ORIGINAL RESEARCH

From waste to value: protein hydrolysates from byproducts of the Argentine hake (*Merluccius hubbsi*) processing using endogenous enzymes and Alcalase® 2.4L

Clara Liebana[1], Nair de los Ángeles Pereira[1], Analia V. Fernández-Gimenez[1] and Maria Florencia Fangio[2,3,*]

[1]Instituto de Investigaciones Marinas y Costeras (IIMyC-CONICET), Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata (UNMdP), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), CC 1260, Mar del Plata, Argentina. [2]Departamento de Química y Bioquímica, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata (UNMdP), Funes 3350, B7602AYL - Mar del Plata, Argentina. [3]Instituto de Investigaciones Físicas de Mar del Plata (IFIMAR-CONICET), Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata (UNMdP), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Funes 3350, B7602AYL - Mar del Plata, Argentina. ORCID *Clara Liebana* https://orcid.org/0000-0002-7062-8675, *Nair de los Ángeles Pereira* https://orcid.org/0000-0002-7341-5333, *Analia V. Fernández-Gimenez* https://orcid.org/0000-0001-9232-4560, *Maria Florencia Fangio* https://orcid.org/0000-0001-5860-6513

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ABSTRACT. The valorization of fishery byproducts is essential to reduce waste and create high-value products. Waste from Argentine hake (*Merluccius hubbsi*) could enhance its functional and antioxidant properties through hydrolysis, releasing peptides with bioactive properties. Protein hydrolysates of Argentine hake were produced through autolysis (Aut) and enzymatic hydrolysis using Alcalase® 2.4L at concentrations of 0.24% and 2% (v/v) (Alc-0.24 and Alc-2), respectively, over 150 min. Alkaline peptidase activity, degree of hydrolysis, and antioxidant activity were assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical $ABTS^{\bullet+}$ scavenging assays. All hydrolysates retained alkaline peptidase activity throughout the process. Alcalase-treated hydrolysates exhibited significantly higher peptidase activity and hydrolysis degree compared to autolysis. At 60 min, Alc-0.24 reached peptidase activity levels similar to Alc-2, and by 30 min, both had comparable degrees of hydrolysis. $ABTS^{\bullet+}$ scavenging activity increased over time for Alc-0.24, with both Alcalase® 2.4L concentrations outperforming autolysis. No significant differences were found between Alc-0.24 and Alc-2. Although all hydrolysates showed DPPH scavenging activity, no significant differences were detected between treatments or reaction times. These findings highlight the potential for producing value-added protein hydrolysates from Argentine hake waste.

Key words: Waste management, fish protein hydrolysate, peptidase activity, hydrolysis degree, antioxidant property.

*Correspondence:
mfangio@mdp.edu.ar

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De residuo a valor: hidrolizados proteicos de subproductos del procesamiento de la merluza argentina (*Merluccius hubbsi*) utilizando enzimas endógenas y Alcalasa® 2.4L

RESUMEN. La valorización de los subproductos pesqueros es fundamental para reducir los residuos y crear productos de alto valor. Los residuos de la merluza argentina (*Merluccius hubbsi*) podrían potenciar sus propiedades funcionales y antioxidantes a través de la hidrólisis, liberando péptidos con propiedades bioactivas. Los hidrolizados proteicos de merluza argentina tienen un gran potencial como ingredientes funcionales debido a sus propiedades bioactivas, pero optimizar los procesos de hidrólisis es esencial para mejorar el rendimiento y las características biofuncionales, como la actividad antioxidante. Se obtuvieron hidrolizados proteicos de merluza argentina mediante autólisis

(Aut) y hidrólisis enzimática utilizando Alcalasa® 2.4 L a concentraciones de 0,24% y 2% (v/v) (Alc-0.24 y Alc-2), respectivamente, durante 150 min. Se evaluó la actividad de peptidasas alcalinas, el grado de hidrólisis y la actividad antioxidante utilizando ensayos de inhibición del radical 2,2-difenil-1-picrilhidrazilo (DPPH) y 2,2'-azino-bis (ácido 3-etilbenzotiazolina-6-sulfónico) $ABTS^{\bullet+}$. Todos los hidrolizados mantuvieron actividad de peptidasas alcalinas a lo largo del proceso. Los hidrolizados tratados con Alcalasa® 2.4L mostraron una actividad de peptidasas e un grado de hidrólisis significativamente mayores en comparación con Aut. A los 60 min, Alc-0.24 alcanzó niveles de actividad de peptidasas similares a los de Alc-2, y a los 30 min, ambos presentaron grados de hidrólisis comparables. La actividad de captura de $ABTS^{\bullet+}$ aumentó con el tiempo para Alc-0.24, siendo ambas concentraciones de Alcalasa® 2.4 L superiores a la autólisis. No se encontraron diferencias significativas entre Alc-0.24 y Alc-2. Aunque todos los hidrolizados mostraron actividad de captura de DPPH, no se detectaron diferencias significativas entre tratamientos o tiempos de reacción. Estos hallazgos destacan el potencial para producir hidrolizados proteicos de valor agregado a partir de residuos de merluza argentina.

Palabras clave: Gestión de residuos, hidrolizado de proteínas de pescado, actividad de peptidasa, grado de hidrólisis, propiedad antioxidante.

INTRODUCTION

Protein hydrolysates are produced through the enzymatic cleavage of peptide bonds in food proteins, resulting in peptides of varying molecular weights. These hydrolysates have broad applications in food formulations, functional foods, animal nutrition, pharmaceuticals, and cosmetics (Gao et al. 2021). They may contain bioactive peptides with diverse beneficial properties, such as antioxidant, antimicrobial, antithrombotic, immunomodulatory, and functional effects (Nirmal et al. 2022). In particular, antioxidant peptides derived from protein sources have garnered significant attention due to their health-promoting benefits and lower toxicity compared to synthetic pharmaceuticals (Wang et al. 2021). Antioxidant activity in protein hydrolysates obtained from various fish species has been widely reported (Gao et al. 2021; Moya-Moreira et al. 2023; Shekoohi et al. 2024). These properties depend on the type of peptides released during enzymatic hydrolysis, which can be carried out using endogenous enzymes (autolysis) or commercial enzymes, such as Alcalase® 2.4L (Gao et al. 2021).

The vast marine coastline of Argentina is homes to a thriving fishing industry, with *Merluccius hubbsi* being the most widely caught species in the country. In 2023, 297,354 t of Argentine hake were captured, making it one of the nation's most valuable fishery resources (MAGyP 2024). Byproducts of this processing represent approximately 50-60% of the total fish weight (Karoud et al. 2019; Ananey-Obiri et al. 2019). If not properly managed, these materials are often discarded, leading to inefficiencies and potential environmental pollution due to the accumulation of organic waste (Ananey-Obiri et al. 2019; Karoud et al. 2019). The substantial volume of byproducts generated during processing highlights the need for more effective strategies to increase the value of this resource (Góngora et al. 2012). Previous efforts have aimed at revalorizing *M. hubbsi* fishery byproducts by producing chemical and biological silages (Góngora et al. 2012; Fernández-Herrero et al. 2015) and extracting digestive enzymes for characterization or biotechnological applications (Lamas et al. 2015; Friedman et al. 2022, 2024). However, to the best of our knowledge, only Martone et al. (2005) have produced a protein hydrolysate from these residues, specifically seeking its use as a culture medium.

Various hake species have been treated enzymatically to produce protein hydrolysates, either by autolysis or by using commercial enzymes (Samaranayaka et al. 2007; Pacheco-Aguilar et al. 2008; Karoud et al. 2019). To enhance the value of fish protein byproduct, this study aimed to produce a protein hydrolysate from *M. hubbsi* processing waste using both endogenous enzymes and the commercial enzyme Alcalase® 2.4L (Novozymes).

The enzymatic treatments were then evaluated and compared on the basis of their degree of hydrolysis and antioxidant activity.

MATERIALS AND METHODS

The processing waste of *M. hubbsi* used in this study was supplied by Grupo Polo Sur (Mar del Plata, Argentina). This waste included heads, skeletons and viscera. Materials were uniformly minced and subsequently stored at -20 °C until needed.

Preparation of hake protein hydrolysate

Hake protein hydrolysate was prepared according to the method of Pereira et al. (2022) with slight modifications. To produce the hake protein hydrolysate by autolysis (Aut), the mixture was digested in a water bath maintained at 45 ± 2 °C for 150 min with stirring, followed by an increase in temperature to 80 °C for 20 min to deactivate the enzymes. Subsequently, the mixture was centrifuged at 10,000 g at 4 °C for 15 min (KIMADI 3H12RI, China), resulting in the protein hydrolysate being present in the supernatant. For the hydrolysate production using 0.24% and 2% (w/v) Alcalase® 2.4L (designated as Alc-0.24 and Alc-2, respectively), the mixture was preheated to 80 °C for 20 min to inactivate endogenous enzymes before adding the commercial enzyme. Finally, the Aut, Alc-0.24, and Alc-2 were lyophilized (RIFICOR L-A-B3, Argentina) and stored at -20 °C until further use. All protein hydrolysates were made in triplicate.

Alkaline peptidase activity

The activity of alkaline peptidases was assessed using the method described by García-Carreño (1992). A 0.5% (w/v) azocasein solution (Sigma A2765) dissolved in a 50 mM Tris-HCl buffer at pH 7.5 was used as substrate. For the assay, 5 µl aliquots of the protein hydrolysates (prior to the enzyme deactivation) were incubated for 30 min with a mixture containing 250 µl of substrate and 250 µl of the same buffer. The reaction was terminated by adding 250 µl of 20% (w/v) trichloroacetic acid (TCA). In control treatments, TCA was added before the substrate. Absorbance readings were taken at a wavelength of 366 nm using a microplate spectrophotometer (Epoch BioTek, Gen5™ Software, USA). Total alkaline peptidase activity was calculated as activity units, representing the change in absorbance per minute measured in the crude extract, expressed per milliliter of crude extract (U ml^{-1}).

Degree of hydrolysis

The degree of hydrolysis (DH) was assessed using the methodology described by Baek and Cadwallader (1996). Aliquots from the three replicates were collected at 15, 30, 60, 90, 120, and 150-min intervals. Enzyme activity was then halted by heating the samples to 80 °C for 20 min. After this, 500 µl of the sample was mixed with 1,000 µl of 0.3 M trichloroacetic acid and incubated at room temperature for 20 min before centrifugation at 2,000 g for 5 min. The resulting supernatant (25 µl) was then combined with 225 µl of distilled water, 125 µl of 0.5 N NaOH and 0.25 ml of 1 N Folin-Ciocalteu reagent (Sigma F 9252). The mixture was stirred and incubated at 30 °C for 30 min, followed by centrifugation at 2,000 g for 10 min. The absorbance of the supernatant was measured at 578 nm. A blank was prepared by adding 1,000 µl of 0.3 M TCA to unhydrolyzed shrimp protein (zero time), representing the baseline concentration of TCA-soluble peptides as tyrosine. The maximum hydrolysis level, indicating the highest concentration of TCA-soluble peptides, was obtained by hydrolyzing 0.1 g of shrimp substrate with 4 ml of 6 N HCl at 110 °C for 24 h. The DH was calculated using the following equation:

$$DH = \frac{Abs\ t - Abs\ t_0}{Abs\ max} \times 100$$

where *Abs t* is the absorbance at a specific time point, *Abs* t_0 is the initial absorbance, and *Abs max* is the absorbance at the final hydrolysis time.

Antioxidant activity

The antioxidant activity was evaluated to determine the hydrolysis reaction time that maximizes antioxidant capacity, with evaluations performed at 0, 30, 60, and 90 min of the reaction. The 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\bullet+}$) scavenging activity was also measured according to the method of Re et al. (1999). The $ABTS^{\bullet+}$ was prepared by mixing 7 mM $ABTS^{\bullet+}$ with 2.45 mM potassium persulfate, allowing it to stand in the dark at room temperature for 16 h. The $ABTS^{\bullet+}$ solution was diluted with 5 mM sodium phosphate buffer (pH 7.4) to achieve an absorbance of 0.70 ± 0.02 at 734 nm. A 20 µl aliquot of hydrolysate at various concentrations (0.5-10 mg ml^{-1}) was combined with 2 ml of the $ABTS^{\bullet+}$ solution and incubated in the dark for 6 min. Absorbance was measured at 734 nm using a spectrophotometer (SPECTROstar Nano BMG LABTECH, Germany). The control solution was prepared similarly, replacing the sample with distilled water. The $ABTS^{\bullet+}$ scavenging activity (sa) was calculated using the following formula:

$$ABTS^{\bullet+}\ sa\ (\%) = 1 - \frac{Abs\ sample}{Abs\ control} \times 100$$

where *Abs control* represents the absorbance of the control and *Abs sample* represents the absorbance of sample. The IC_{50}, defined as the concentration of the sample required to scavenge 50% of DPPH, was subsequently calculated for each protein hydrolysate from the plot of sample concentration versus percentage inhibition.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined following the method of Shimada et al. (1992). A 1 ml aliquot of each SPH hydrolysate (0.25-10 mg ml^{-1}) was combined with 1 ml of DPPH solution (0.1 mM DPPH in 95% ethanol). The mixture was kept in the dark at room temperature for 30 min. Absorbance was measured at 517 nm using a spectrophotometer (SPECTROstar Nano BMG LABTECH, Germany). A control sample was prepared using distilled water in place of the hydrolysate solution. The DPPH radical scavenging activity (sa) was calculated as the percentage inhibition of DPPH according to the following equation:

$$DPPH\ sa\ (\%) = 1 - \frac{Abs\ sample}{Abs\ control} \times 100$$

where *Abs sample* and *Abs control* refer to the absorbance of sample and control, respectively. The IC_{50} value for this assay was also determined as previously described.

Statistical analysis

All experiments were conducted in triplicate. Results were reported as mean ± SE. Analysis of variance (ANOVA) was carried out followed by Tukey's test for *post-hoc* pairwise comparisons of mean values, using R statistical software (version 4.3.1) (R Core Team 2022). A significance criterion of $p < 0.05$ was applied.

RESULTS

Enzymatic activity of alkaline peptidases

The enzymatic activity in Aut (with an initial activity of 0.53 ± 0.041 U ml^{-1}) decreased significantly after 90 min, reaching a value of 0.23 ± 0.024 U ml^{-1}, and remained stable for the rest of the 150 min (Figure 1). The enzymatic activity in the hydrolysates treated with Alcalase® 2.4L was significantly higher at all evaluated times compared to Aut. Although Alc-0.24 initially showed enzy-

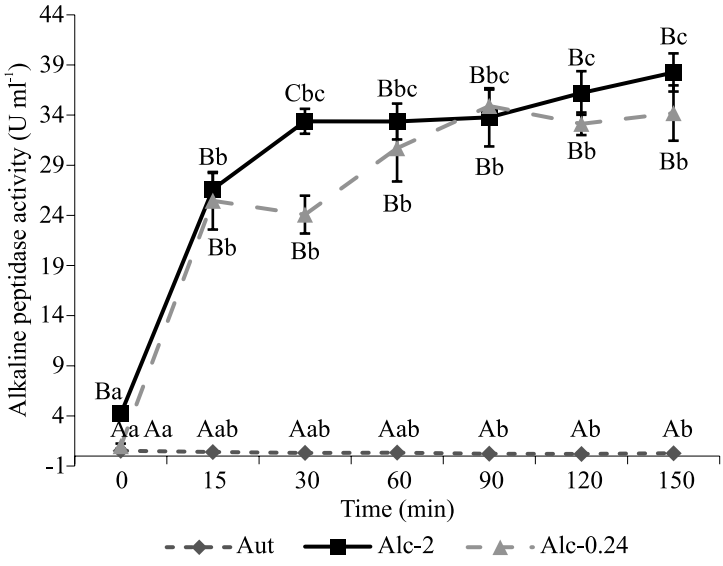

Figure 1. Alkaline peptidase activity (U ml^{-1}) of protein hydrolysates from *Merluccius hubbsi* over the hydrolytic reaction time. Different uppercase letters indicate significant differences ($p < 0.05$) between treatments at the same time point, while different lowercase letters indicate significant differences ($p < 0.05$) between time points within the same treatment. Aut: *M. hubbsi* hydrolysate obtained through autolysis; Alc-0.24: *M. hubbsi* hydrolysate obtained using 0.24% commercial Alcalase® 2.4L enzyme; Alc-2: *M. hubbsi* hydrolysate obtained using 2% commercial Alcalase® 2.4L enzyme.

matic activity comparable to Aut, it experienced a sharp increase after 15 min (25.46 ± 2.87 U ml^{-1}) and remained stable throughout the reaction. At 15 min, the activity was similar to Alc-2 (26.61 ± 1.62 U ml^{-1}), and this similarity persisted after 60 min (30.70 ± 3.32 and 33.36 ± 1.79 U ml^{-1} for 0.24% and 2%, respectively), continuing until the end of the reaction.

Degree of hydrolysis

The DH of Aut began to increase after 120 min, reaching 14.09 ± 0.451%, remaining stable until the end of the 150-min study (Figure 2). In contrast, protein hydrolysates obtained with Alcalase® 2.4L exhibited significantly higher degrees of hydrolysis at all time points, indicating a marked difference in enzymatic activity between *M. hubbsi* endogenous enzymes and Alcalase® 2.4L. Notably, Alc-0.24 reached a DH comparable to Alc-2 starting at 30 min, maintaining this level for the remainder of the reaction. At 120 min, both Alcalase-treated hydrolysates reached a plateau that persisted until the end of the reaction, with values of 64.39 ± 2.56% for Alc-0.24 and 72.84 ± 1.79% for Alc-2.

Antioxidant capacity

ABTS$^{\bullet+}$ radical scavenging activity

Both enzymatic treatment and reaction time had a significant impact on the ABTS$^{\bullet+}$ radical scavenging activity (Table 1). Regarding differences between time points within the same enzymatic treatment, a significant increase in the antioxidant capacity of the *M. hubbsi* hydrolysate treated with 0.24% Alcalase® 2.4L was observed at 30 and 60 min. Additionally, when comparing different enzymatic treatments, hydrolysates treated with Alcalase® 2.4L at 0.24% and 2% exhibited a greater inhibitory effect on the ABTS$^{\bullet+}$ radical compared to the hydrolysate obtained by autolysis at all evaluated time points. Furthermore, no significant differences were found between hydrolysates treated with different concentrations of Alcalase® 2.4L.

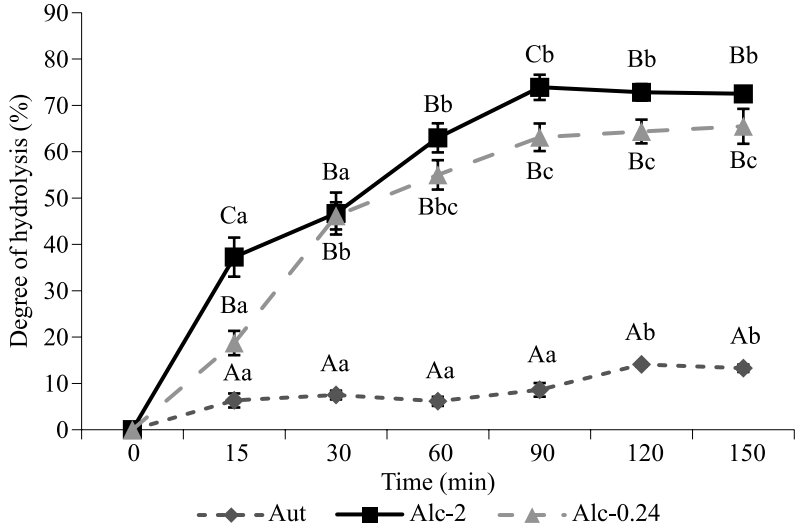

Figure 2. Degree of hydrolysis (%) of protein hydrolysates from *Merluccius hubbsi* over the hydrolytic reaction time. Different uppercase letters indicate significant differences ($p < 0.05$) between treatments at the same time point, while different lowercase letters indicate significant differences ($p < 0.05$) between time points within the same treatment. Aut: *M. hubbsi* hydrolysate obtained through autolysis; Alc-0.24: *M. hubbsi* hydrolysate obtained using 0.24% commercial Alcalase® 2.4L enzyme; Alc-2: *M. hubbsi* hydrolysate obtained using 2% commercial Alcalase® 2.4L enzyme.

DPPH scavenging assay

Results showed no significant differences ($p < 0.05$) either across various reaction times within the same enzymatic treatment or among different enzymatic treatments at any given time point. This suggested that neither reaction time nor the type of enzymatic treatment had a significant effect on antioxidant activity (Table 1).

DISCUSSION

In this study, wastes from Argentine hake (*M. hubbsi*) were hydrolyzed using endogenous enzymes or Alcalase® 2.4L at concentrations of 0.24% and 2% (v/v) for 150 min. Based on results of the peptidase activity of different protein hydrolysates (Aut, Alc-0.24 and Alc-2), it can be concluded that enzymes remained active in all cases, suggesting that the hydrolytic reaction was sustained over time. The high stability of endogenous enzymes from *M. hubbsi* is consistent with the findings of Friedman et al. (2022), who evaluated intestinal peptidase activity in this species and observed that these enzymes were highly stable within a pH range of 7 to 11.5 and temperatures between 10 and 50 °C over a period of 150 min. In the same way, Samaranayaka et al. (2007), Mazorra-Manzano (2008, 2012), and Cheung et al. (2012) reported peptidase activity in endogenous enzymes of the Pacific whiting (*M. productus*). Overall, enzymatic activity was higher in protein hydrolysates produced with Alcalase® 2.4L than those obtained through autolysis. Additionally, the enzymatic activity of the hydrolysate produced with Alcalase® 2.4L 0.24% v/v reached a comparable level to that of Alcalase® 2.4L 2% hydrolysate after 60 min of reaction.

The DH is a key indicator of the progress of protein hydrolysis and the extent of product degradation. Generally, a higher DH is linked to improved bioactive properties due to the release of smaller bioactive peptides, while a lower degree is associated with better sensory qualities as shorter peptides often contribute to increased bitterness (Idowu and Benjakul 2019; Nirmal et al. 2022). In

Table 1. Antioxidant activity of *Merluccius hubbsi* hydrolysates obtained through autolysis (Aut) or with 0.24% and 2% v/v Alcalase® 2.4L (Alc-0.24 and Alc-2, respectively), determined by DPPH and $ABTS^{\bullet+}$ inhibition assays.

Time (min)	Aut	Alc-0.24	Alc-2
$ABTS^{\bullet+}$ radical inhibition assay (IC_{50})			
0	1.60 ± 0.11^{Aa}	1.45 ± 0.179^{ABa}	0.98 ± 0.210^{B}
30	1.34 ± 0.13^{Aab}	1.47 ± 0.301^{Aa}	0.86 ± 0.113^{B}
60	1.47 ± 0.116^{Aab}	1.09 ± 0.140^{ABb}	0.83 ± 0.158^{B}
90	1.02 ± 0.026^{Ab}	0.90 ± 0.125^{Ab}	0.83 ± 0.012^{A}
DPPH radical inhibition assay (IC_{50})			
0	3.52 ± 0.238^{Aa}	3.89 ± 0.261^{Aa}	3.54 ± 0.291^{Aa}
30	3.64 ± 0.050^{Aa}	3.96 ± 0.141^{Aa}	3.58 ± 0.221^{Aa}
60	3.50 ± 0.046^{Aa}	3.79 ± 0.369^{Aa}	3.69 ± 0.148^{Aa}
90	3.22 ± 0.533^{Aa}	3.27 ± 0.623^{Aa}	3.29 ± 0.310^{Aa}

Values are expressed as IC_{50} (mg ml^{-1}) (mean ± SE). Lowercase letters within the same column indicate significant differences ($p < 0.05$) between time points for each treatment. Uppercase letters within the same row indicate significant differences among treatments at the same hydrolytic reaction time ($p < 0.05$).

our study, according to results of enzymatic activity, DH was significantly lower in protein hydrolysates obtained by autolysis compared to those treated with Alcalase® 2.4L.

The hydrolysate obtained through autolysis achieved a peak of around 14% after 120 min of reaction, which is greater than the percentage reported by Mazorra-Manzano et al. (2012) for protein hydrolysates from *M. productus*. On the other hand, Alc-0.24 and Alc-2 showed values of approximately 64% and 72% of DH. These values were higher than those reported by Pires et al. (2013, 2024), who found a degree of hydrolysis ranging from 10.7% to 36.4% for hake hydrolysates treated with Alcalase® 2.4L. However, other studies have reported closer values of DH, ranging from 42.5% to 78.26%, in hydrolysates of other fish species treated with Alcalase® 2.4L (Piotrowicz and Mellado 2015; Gómez et al. 2020). In agreement with results of this study, Ovissipour et al. (2013) reported a significantly lower DH in protein hydrolysates of the anchovy sprat (*Clupeonella engrauliformis*) produced by autolysis (17.4%) compared to those treated with Alcalase® 2.4L (55.8%). Furthermore, the low enzymatic activity and DH observed in the Aut treatment could be associated with the initial quality of byproducts, which may have impacted the activity of endogenous enzymes, as indicated in studies in rainbow trout byproducts and other marine resources (Nikoo et al. 2021, 2022).

The significantly higher alkaline peptidase activity and DH observed in the protein hydrolysate of hake prepared with Alcalase® 2.4L, compared to the autolysate ($p < 0.05$), can be attributed to the broad specificity for peptide bond hydrolysis of Alcalase® 2.4L (Sbroggio et al. 2016) and its high efficiency in achieving elevated degrees of hydrolysis in a short time (Ovissipour et al. 2009). Similarly, Cheung et al. (2012) found that although endogenous enzymes in *M. productus* exhibited activity, commercial enzymes achieved a greater degree of hydrolysis in their hydrolysates.

Consistent with the enzymatic activity findings, the protein hydrolysate produced with Alcalase® 2.4L 0.24% reached a DH similar to that of the Alcalase® 2.4L 2% hydrolysate starting at 30 min,

maintaining this level throughout the remainder of the reaction, with both hydrolysates reaching a plateau at 120 min.

Antioxidants are stable compounds that neutralize reactive oxygen species, helping to prevent diseases through various mechanisms (Ananey-Obiri et al. 2019). Protein hydrolysates or peptides are commonly evaluated for their antioxidant capacity using methods such as DPPH and $ABTS^{\bullet+}$ radical inhibition, which indicates their ability to scavenge radicals through hydrogen donation or electron transfer (Sila and Bougatef 2016; Idowu and Benjakul, 2019).

The $ABTS^{\bullet+}$ radical inhibition assay revealed that only Alc-0.24 exhibited an increase in antioxidant activity at 30 and 60 min, while the $ABTS^{\bullet+}$ inhibition remained stable over time for both Aut and Alc-2. Regarding the comparison between different enzymatic treatments, although Aut showed a significant inhibitory effect on the $ABTS^{\bullet+}$ radical, Alc-0.24 and Alc-2 demonstrated a superior inhibitory effect on this radical at all evaluated time points. These results align with the findings of Cheung et al. (2012), who reported improved $ABTS^{\bullet+}$ radical inhibition with the addition of exogenous peptidases. Additionally, no significant differences were observed between the hydrolysates treated with the two concentrations of Alcalase® 2.4L, suggesting that a lower concentration of Alcalase® 2.4L can produce a protein hydrolysate with the same $ABTS^{\bullet+}$ radical inhibitory activity as a higher concentration.

Results of the $ABTS^{\bullet+}$ assay indicate a strong correlation between the DH and $ABTS^{\bullet+}$ radical scavenging activity in the Argentine hake. These findings are in line with those of Raghavan et al. (2008) and Phanturat et al. (2010), who observed that an increase in the DH enhances the $ABTS^{\bullet+}$ free radical scavenging activity in hydrolysates from other fish species. On the other hand, it is generally reported that a higher DH releases a greater number of low molecular weight peptides, which exhibit higher DPPH scavenging activity (Chalamaiah et al. 2015; Henriques et al. 2021). For example, Karoud et al. (2019) found an increase in DPPH scavenging activity as the DH increased in protein hydrolysates from *M. merluccius* heads. However, in this study, no significant differences were observed among hydrolysates produced with different enzymatic treatments, despite their varying DH levels. This lack of correlation between DPPH scavenging activity and DH has also been reported in other studies. Klompong et al. (2007), for instance, found that hydrolysates with a low DH exhibited higher antioxidant power in the yellow stripe trevally (*Selaroides leptolepis*). Similarly, Pires et al. (2024) observed that smaller peptides had lower DPPH scavenging activity in protein hydrolysates from Atlantic salmon (*Salmo salar*) and Cape hake (*M. capensis*) byproducts.

In this study, although hydrolysates did not show improvements in DPPH inhibitory activity during the hydrolysis process, they maintained a high activity level from the beginning. This initial scavenging ability, sustained over time, may be attributed to the natural presence of various antioxidant compounds in the hake byproducts (Ognjanović et al. 2008; Karoud et al. 2020). On the other hand, while the DH, the enzyme used, and the protein substrate play significant roles in the antioxidant capacity of protein hydrolysates (Klompong et al. 2007), the net hydrophobicity of peptides and free amino acids also influences this activity (Asaduzzaman et al. 2020). It is important to note that the DPPH assay is conducted in a lipophilic medium, whereas the $ABTS^{\bullet+}$ assay is performed in an aqueous medium. This difference influences the radical scavenging capabilities: hydrophobic peptides demonstrate greater efficacy in the DPPH scavenging assay, whereas less hydrophobic peptides perform better in the $ABTS^{\bullet+}$ scavenging assay (Latorres et al. 2018; Singh et al. 2024). Therefore, it is possible that the hydrophobic peptides released in this study did not demonstrate a greater antioxidant capacity than natural compounds present in the hake byproducts.

This study offers valuable insights into the hydrolysis process and the antioxidant activity of hake hydrolysates. The complexity of the relation-

ship between hydrolysis and antioxidant properties is highlighted by the absence of significant differences in DPPH radical scavenging activity among treatments, despite notable variations in DH and peptidase activity. Future research should focus on analyzing the molecular weight distribution and amino acid composition of these protein hydrolysates to provide a deeper understanding of the results and inform their potential applications in feed or food-grade products.

Results showed that protein hydrolysates of Argentine hake obtained with Alcalase® 2.4L exhibited higher alkaline peptidase activity, degree of hydrolysis, and $ABTS^{\bullet+}$ scavenging activity compared to autolysis. Notably, Alc-0.24 produced a hydrolysate with similar characteristics to Alc-2 after 120 min of hydrolysis. This finding is particularly interesting since it implies that less commercial enzyme may be needed to make protein hydrolysates with comparable characteristics, which could minimize the cost associated to the enzymatic hydrolysis of these residues. It also highlights the potential of using Argentine hake waste to produce value-added protein hydrolysates, offering a more efficient and sustainable approach to the use of fishery byproducts.

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Conflict of interest

The authors declare that there is no conflict of interest and that the research meets the required ethical guidelines.

Author contributions

Clara Liebana: data curation; formal analysis; writing-original draft; project administration. Nair de los Ángeles Pereira: funding acquisition; project administration; supervision; writing-review and editing. Analia V. Fernández Gimenez: funding acquisition; project administration; supervision; writing-review and editing. Maria Florencia Fangio: funding acquisition; project administration; supervision, writing-review and editing.

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