. Nevertheless, FPH with higher DH (12%) presenting a higher proportion of short peptides was required to better inhibit ACE ($\text{IC}_{50}=172 \text{ mg protein mL}^{-1}$). As expected, the antigenic levels of the FPH were reduced with DH, particularly for the subtilisin hydrolysates.

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Tables and Figures captions

- Table 1. Molecular weight fractions of the hydrolysates.

- Figure 1. Gel filtration chromatograms showing the molecular mass weight distribution of the hydrolysates obtained by: a) subtilisin, b) trypsin, and c) combination of subtilisin and trypsin.
- Figure 2. Protein solubility of the powdered hydrolysates.
- Figure 3. Emulsifying capacity EC (a) and emulsion stability ES (b) of the powdered hydrolysates.
- Figure 4. Foaming capacity FC (a) and foaming stability FS (b) of the powdered hydrolysates.
- Figure 5. Water holding capacity (a) and oil binding capacity (b) of the powdered hydrolysates.
- Figure 6. DPPH scavenging activity (a) and ACE-inhibitory activity (b) of the hydrolysates.
- Figure 7. Inhibition curves against anti-Parvalbumin for the native protein (▲) and the powdered hydrolysates at DH 4% and the enzymatic treatments: subtilisin (△), trypsin (□) and combination subtilisin and trypsin (○).

TABLES

Table 1. Molecular weight fractions of the hydrolysates.

DH, %	Enzymatic treatment	Peak Fraction, %			
		A (>903 Da)	B (903-337 Da)	C (337-92 Da)	D (>92 Da)
4	Subtilisin	22.5±0.3 ^a	12.6±0.1 ^a	24.0±0.6 ^a	40.9±0.3 ^a
	Trypsin	25.8±0.6 ^b	9.7±0.1 ^b	23.1±0.5 ^b	41.4±1.1 ^a
	S-T	19.0±0.4 ^c	9.9±0.1 ^b	25.4±0.7 ^c	45.7±0.3 ^{bc}
8	Subtilisin	16.2±0.6 ^d	15.6±0.7 ^c	24.0±0.3 ^a	43.3±0.7 ^d
	Trypsin	15.3±0.3 ^d	13.6±0.1 ^d	28.2±0.3 ^d	42.9±0.6 ^d
	S-T	13.4±0.3 ^e	14.6±0.0 ^e	25.5±0.3 ^c	46.5±0.6 ^{ce}
12	Subtilisin	9.7±0.2 ^f	15.2±0.1 ^c	31.8±0.7 ^e	43.4±1.0 ^d
	Trypsin	10.1±0.5 ^f	12.9±0.2 ^a	31.8±0.2 ^e	45.1±0.9 ^b
	S-T	8.8±0.8 ^f	14.3±0.1 ^e	29.8±0.4 ^f	47.2±0.4 ^e

S-T: combination of subtilisin and trypsin. Results are the average of triplicate determinations ± standard deviation. For each column, mean values with different superscript letters are significantly different (p<0.05).

FIGURES

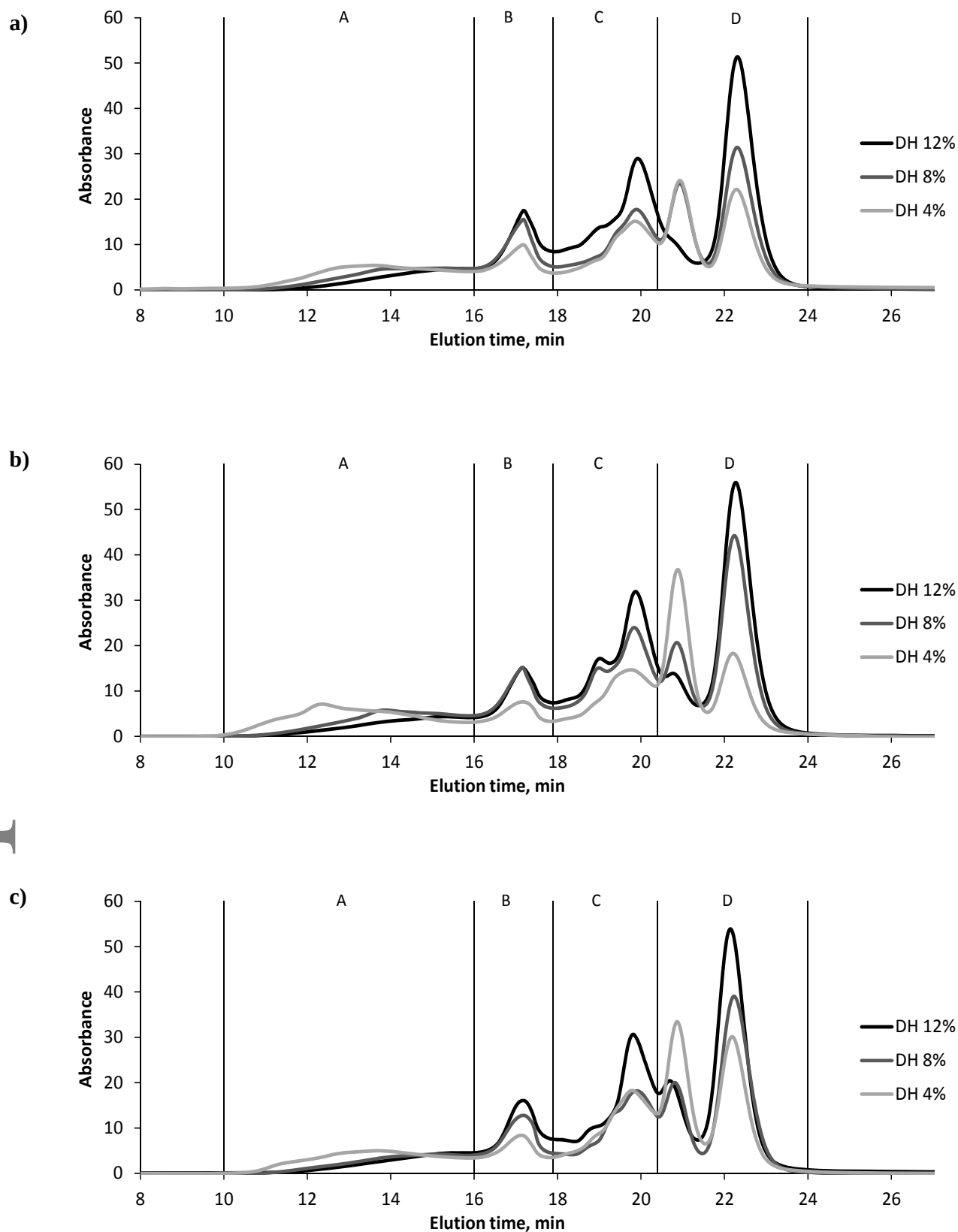


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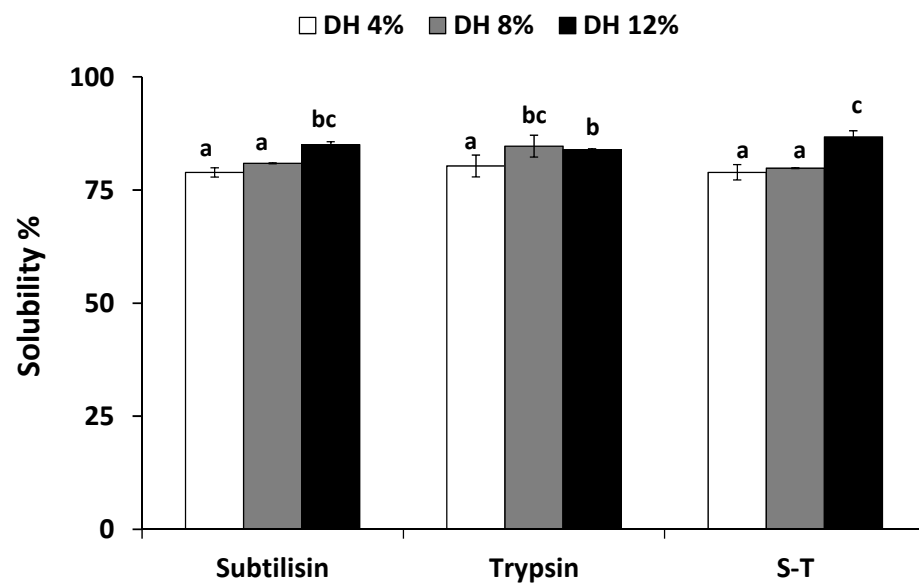


Fig. 2. Protein solubility of the powdered hydrolysates.

S-T: combination of subtilisin and trypsin. Results are the average of triplicate determinations $\pm$ standard deviation. Mean values with different superscript letters are significantly different ($p < 0.05$).

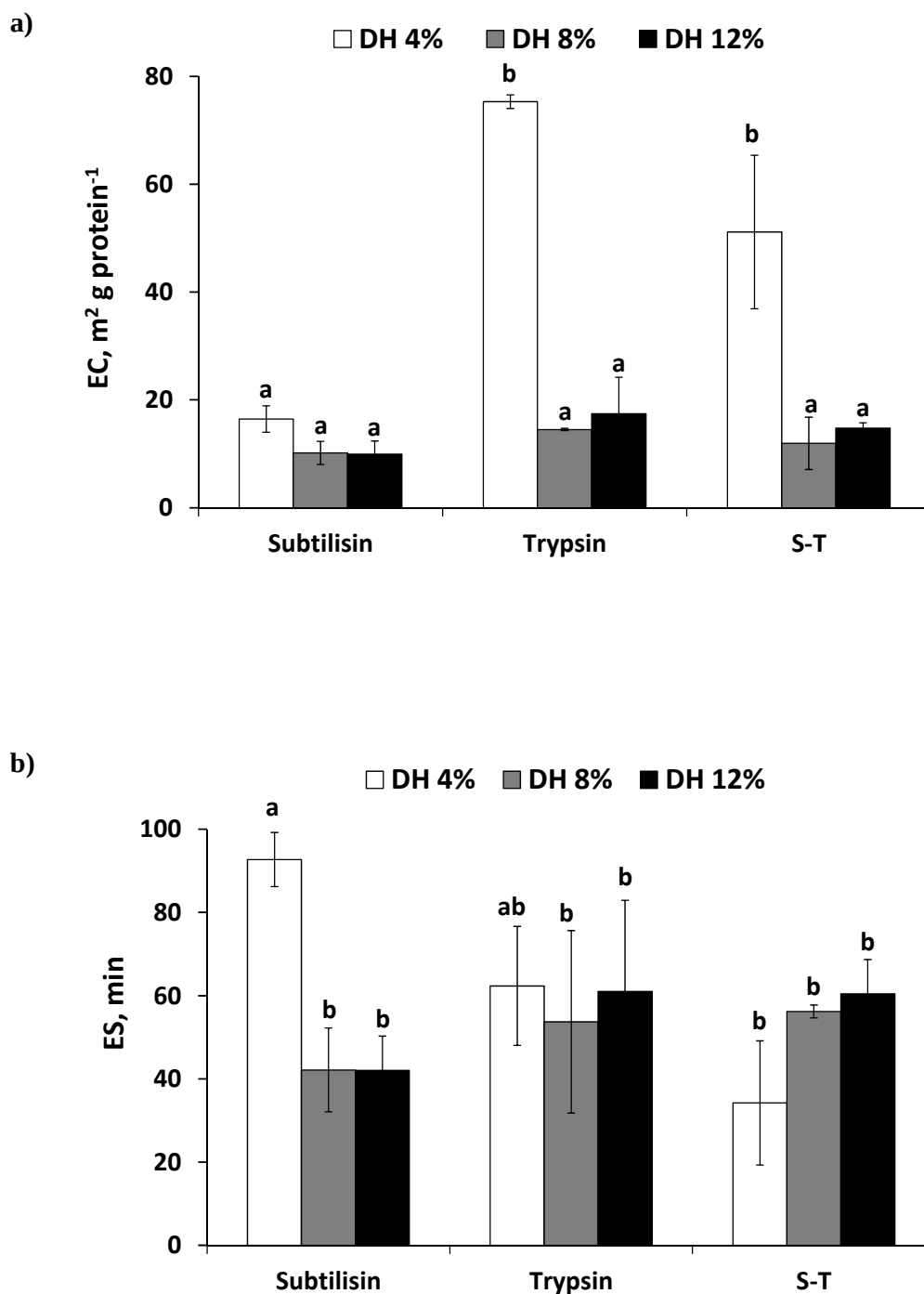
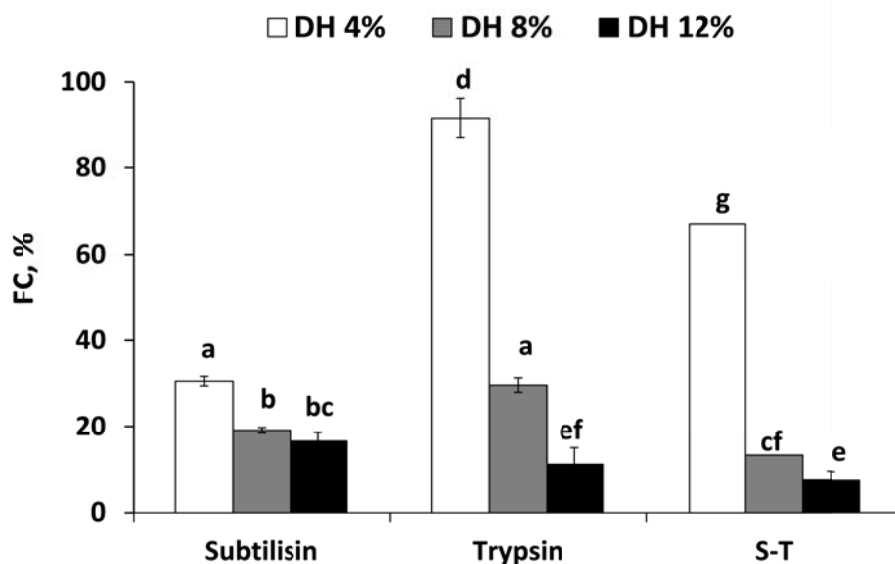


Fig. 3. Emulsifying capacity EC (a) and emulsion stability ES (b) of the powdered hydrolysates.

S-T: combination of subtilisin and trypsin. Results are the average of triplicate determinations $\pm$ standard deviation. Mean values with different superscript letters are significantly different ($p < 0.05$).

a)



b)

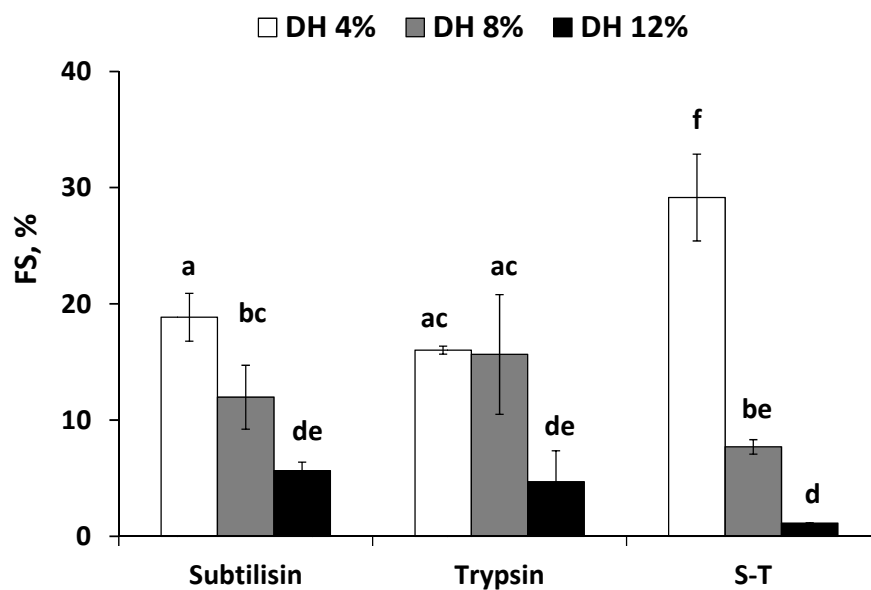


Fig. 4. Foaming capacity FC (a) and foaming stability FS (b) of the powdered hydrolysates.

S-T: combination of subtilisin and trypsin. Results are the average of triplicate determinations $\pm$ standard deviation. Mean values with different superscript letters are significantly different ($p < 0.05$).

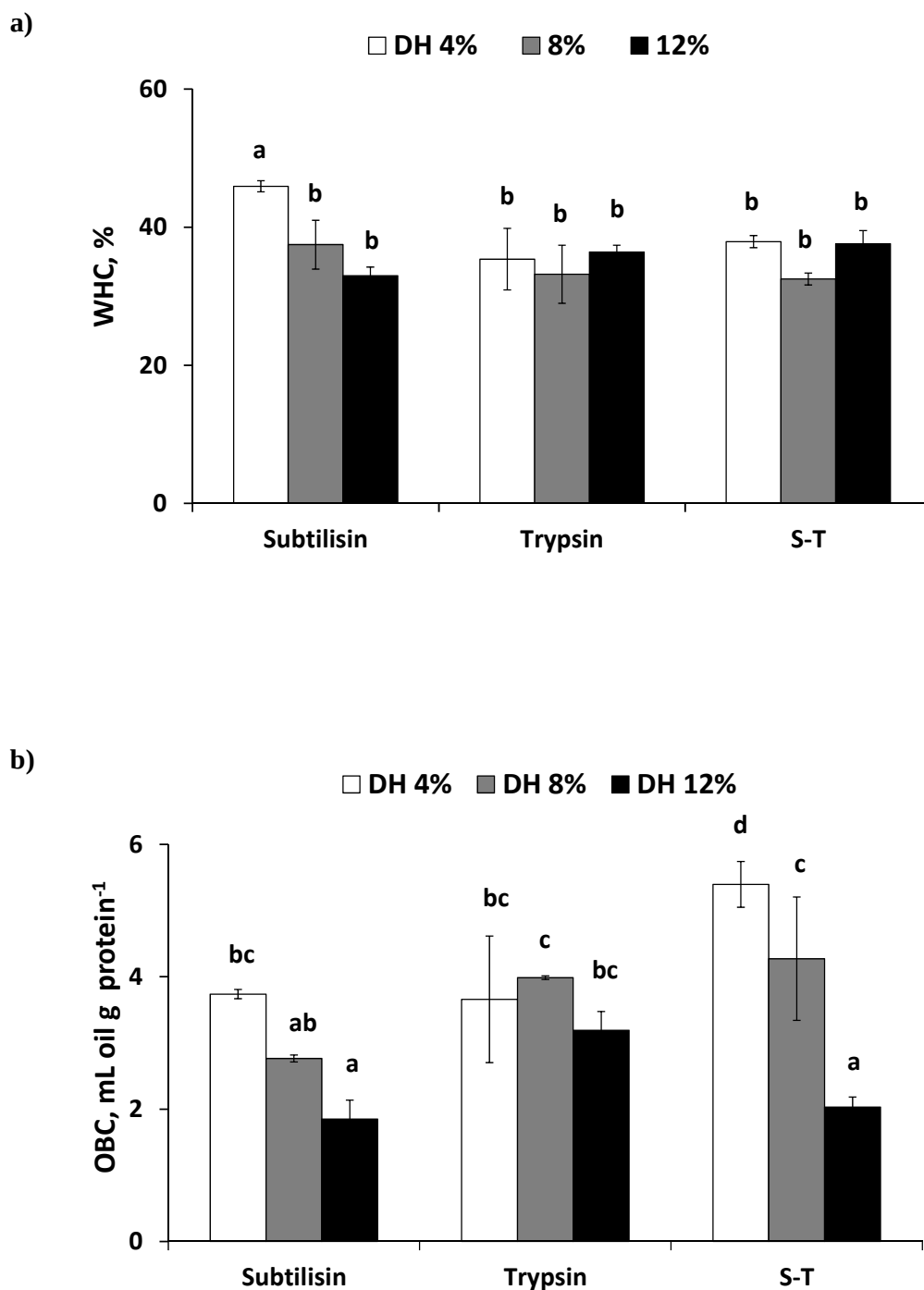
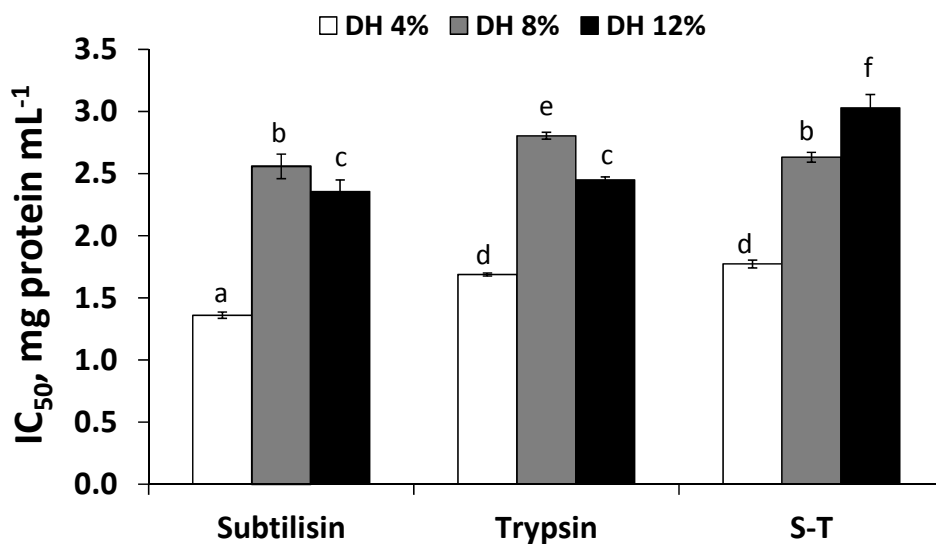


Fig. 5. Water holding capacity (a) and oil binding capacity (b) of the powdered hydrolysates.

S-T: combination of subtilisin and trypsin. Results are the average of triplicate determinations $\pm$ standard deviation. Mean values with different superscript letters are significantly different ($p < 0.05$).

a)



b)

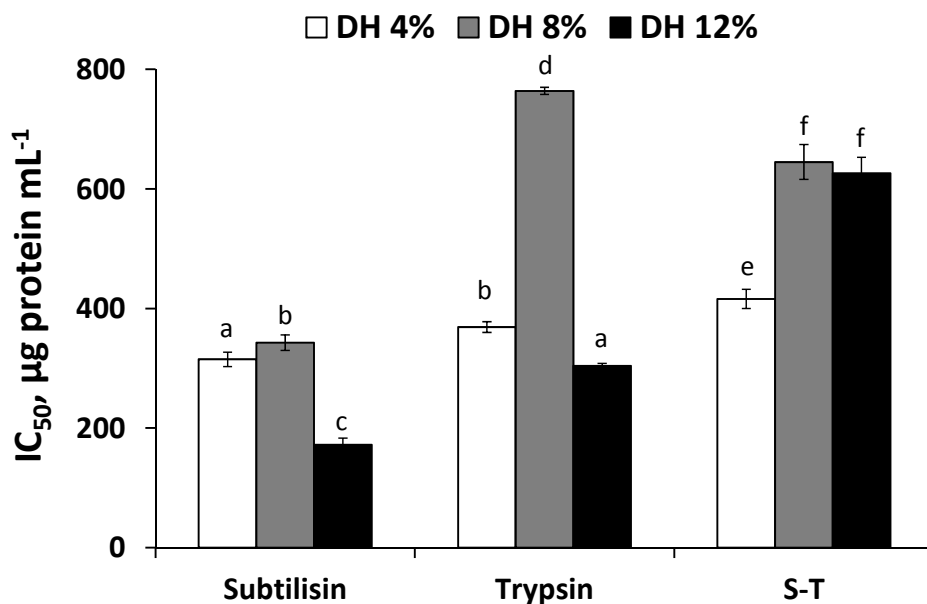


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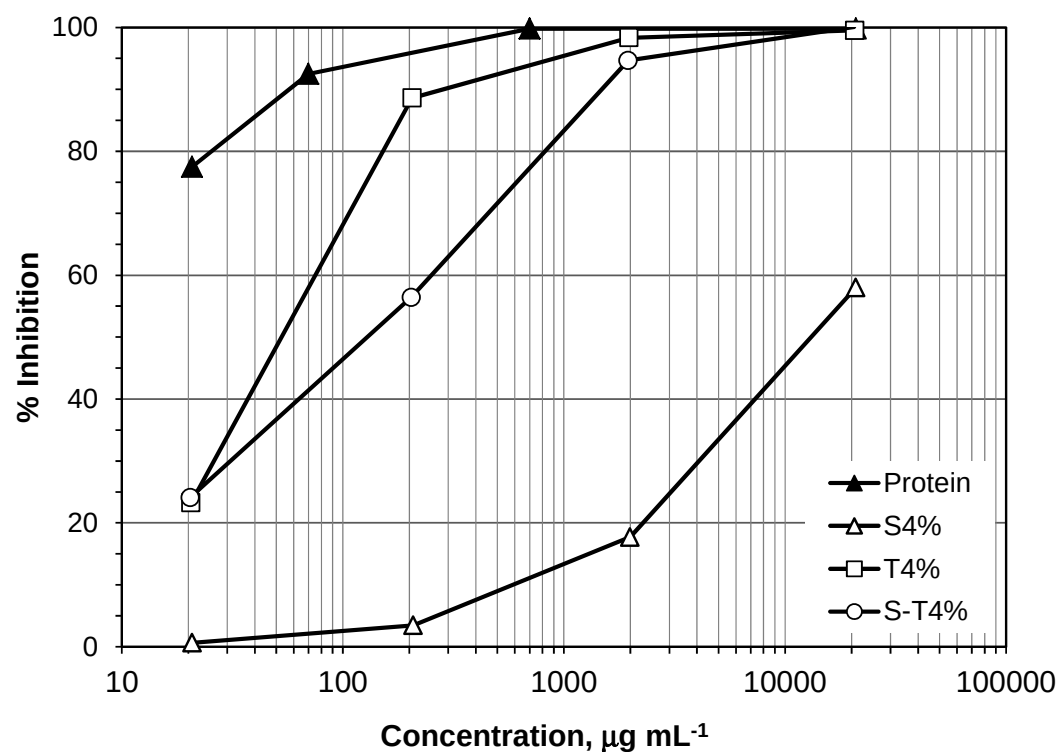


Fig. 7. Inhibition curves against anti-Parvalbumin for the native protein ($\blacktriangle$) and the powdered hydrolysates at DH 4% and the enzymatic treatments: subtilisin ($\triangle$), trypsin ($\square$) and combination subtilisin and trypsin ($\circ$).