Food Research International 188 (2024) 114499

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Effect of enzymatic hydrolysis with Alcalase or Protamex on technological and antioxidant properties of whey protein hydrolysates

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ARTICLE INFO

Keywords:
Enzymatic hydrolysis
Hydrolysis degree
Technological properties
Antioxidant capacity

ABSTRACT

The aim of this study was to evaluate the effect of the enzymatic hydrolysis, performed using Alcalase and Protamex enzymes, on the technological functionalities and the antioxidant capacity of whey protein hydrolysates (WPHs) to identify the conditions allowing to obtain target functionality/ies. Samples were characterized for hydrolysis degree (DH), molecular weight distribution, structural properties, and food-related functionalities. Free sulfhydryl groups and surface hydrophobicity significantly decreased with the increase in DH, regardless of the used enzyme. The foaming and antioxidant properties of Alcalase WPHs were higher as compared to those of WPI, reaching the maximum value at DH = 18–20 %, while higher DH resulted in impaired functionality. Gelling properties were guaranteed when WPI was hydrolysed by Protamex at DH < 15 % while foaming and antioxidant abilities were fostered at 15 < DH < 21 %. These results were well correlated with MW distribution and were rationalized into a road map which represents a useful tool in the selection of proper hydrolysis conditions (time, DH, enzyme type) to obtain WPHs with tailored functionalities. Research outcomes highlighted the possibility to drive protein hydrolysis to optimize the desired functionality/ies.

1. Introduction

Different physical, chemical, and biological approaches can be used to steer the protein functionalities intended as technological (i.e., solubility, emulsifying, foaming, and gelling), nutritional (i.e., digestibility) or biological (i.e., antioxidant, antihypertensive, antidiabetic, antimicrobial) properties (Nisov et al., 2020; Yuan et al., 2012). Among biological strategies, enzymatic hydrolysis has received great attention in recent years because of the possibility of tailoring the characteristics of the final product. Extended hydrolysis leading to the formation of small peptides has been demonstrated to improve protein digestibility and reduce allergenicity since fragmentation allows easier access to digestive enzymes and more rapid absorption and transport of peptides into the bloodstream (Minj & Anand, 2020). Moreover, small peptides have demonstrated interesting biological activities, such as antihypertensive, antioxidant, and antimicrobial (Dullius et al., 2018; Innocente et al., 2019; Jeewanthi et al., 2015). These effects of extended hydrolysis have already been exploited at an industrial scale to respond to the growing consumer demand for functional foods (Dullius et al., 2018).

On the other hand, hydrolysis could also be considered as a tool to steer protein technological functionalities, i.e. solubility, foaming, gelling and emulsifying properties. In this case, a high degree of hydrolysis (DH) is reported to impair these functionalities due to the excessive reduction in chain length (Yin et al., 2008; Yu et al., 2018). For instance, peanut protein hydrolysates with a DH of 40 % have been demonstrated to exhibit poorer emulsifying and foaming properties than the corresponding isolate, but higher angiotensin-converting enzyme (ACE) and antioxidant activities (Jamdar et al., 2010; Yin et al., 2008). Similarly, Kheroufi et al. (2022) reported that whey protein concentrate hydrolysed for up to 6 h exhibited higher antioxidant activity than the corresponding unhydrolyzed sample, but lower technological properties. Moreover, small peptides are reported to be unable to form the network necessary to obtain hydrogels (Nisov et al., 2020). Differently, the partial or limited hydrolysis of proteins results in polypeptides with size and flexibility that can lead to an improvement in technological functionalities. This approach has been demonstrated to remarkably enhance the functional properties of proteins from whey, soy, sunflower, oat bran, faba bean, and peanuts (Nawaz et al., 2022; Yin et al., 2008; Zhao et al., 2011). In summary, the proteolytic process results in the exposure of hydrophobic groups, originally buried in the internal structure of the native protein structure, as well as MW reduction and increase in ionizable groups of peptides. These changes lead to a

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https://doi.org/10.1016/j.foodres.2024.114499
Received 20 November 2023; Received in revised form 30 April 2024; Accepted 7 May 2024
Available online 9 May 2024

modification of the protein interfacial properties, associated with foaming and emulsifying properties, gelling ability as well as the solubility of the hydrolysates. Only through the precise design of the hydrolysis process it could be possible to deliver biological functionalities exploiting at the same time the technological functionalities of protein hydrolysates. The present study aimed to assess the effect of enzymatic hydrolysis on the technological (solubility, emulsifying, gelling, and foaming capacity) and biological (antioxidant) functionalities of a whey protein isolate to finally identify the hydrolysis degree able to maximise the desired functionalities.

In this study, a commercial whey protein isolate (WPI) was subjected to enzymatic *in vitro* hydrolysis by using Protamex and Alcalase proteases for increasing times (from 10 to 480 min) and by using predetermined processing conditions able to maximise the enzymatic activity.

Freeze-dried WP hydrolysates (WPHs) were then characterised for the degree of hydrolysis (DH), molecular weight distribution, free sulfhydryl groups (SH), and surface hydrophobicity as well as solubility, foaming, emulsifying and gelling ability and antioxidant activity.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) was obtained from Davisco Food International Inc. (Le Sueur, MN, USA). The WPI content in dry matter, total nitrogen, ash, and fat was determined in our previous study (Innocente et al., 2023). In particular, the protein content (%, w/w) was 94.70 ± 0.12 and the protein composition included 74.6 % β-lactoglobulin, 23.8 % α-lactalbumin, and 1.6 % bovine serum albumin. Alcalase® 2.4L (Protease from *B. licheniformis*, declared activity ≥ 2.4 AU/g; EC 3.4.21.62), Protamex® (Protease from *Bacillus* sp., declared activity ≥ 1.5 AU/g; EC 3.4.21.62), sodium tetraborate, sodium dodecyl sulfate (SDS), *o*-phtaldialdehyde (OPA), L-serine, bovine serum albumin (BSA), β-Lactoglobulin (β-Lg), C-peptide, aprotinin, Lys-Lys-Lys, L-Ser, L-glutathione oxidised, dithiothreitol, 5′,5-dithiobis (2-nitrobenzoic acid), (DTNB), tris base, glycine, Ethylenediaminetetraacetic acid (EDTA), 8-Anilino-1-naphthalenesulfonic acid (ANS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Trolox® and potassium-phosphate buffer were purchased from Sigma-Aldrich (Milan, Italy). Absolute ethanol and N, N-dimethylformamide were purchased from VWR International (Milan, Italy). Deionized water System advantage A10® (Millipore S.A.S, Molsheim, F) was used. All other chemicals used were of analytical reagent grade.

2.2. Preparation of whey protein hydrolysates

Hydrolysis of WPI was carried out using Alcalase 2.4L or Protamex under the conditions previously optimised and reported in our previous work (Innocente et al., 2023). Briefly, 3 % (w/w, protein basis) WPI aqueous solution was prepared in 0.1 M potassium-phosphate buffer at the appropriate pH for each enzyme. For Alcalase-mediated hydrolysis, the pH was set to 8.0 and the solution was equilibrated at 50 °C using a water bath. Alcalase was added at a 1:10 (w/w) enzyme-substrate ratio. For Protamex hydrolysis, the pH of the potassium-phosphate buffer was 7.0, and the solution was equilibrated at 55 °C, with Protamex added at a 1:5 enzyme-substrate ratio (w/w). For each enzyme, 8 batches of 200 mL were prepared. Each batch underwent hydrolysis for a predetermined duration, specifically 10, 20, 30, 60, 120, 240, 360, or 480 min. The unhydrolyzed WPI solution was considered the control. The enzymatic reactions were stopped using HCl 1 M to reach a pH value of 2.3 ± 0.2, at which both enzymes were found to be irreversibly inactivated based on preliminary trials. Afterwards, each batch was adjusted to pH 7.0 with NaOH 6 M before freeze-drying (Epsilon 2-4LSCplus, Christ, Osterode am Harz, Germany). The dried samples were stored at room temperature in vacuum-sealed plastic pouches until further analysis.

2.3. Analytical determinations

2.3.1. Degree of hydrolysis

The degree of hydrolysis (DH) of the whey protein hydrolysates (WPHs) was determined using the method modified by Bavaro et al. (2021), using the O-phthaldialdehyde (OPA) reagent. The OPA reagent was prepared by mixing 80 mL of 3.81 g sodium tetraborate, 100 mg sodium dodecyl sulfate (SDS), 2 mL absolute ethanol containing 80 mg OPA, and 88 mg dithiothreitol. The final volume was made up to 100 mL with deionized water. Briefly, 150 μL of WPH (2.5 mg/mL) was mixed with 3 mL of OPA reagent and allowed to react in the dark for 2 min. Absorbance was measured at 340 nm using an ultraviolet-visible spectrophotometer (UV-1800, Shimadzu Kyoto, Japan). A calibration curve was constructed using L-serine as a reference. DH was calculated according to Eq. (1):

$$DH(\%) = \frac{NH_2}{NH_2 total} \quad (1)$$

where NH_2 is the concentration of free amino groups in the WPHs and NH_{2total} is the total content of free amino groups in the unhydrolyzed sample and the value was obtained upon total acid hydrolysis. The latter was determined by heating 100 mg of WPI and 2.5 mL of 6 M HCl at 110 °C for 24 h and then adding 7.5 mL of 2 M NaOH.

2.3.2. Molecular weight distribution

The molecular weight (MW) distribution was estimated with the method described by Cui et al. (2022) and Innocente et al. (2023) high-performance gel-filtration chromatography. A TSKgel 2000 SWXL 300 mm × 7.8 mm column (Tosoh Bioscience, Griesheim, Germany) was used with a mobile phase consisting of water/acetonitrile/TFA (55/45/0.1, v/v/v). The flow rate was 0.5 mL/min, column temperature was 30 °C. 10 μL of the sample at a concentration of 1 mg/mL was injected into the HPLC system (LC 4000, Jasco Europe, Cremella, Italy) with a PDA detector set at 220 nm. BSA (66,463 Da), β-Lg (18,400 Da), aprotinin (6,511 Da), C-peptide (3,183 Da), L-glutathione oxidised (612 Da), and L-Ser (106 Da) were run as standards. MW distributions are reported taking into account β-Lg, C-peptide, and L-glutathione oxidised as more representative standards. Data analysis was performed using the chromatography software ChromNAV2 (Jasco Europe, Cremella, Italy).

2.3.3. Free sulfhydryl groups

The content of total free sulfhydryl (SH) groups was quantified using Ellman's reagent (5′,5-dithiobis (2-nitrobenzoic acid), DTNB) following the method described by Panozzo et al. (2014). 5 mg freeze-dried was added to 1.5 mL SDS-TGE solution, obtained by mixing 5 mL SDS solution (25 % w/v) with 45 mL TGE buffer (10.4 g Tris, 6.9 g glycine, 1.2 g EDTA per litre, pH 8). 30 μL Ellman's reagent (4 mg/mL DTNB in TGE) was added, and differences in the colour developed by each sample were measured at 412 nm with a spectrophotometer and corrected using a blank measured without the sample. Free SH groups were determined according to the following equation (Eq. (2)):

$$\mathrm{m}M\,SH/g = \frac{73.53 \times A_{412} \times D}{C} \quad (2)$$

where A_{412} is the absorbance at 412 nm, C is the sample concentration (mg/mL), D is the dilution factor (10) and 73.53 is derived from $10^6/(1.36\times10^{-4})$, with 1.36×10^{-4} representing the molar absorptivity of Ellman's reagent.

2.3.4. Surface hydrophobicity

Protein surface hydrophobicity was determined by fluorescence testing according to the method of Cui et al. (2012). Protein dispersions (10 mL) at 2 % (w/v) in 50 mM phosphate buffer were prepared. After complete hydration of the sample by stirring overnight at 4 °C, 0.6 mL of the fluorescent probe ANS (8 mM in 50 mM phosphate buffer, pH 6.8)

was added and allowed to react for 15 min in the dark (F). A reference blank was also prepared without the sample (F_0). A fluorescence spectrometer (Cary Eclipse model, Agilent Technologies, Santa Clara, CA, United States) was used to determine the relative fluorescence intensity (RFI) of each sample, which was set as follows: excitation at 390 nm and emission at 480 nm. The RFI is defined by the following equation (Eq. (3)):

$$RFI = \frac{(F - F_0)}{F_0} \quad (3)$$

Where F is the fluorescence intensity of the sample and F_0 is the fluorescence intensity of the reference blank. The surface hydrophobicity index was determined from the slope obtained by plotting RFI versus protein concentration.

2.3.5. Solubility

Freeze-dried samples were suspended in deionized water (3 % w/w) to determine the solubility according to Gao et al. (2023). The solutions were stirred at room temperature for 24 h, centrifuged (Mikro 120, Hettich Italia srl, Milan, Italy) at 20,000 × *g* for 20 min at 4 °C and the supernatant was subjected to protein quantification using the Kjeldahl method (N×6.5) (Method 920.87, AOAC, 1997). Sample solubility was calculated based on the total protein content of the WPI (94.7 %, Section 2.1) according to Eq. (4):

$$Protein\ solubility\ (\%) = \frac{Proteins\ in\ the\ supernatant}{Total\ proteins} \times 100 \quad (4)$$

2.3.6. Foaming properties

Protein dispersions (10 mL) at a concentration of 1 % (w/v) in distilled water were hydrated at 4 °C for 12 h. The next day, the foaming activity (FAI) and foaming stability (FSI) indices were determined by placing the samples in a 50 mL graduated cylinder. The generated foam was mixed with Ultraturrax (Ika-Werke, DI 25 basic, Staufen, Germany) at 800 × *g* for 3 min and was measured over 1 h. FAI and FSI were calculated by Eq. (5) and Eq. (6):

$$FAI\ (\%) = \frac{V_1}{V_0} \times 100 \quad (5)$$

$$FSI(\%) = \frac{V_{60}}{V_0} \times 100 \quad (6)$$

Where V_1 is the volume of the foam measured immediately after the foaming process, V_{60} after 60 min and V_0 is the volume of the initial liquid phase.

2.3.7. Emulsifying properties

Samples were dispersed in deionized water (0.01 g/mL) and stirred overnight at 4 °C. Emulsions were prepared using sunflower oil (9:1 w/v sample:oil ratio) and to homogenize with an Ultraturrax at 1000 × *g* for 3 min. 250 μL of the obtained emulsions were taken from the bottom of the homogenized sample, diluted (1:100, v/v) in 0.1 % SDS solution and used to determine emulsifying activity (EAI) and stability (ESI) indices at 0 and 120 min respectively by spectrophotometric analysis at 500 nm (Pearce & Kinsella, 1978). The indices were calculated as follows (Eq. (7), Eq. (8)):

$$EAI\left(\frac{m^2}{g}\right) = \frac{2 \times 2.303 \times A \times DF}{C \times (1 - \theta) \times \Phi \times 10000} \quad (7)$$

$$ESI(\min) = \frac{A_0}{A_0 - A_{120}} \times 120 \quad (8)$$

where DF is the dilution factor (100), C is the initial protein concentration (0.01 g/mL), θ is the emulsion oil fraction (0.1), Φ is the optical path (1), and A_0 and A_{120} are the absorbances of the diluted emulsion at time 0 and after 120 min, respectively.

2.3.8. Gelling properties

Gelling properties were determined by the method of Zhao et al. (2017) using 5 mL sample suspensions in distilled water and protein concentrations of 5, 10, 15, and 20 % (w/v). The suspensions were placed into plastic tubes, heated at 90 °C for 30 min, and then cooled at 4 °C for 12 h. The least gelling concentration was determined visually by inverting the tube when the sample did not fall or slip. Gelled samples were further tested for viscoelastic properties (moduli G′ and G′′) using an RS6000 Rheometer (Thermo Scientific RheoStress, Haake, Germany), equipped with a Peltier system for temperature control (20 °C). To this aim, gelled samples were gently removed from the tube and portioned before being transferred to a rheometer plate. A resting time of 5 min was used before the analysis. A parallel plate geometry (25 mm diameter) with a gap of 1.0 mm was used. Amplitude sweep tests were performed to identify the linear viscoelastic region (LVR) by increasing the stress from 0.1 to 5000 Pa at 1 Hz. Frequency sweep tests were then performed by increasing the frequency from 0.1 to 10 Hz at stress values selected in the LVR.

2.4. Antioxidant activity

Determination of the scavenging activity of whey protein hydrolysates was performed against the DPPH radical using the method of (Cui et al., 2022) with some modifications for a 96-well microplate reader. The samples were dissolved in deionized water (3 % w/v) and hydrated for 12 h at 4 °C. DPPH was prepared at a concentration of 0.05 mg/mL in ethanol under dark conditions and stored for a maximum of 3 h at ambient temperature. Each sample (50 μL) was mixed with 150 μL DPPH solution in a 96–well microplate and incubated for 20 min at 25 °C in a microplate reader (Sunrise, Tecan Italy S.r.L., Milan, Italy). The absorbance values were read at 517 nm every 30 s, before shaking for 5 s, for samples and blanks, the latter consisting of distilled water and DPPH. Trolox was used as a positive control and was diluted from 0 to 1.2 μM. The antioxidant activity was expressed as Trolox equivalents (TE) per g of the sample.

2.5. Data analysis

All determinations are expressed as the mean ± standard deviation (SD) of at least three repeated measurements from two experimental replicates (n = 2). Statistical analysis was performed using R v. 2.15.0 (The R Foundation for Statistical Computing, Wien, Austria). Bartlett's test was used to check the homogeneity of variance, one-way ANOVA and *t*-test were carried out, and the Tukey test was used to determine statistically significant differences among means ($p < 0.05$). Correlation analysis was performed between the amount of selected MW fractions and functionalities and expressed in terms of linear correlation coefficient (R), relative slope and p-value. The functionality index (FI) was computed by the ratio between the functional properties of WPH and WPI at each hydrolysis time.

3. Results and discussion

3.1. Characterization of whey protein hydrolysates

Figure 1 shows the degree of hydrolysis (DH) of samples obtained using Alcalase and Protamex enzymes at increasing hydrolysis time. The DH significantly increased just after 10 min of hydrolysis, showing a further increase after 20 min. Afterwards, DH did not significantly change up to 120 min and 60 min, for Alcalase and Protamex, respectively. By further increasing time, the hydrolysis degree progressively rose reaching DH of 25.65 % ± 1.13 and 26.71 % ± 1.37 after 480 min using Alcalase and Protamex, respectively. These data are in agreement with Li-jun et al. (2008), who observed a DH of approximately 20 % after

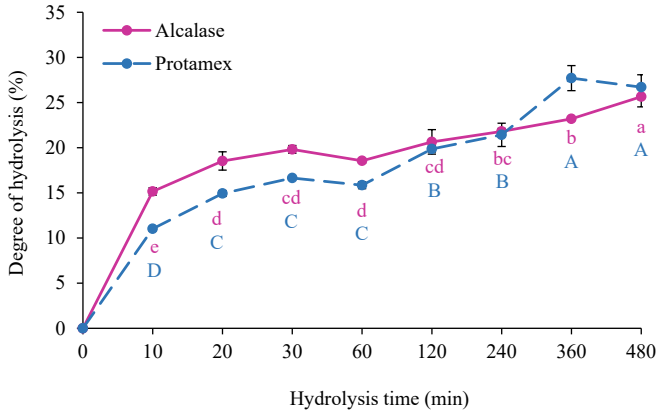

Fig. 1. Degree of hydrolysis (DH) of whey protein hydrolysates (WPHs) obtained from whey protein isolate (WPI) hydrolysed with Alcalase and Protamex as a function of time. [a–e]: means indicated by different letters are statistically different ($p < 0.05$) for Alcalase hydrolysates. [A–D]: means indicated by different letters are statistically different ($p < 0.05$) for Protamex hydrolysates.

240 min of WPC hydrolysis, and Perea et al. (1993), who detected almost 25 % DH when Alcalase was used on WPI. In agreement with previous research (Innocente et al., 2023), Alcalase exhibited a higher proteolytic activity up to 60 min of hydrolysis as compared to Protamex ($p < 0.05$), probably due to the presence of several proteinases with different specificities that confer a higher endopeptidase activity (Hoa & Dao, 2017; Osman et al., 2016; Severin & Xia, 2006; Zhang et al., 2013). However, at the end of the reaction, the hydrolysis degree between the two enzymes was not significantly different ($p > 0.05$).

To further investigate the effect of the hydrolysis process on WPHs, the molecular weight (MW) distribution of peptides was obtained by high-performance gel-filtration chromatography (Figure 2 and Fig. S1). As expected, unhydrolyzed WPI showed the presence of proteins with MW > 18 kDa, attributed to BSA (66.4 kDa) and in the range of 3–18 kDa, attributed to β-lactoglobulin monomers formed during acidic HPLC elution (18.4 kDa), and α-lactalbumin (14 kDa) (Jambrak et al., 2014). By increasing hydrolysis time, both enzymes induced the progressive hydrolysis of the high MW native proteins. Just after 10 min, the amount of the > 18 kDa MW class was significantly reduced by 33 % and 26 % in the case of Alcalase and Protamex respectively, leading to the formation of peptides with lower dimensions, in accordance with previous data (Innocente et al., 2023). The progressive reduction of the amount of high and intermediate MW peptides was maintained for the entire hydrolysis time. At the end of the hydrolysis, peptides with MW < 3 kDa

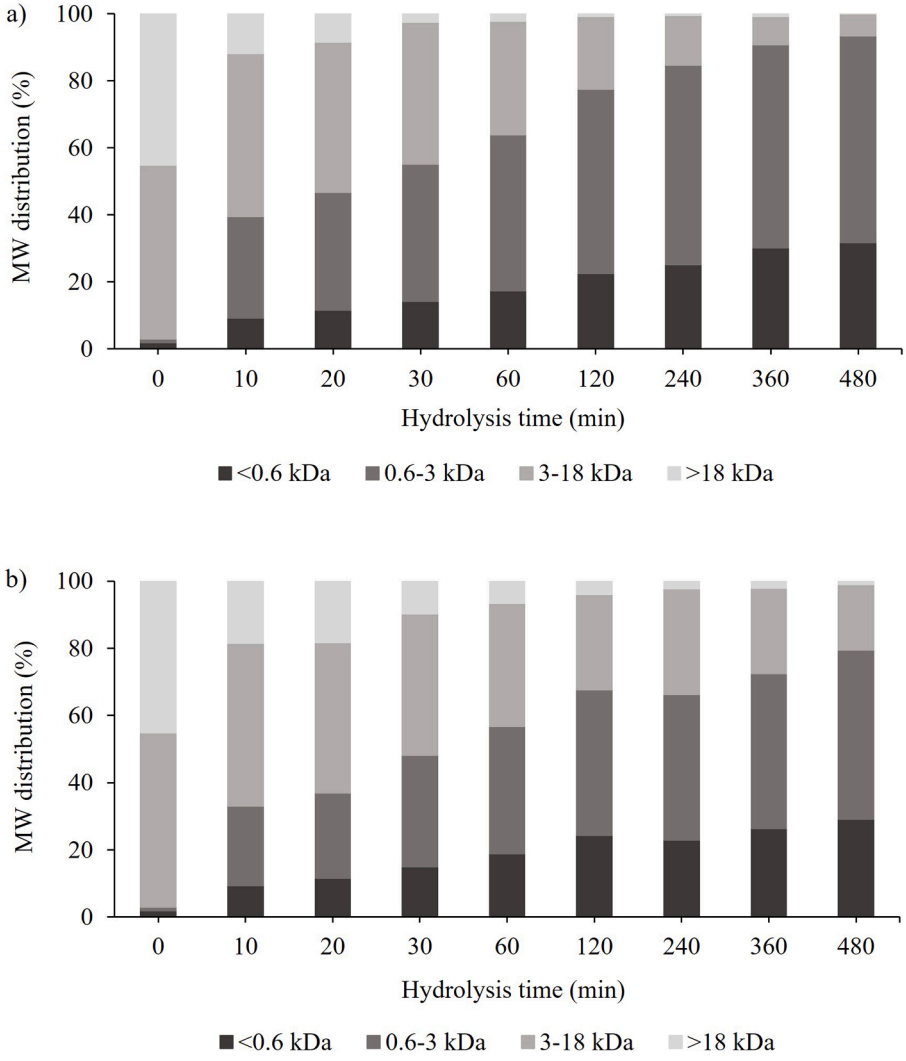

Fig. 2. Molecular weight (MW) distribution of whey protein hydrolysates (WPHs) obtained from whey protein isolate (WPI) hydrolysed with Alcalase (a) and Protamex (b). WPI data were reported for both enzymes to allow the comparison with WPHs.

represented almost 93 % of the hydrolysate sample for Alcalase and 79 % for Protamex, whereas native proteins were almost absent. These results indicate that Alcalase was more prone to form small peptides than Protamex ($p < 0.05$), in agreement with literature studies on WPI, soybean, and fish protein (Da Rocha et al., 2018; Hoa & Dao, 2017; Innocente et al., 2023). This behaviour could be attributed to the enzyme type. Alcalase exhibited greater activity in comparison with Protamex due to its higher specificity towards serine residues, which are abundant in WP (Klompong et al., 2007), and its endoproteolytic activity (Schlegel et al., 2019).

To further investigate the structural modifications occurring upon hydrolysis of the WPHs, free SH groups were analysed (Table 1). The WPI showed a free SH content of approximately 18 μM/g, which is comparable to the range reported in the literature (Shen et al., 2017). SH groups are associated with the presence of sulfur-containing amino acids, mainly cysteine, which are abundant in the primary structure of WPI β-lactoglobulin (Le Maux et al., 2015). The free SH groups content progressively decreased upon hydrolysis, independently of the enzyme used, and no significant differences were detected after 240 min. Free SH groups are expected to increase with increasing DH due to peptide bond cleavage and exposure of inner protein residues (Adjonu et al., 2013; Xu et al., 2016). However, the results acquired in this study could be attributed to reassembling mechanisms occurring during hydrolysis. The oxidation of the exposed SH residues as well as the aggregation phenomena through disulfide linkages within and among whey peptides has been previously indicated as a major factor for the reduction of free SH groups in hydrolysate samples (Mohan et al., 2015). Similarly, Zhao et al. (2011) detected the reduction in SH groups and the consequent easy formation of S–S bridges in peptides when hydrolysis was conducted with Alcalase under mild temperature conditions (50 °C) (Van Lancker, Adams, & De Kimpe, 2011).

Additionally, the surface hydrophobicity of WPHs was assessed (Table 1). Independently of the enzyme type, a progressive decrease in surface hydrophobicity was observed with increasing hydrolysis time. This reduction suggests that hydrolysis resulted in the burying of hydrophobic amino acids in the inner peptide structure (Banach et al., 2013). Similar results were previously obtained with increasing DH probably due to the exposure of ionizable amino and carboxyl groups upon enzymatic treatment, which may increase the polarity of WPHs (Yang et al., 2022). Moreover, the effect of the peptide size could not be excluded. As observed by Wu et al. (1998) in the case of soy protein hydrolysed by papain, such a decreasing trend occurred when MW was below 100 kDa. Finally, the hydrolysis process with the concomitant reduction of peptides MW may be responsible for the modification/reduction of the binding sites for ANS resulting in lower surface hydrophobicity (Nisov et al., 2020).

Overall, the results acquired indicate that hydrolysis induced complex structural modifications. On one hand, peptides formed during hydrolysis can interact to form new disulfide bridges at both intra- and intermolecular structural levels leading to the reduction of free SH groups (Table 1). On the other hand, the resulting low-MW peptides (Figure 2) may expose hydrophilic groups on their surface probably affecting the functionalities of peptides.

Table 1
Free sulfhydryl groups (μM/g) and surface hydrophobicity index of whey protein hydrolysates (WPHs) obtained from whey protein isolate (WPI) hydrolysed with Alcalase and Protamex enzymes.

Time (min)	Free −SH groups (μM/g)		Surface hydrophobicity index	
	Alcalase	Protamex	Alcalase	Protamex
0	$17.96 \pm 1.53^{a,B}$		$222.15 \pm 0.66^{a,A}$	
10	11.72 ± 0.31^{c}	20.32 ± 0.37^{A}	66.70 ± 0.12^{b}	140.49 ± 0.51^{B}
20	12.37 ± 0.97^{bc}	16.67 ± 0.27^{BC}	31.17 ± 0.18^{e}	58.94 ± 0.09^{D}
30	12.98 ± 0.27^{bc}	15.92 ± 0.06^{C}	42.41 ± 0.23^{c}	48.31 ± 0.14^{E}
60	13.87 ± 0.39^{b}	15.35 ± 0.38^{D}	36.30 ± 0.29^{d}	63.60 ± 0.15^{C}
120	8.68 ± 0.07^{d}	9.66 ± 0.31^{E}	31.34 ± 0.35^{e}	22.27 ± 0.10^{F}
240	2.28 ± 0.11^{e}	2.18 ± 0.12^{F}	13.34 ± 0.09^{g}	23.12 ± 0.12^{F}
360	2.23 ± 0.76^{e}	0.16 ± 0.14^{F}	20.06 ± 0.31^{g}	17.48 ± 0.17^{G}
480	0.92 ± 0.26^{e}	1.43 ± 0.89^{E}	10.44 ± 0.16^{h}	16.34 ± 0.27^{G}

Data show mean values (±SD) for three replicates. WPI data were reported for both enzymes to allow the comparison with WPHs.
[a–f]: means indicated by different letters are statistically different ($p < 0.05$) for Alcalase hydrolysates.
[A–F]: means indicated by different letters are statistically different ($p < 0.05$) for Protamex hydrolysates.

3.2. Functionalities of whey protein hydrolysates

In the second part of the study, WPHs were analysed for their functionalities. Results regarding solubility, emulsifying (emulsion ability index, EAI, and emulsion stability index, ESI) and foaming abilities (foaming ability index, FAI, and foaming stability index, FSI) are reported in Table 2. In agreement with the literature, WPI showed a solubility higher than 95 % (Segat et al., 2014) and slightly increased after 10 min hydrolysis with both enzymes, probably due to the reduction of peptide dimensions (Figure 2). Moreover, the decrease in surface hydrophobicity (Table 1) is expected to favour WPHs-water interactions (Banach et al., 2013; Severin & Xia, 2006). Similar results were obtained by Severin and Xia (2006), who detected a solubility increase of WPC hydrolysates obtained by both Alcalase and Protamex. No further increase in solubility was detected upon further increasing the hydrolysis time.

Regarding emulsifying properties (Table 2), EAI progressively increased within 20 min-hydrolysis with Alcalase, whereas a further increase in hydrolysis time resulted in a reduction of the emulsion ability below that of WPI. On the contrary, in the case of Protamex, a general decreasing trend in EAI as compared with WPI was observed. No significant differences in ESI were found between WPI and the Protamex hydrolysates. By contrast, a slight decrease in ESI was observed for Alcalase WPHs obtained after 240 and 480 min (Table 1).

These results may be attributed to the fact that limited hydrolysed WPHs may still exert the well-known surfactant properties of native proteins. Independently of the enzyme used, WPHs with 10–20 % of polypeptides with MW > 18 kDa and approximately 45 % of polypeptides with MW between 3 kDa and 18 kDa (Figure 2) presented emulsifying properties higher or comparable to those of WPI. These results agree with Liu et al. (2013), who reported enhanced emulsifying properties of hydrolysates containing residual native proteins. Moreover, the increasing presence of soluble peptides with low (< 0.6 kDa) and intermediate (< 3 kDa) MW may reduce the interfacial tension at the water–oil interface and favour the formation of stable films around the oil droplets (Jamdar et al., 2010). Overall, solubility and MW distribution of peptides were found to be the main factors determining the emulsion properties of WPHs (Table 1), as previously observed by other authors (Nisov et al., 2020).

Considering the foaming properties of WPHs (Table 2), both FAI and FSI were higher than those of WPI for all hydrolysis times considered. Alcalase hydrolysis promoted a sharp and constant increase in FAI from 10 to 30 min of hydrolysis, the range in which the highest value was observed. At higher hydrolysis times, a gradual decrease in FAI was observed. On the other hand, WPHs obtained by Protamex exhibited a progressive increase in FAI over the entire hydrolysis time. Similar foaming behaviour was detected in WPHs due to the ability of low–MW peptides to effectively place themselves at the water–air interface (Jamdar et al., 2010; Sindayikengera & Xia, 2006; Sinha et al., 2007).

Table 2
Solubility (%), emulsion ability index (EAI, m^2/g), emulsion stability index (ESI, min), foaming ability index (FAI, %), and foaming stability index (FSI, %) of whey protein isolate (WPI) and hydrolysates (WPHs) obtained by Alcalase and Protamex at different hydrolysis times.

Hydrolysis time (min)	Solubility (%)		EAI (m^2/g)		ESI (min)		FAI (%)		FSI (%)	
	Alcalase	Protamex	Alcalase	Protamex	Alcalase	Protamex	Alcalase	Protamex	Alcalase	Protamex
0	$95.2 \pm 1.1^{d,B}$		$20.3 \pm 0.5^{bc,A}$		$177.9 \pm 33.1^{ab,A}$		$20.4 \pm 3.9^{f,D}$		$12.5 \pm 2.8^{c,D}$	
10	99.8 ± 0.8^{ab}	99.0 ± 0.4^{AB}	22.1 ± 1.2^{ab}	15.3 ± 2.4^{BC}	233.4 ± 39.8^{a}	180.7 ± 5.6^{A}	76.2 ± 3.0^{e}	72.2 ± 2.6^{C}	26.0 ± 0.1^{bc}	25.9 ± 10.5^{C}
20	99.3 ± 0.7^{ac}	99.8 ± 0.9^{A}	24.0 ± 0.0^{a}	17.2 ± 1.7^{B}	163.5 ± 7.7^{ab}	171.2 ± 39.8^{A}	129.6 ± 0.0^{b}	100.3 ± 10.9^{BC}	44.4 ± 10.5^{bc}	38.5 ± 2.1^{BC}
30	99.9 ± 0.9^{a}	98.9 ± 0.9^{AB}	19.2 ± 1.3^{cd}	18.0 ± 0.0^{AB}	178.0 ± 10.3^{ab}	170.0 ± 24.6^{A}	176.5 ± 4.9^{a}	104.0 ± 5.7^{B}	29.5 ± 3.6^{bc}	36.0 ± 11.3^{BC}
60	99.8 ± 0.3^{a}	99.8 ± 1.7^{AB}	18.4 ± 0.5^{d}	16.3 ± 0.4^{B}	173.0 ± 10.5^{ab}	181.5 ± 31.6^{A}	139.7 ± 3.7^{b}	104.0 ± 5.7^{B}	56.7 ± 6.9^{ab}	30.0 ± 2.8^{C}
120	97.4 ± 0.7^{c}	99.8 ± 0.3^{A}	17.5 ± 0.0^{cd}	13.3 ± 1.2^{C}	155.4 ± 9.0^{ab}	176.4 ± 14.7^{A}	94.4 ± 2.5^{d}	136.0 ± 5.7^{A}	47.4 ± 14.6^{bc}	56.0 ± 00.0^{AB}
240	97.8 ± 0.1^{bc}	99.5 ± 0.6^{A}	7.5 ± 0.3^{e}	10.0 ± 0.7^{D}	138.1 ± 7.2^{b}	171.1 ± 15.3^{A}	111.7 ± 4.2^{c}	120.0 ± 10.0^{AB}	85.0 ± 15.5^{a}	70.0 ± 8.5^{A}
360	97.5 ± 0.2^{bc}	99.6 ± 1.4^{AB}	5.5 ± 0.2^{e}	8.5 ± 0.0^{D}	174.4 ± 30.2^{ab}	234.1 ± 3.8^{A}	106.0 ± 2.8^{cd}	108.0 ± 17.0^{AB}	42.0 ± 8.5^{bc}	66.0 ± 14.1^{A}
480	97.7 ± 0.5^{c}	97.6 ± 0.8^{AB}	2.7 ± 0.9^{f}	8.0 ± 0.3^{D}	131.4 ± 2.3^{b}	175.2 ± 1.4^{A}	102.2 ± 3.1^{cd}	122.0 ± 19.8^{AB}	54.1 ± 2.7^{ab}	54.0 ± 2.8^{AB}

Data show mean values (±SD) for three replicates. WPI data were reported for both enzymes to allow the comparison with WPHs.
[a–f]: means indicated by different letters are statistically different ($p<0.05$) for Alcalase hydrolysates.
[A–D]: means indicated by different letters are statistically different ($p<0.05$) for Protamex hydrolysates.

Peptide fragments generated upon proteolysis present higher adsorption rates and structural flexibility as compared to intact proteins (Foegeding et al., 2006). Accordingly, a linear increase in foaming properties was observed with the decrease in MW up to hydrolysis times of 30 and 120 min for Alcalase and Protamex, respectively, which are the hydrolysis times corresponding to the highest FAI values. Such a linear increase in FAI correlated well with the decrease in the amount of peptides with dimensions higher than 3 kDa, and the concomitant increase in those with dimensions below 3 kDa (Table S1). Moreover, the high slope values of the functions correlating FAI with peptides with MW in the range 3–18 kDa ($m = -16.0$ and -4.1 for Alcalase and Protamex, respectively, Table S1) and < 0.6 kDa ($m = 12.2$ and 4.7, respectively, Table S1), possibly indicate that the disappearance of the first fraction and the formation of the second one highly contributed to the observed foaming activity.

The gelation ability of WPH samples at different concentrations (10, 15, and 20 % w/v) was assessed and compared to that of WPI. The first screening step was performed by visually observing the samples. All WPHs obtained by Alcalase were unable to form a gel network, probably due to the small size of the peptides. On the other hand, WPHs produced by Protamex exhibited gelling properties when the hydrolysis times were 10, 20, and 30 min, while further increasing the process time did not contribute to this property. In this regard, the nature of the polypeptides specifically generated by the two hydrolytic enzymes has been reported to play a key role in the gelling functionality of the hydrolysates (Severin & Xia, 2006). In particular, the presence of reactive free SH groups, which are higher in Protamex WPHs than in Alcalase ones (Table 1), is probably responsible for the formation of a network stabilized by strong covalent interactions such as disulfide bridges (Zhao et al., 2011). Moreover, although hydrolysates retain the ability to form networks, the extended reduction of MW could affect gel strength and gelling properties (Lamsal et al., 2007).

Samples showing gelling properties were further analysed for their rheological properties (Table 3 and Fig. S2). Both WPI and WPHs gels presented G′ parallel to G″ and dependent on the applied frequency, indicating weak gel behaviour. As expected, WPI formed gels at all the tested concentrations. Large protein molecules are known to present good gelation properties due to their ability to create extensive 3D networks by cross-linking. In particular, in the case of thermal gelation of WPI, the formation of disulfide bonds contributes significantly to the overall gel strength. Such protein–protein interactions are stronger than peptide-peptide interactions because of the lower availability of cross–linking sites on smaller MW molecules (Huang et al., 1999). In fact, in the case of WPH gels, the magnitude of the rheological modulus was lower than that observed for WPI with the only exception of the sample prepared with 20 % WPH obtained after 10 min of hydrolysis. The rheological properties were strongly dependent on DH rather than the concentrations of the sample, being lower when hydrolysis was prolonged. Such results could again be attributed to the peptide dimensions as well as to the reduction of free SH groups and hydrophobicity upon enzymatic treatment (Table 1). The small molecular size, as well as the increase in charge repulsion among peptide molecules, are likely to further contribute to the reduction of WPH's networking ability (Jeewanthi et al., 2015; Severin & Xia, 2006). These hypotheses were also confirmed by Banach et al. (2013), who observed a significant reduction in gel strength when milk proteins were hydrolysed with different

Table 3
Storage (G′), loss (G″) moduli and loss tangent (tan δ) of gels from whey protein isolate (WPI) and whey protein hydrolysates (WPHs) obtained with Protamex at 1 Hz as a function of hydrolysis time and protein or hydrolysate concentration.

Sample	Concentration (% w/v)								
	10			15			20		
	G′ (kPa)	G″ (kPa)	tan δ	G′ (kPa)	G″ (kPa)	tan δ	G′ (kPa)	G″ (kPa)	tan δ
WPI	8.30 ± 0.29^{a}	1.05 ± 0.07^{a}	0.126^{b}	31.29 ± 3.01^{a}	5.00 ± 0.21^{a}	0.160^{b}	71.54 ± 8.88^{a}	8.22 ± 0.79^{b}	0.115^{b}
P10	3.42 ± 0.19^{b}	0.55 ± 0.24^{b}	0.161^{a}	15.05 ± 0.45^{b}	2.53 ± 0.09^{b}	0.168^{ab}	72.94 ± 3.88^{a}	13.17 ± 0.66^{a}	0.180^{a}
P20	1.51 ± 0.21^{c}	0.25 ± 0.03^{c}	0.166^{a}	10.70 ± 0.01^{b}	1.88 ± 0.02^{c}	0.176^{a}	12.93 ± 0.34^{b}	2.16 ± 0.02^{c}	0.167^{a}
P30	0.31 ± 0.05^{d}	0.06 ± 0.01^{d}	0.194^{a}	2.26 ± 0.24^{c}	0.38 ± 004^{d}	0.167^{ab}	3.63 ± 0.13^{c}	0.61 ± 0.04^{d}	0.168^{a}

Data show mean values (±SD) for three replicates.
[a–d]: in the same column, means indicated by different letters are statistically different ($p < 0.05$).

enzymes. These authors suggested that the small peptides were responsible for the production of softer gels owing to the aggregation of their hydrophobic residues, thus resulting in reduced hydrophobic interactions. This hypothesis was corroborated by correlation analysis between G′ values and the amount of each MW fraction (Table S2) exhibiting the highest slope and linear correlation coefficient (R) when peptides with MW of 18–3 kDa decreased and those with MW of 3–0.6 kDa increased.

Finally, the effect of hydrolysis on the antioxidant activity, of WP and the derived hydrolysates was evaluated, chosen as the target biological functionality (Figure 3). The antioxidant activity of WPI was in the range reported in the literature and can be attributed to the intrinsic ability of WPI residues to scavenge free radicals due to the presence of tyrosine and cysteine amino acids (Nwachukwu & Aluko, 2019). The hydrolysis process induced a significant increase in the antioxidant activity of WPHs in accordance with other authors who reported the same behaviour when the process was conducted with Alcalase 2.4L, Flavourzyme, Protamex, and Neutrase enzymes (Dryáková et al., 2010). Looking at the enzyme effect, a progressive increase in antioxidant activity was observed for peptides obtained by Alcalase up to 120 min reaching the maximum value of 8.83 mgTE/g ± 0.08 while a significant reduction of this biological property was detected by further increasing the hydrolysis time. When hydrolysis was conducted with Protamex, the highest antioxidant activity was detected after 60 min, reaching a value of 8.01 ± 0.35, followed by a slight decrease. Antioxidant activity was not correlated with DH. Rather, the dimensions of the generated peptides and amino acid sequences probably played a role in providing antioxidant activity to the hydrolysates. Indeed, the scavenging activity of WPHs can be attributed to the release of peptides that act as electron donors to form more stable products and terminate radical chain reactions (Foh et al., 2010). The obtained results suggest that peptides exposing aromatic and sulfur amino acids in the N–terminal regions were released upon Alcalase and Protamex hydrolysis for 120 and 60 min, respectively. In fact, these peptides are known to exert prominent antioxidant activities (Zapata Bustamante et al., 2021). In the literature, conflicting information is available regarding the effect of peptide size on antioxidant activity. Even though it is generally recognized that antioxidant activity increases with decreasing peptide size, the threshold peptide MW, under which an increase in antioxidant activity is detected, is ambiguously defined. For instance, Foh et al. (2010) and Chen et al. (2022) identified thresholds of 2 kDa and 3 kDa, respectively.

Based on these considerations, the correlation between the antioxidant activity values and the content of peptides with MW < 3 kDa was evaluated to understand the role of this MW class on this biological property. Results revealed a linear increase in antioxidant activity with an increase in the peptide fraction with MW < 3 kDa for both Alcalase (R = 0.958, p < 0.001) and Protamex (R = 0.987, p < 0.001) up to 120 and 60 min, respectively. On the other hand, the downward trend in the scavenging activity observed after 120 min for Alcalase and 60 min for Protamex could be attributed to the excessive formation of low MW peptides. As previously reported by You et al. (2009), the peptide fraction with MW < 0.9 kDa is responsible for reduced antioxidant activity probably due to the chain length and the exposure of terminal amino groups. In our case, when the peptide fraction with MW < 0.6 exceeded 22.6 % for Alcalase and 18.84 % for Protamex the antioxidant activity tended to decrease indicating that such percentages represented the limit for ensuring scavenging activity.

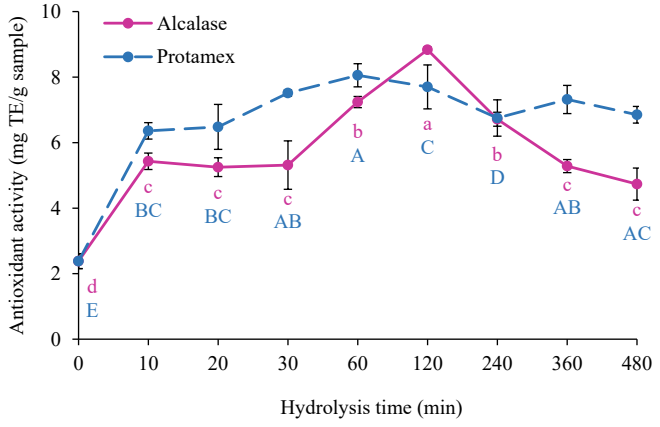

Fig. 3. Antioxidant activity of whey protein isolate (WPI) and whey protein hydrolysates (WPHs) obtained with Alcalase and Protamex at different hydrolysis times. [a–d]: means indicated by different letters are statistically different ($p < 0.05$) for Alcalase hydrolysates. [A–E]: means indicated by different letters are statistically different ($p < 0.05$) for Protamex hydrolysates.

Acquired results showed that specific hydrolysis conditions should be applied to maximize a target functionality. For instance, foaming properties can be maximized using Alcalase for 20 min (Table 1), whereas the highest antioxidant activity was observed upon hydrolysis for 120 min (Figure 3).

In view of the selection of tailored hydrolysis conditions according to the target functionalities of the final ingredient, an indicator, defined as "functionality index" (FI) was computed, based on the ratio between the functionality of WPHs and WPI for each hydrolysis time. When FI is higher or lower than 1 it indicates an increased or decreased functionality of the WPH compared to the WPI, respectively. By contrast, an FI of approximately 1 indicates that hydrolysis had no significant effect on the considered functionality, which resulted comparable to that of the intact WPI. FIs relevant to each functional property were then used to obtain a road map of functionalities for both enzymes as a function of DH (Figure 4). FI allows a quick comparison of the functionalities of hydrolysates with those of the unhydrolyzed proteins and allows the identification of a target DH, which, in turn, is the result of specific processing conditions.

Compared with WPI, Alcalase WPHs (Figure 4a) were unable to form a gel network independently of the DH, and solubility was almost unchanged. For emulsifying properties, only a slight increase in FI was observed at a DH of 18.5 %, for foaming and antioxidant properties, FIs progressively increased with the DH, leading to the maximum value at DH of around 18–20 %, (FI of 8.6 and 3.3 for foaming and antioxidant activity, respectively). A further DH increase impaired both functionalities even if their values were always higher than those of WPI, as indicated by the FI higher than 1. Similarly, when Protamex was used, independent of the DH, FIs (Figure 4b) of foaming and antioxidant activity were higher than 1, indicating higher foaming and antioxidant activity of the WPHs as compared to the WPI. In particular, the highest FIs (6.6 and 2.8) were found in correspondence with DH values of 19.9 and 16.6 % for foaming and antioxidant activity, respectively. At DH below 15 %, gelling properties were comparable to those of WPI, being FI around 1, while emulsifying properties were hampered by the hydrolysis process, regardless of the DH.

Overall, these results indicate that in the case of Alcalase, all functionalities, with the only exception of gelling properties, could be concomitantly enhanced until a DH of approximately 20 %, and when Protamex is used, hydrolysis should be accurately controlled based on the desired properties.

4. Conclusions

Evidence gathered in the present study pointed out that the enzymatic hydrolysis process can be finely steered to obtain hydrolysed whey proteins with specific functionalities. Although solubility and emulsifying capacity were only slightly modified, and gelling properties were generally decreased, hydrolysis greatly favoured foaming and antioxidant properties independently of the used enzyme. Such properties were enhanced in concomitance with specific hydrolysis degrees and peptide sizes depending on the used enzyme. These results evidence the complexity of factors involved in the production of a functional

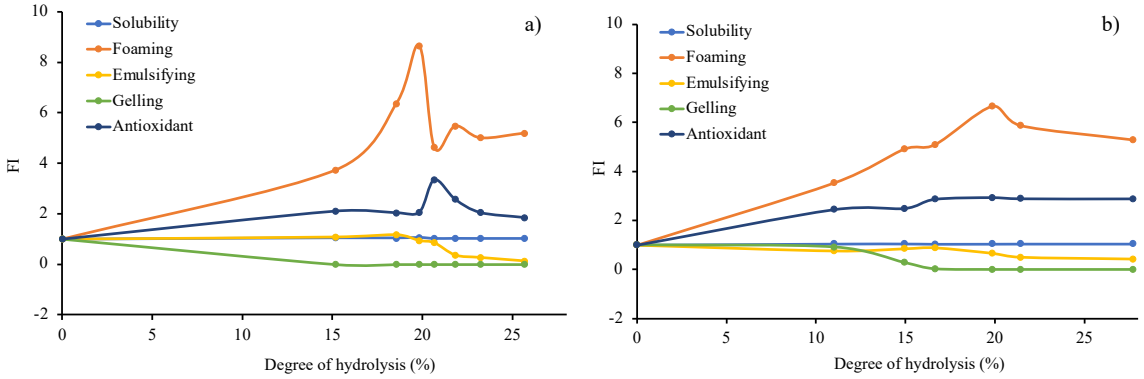

Fig. 4. Road map of the functional index (FI) of whey protein isolate (WPI) and whey protein hydrolysates (WPHs) obtained by Alcalase (a) and Protamex (b) as a function of hydrolysis degree (DH).

hydrolysate, whose performances are determined by the selection of the enzyme, the degree of hydrolysis and the dimensions of the generated peptides. To account for these complex interactions, the "functionality index", defined as the ratio between the functionality of hydrolysates and that of the intact proteins, was introduced to guide the identification of the target hydrolysis degree. By using this tool, the process can be properly steered to beget hydrolysates with tailored properties.

Results acquired open new opportunities in the production of functional ingredients from milk whey, through the application of the hydrolysis process, resulting in a green and biological approach. Overall, the outcomes of this research highlighted the importance of systematically evaluating the effect of proteolysis on structure and functionalities when dealing with protein hydrolysates.

CRediT authorship contribution statement

Giulia Di Filippo: Writing – original draft, Visualization, Validation, Investigation, Formal analysis. **Sofia Melchior:** Writing – review & editing, Writing – original draft, Visualization, Validation, Conceptualization. **Stella Plazzotta:** Writing – review & editing, Writing – original draft, Visualization, Validation, Conceptualization. **Sonia Calligaris:** Writing – review & editing, Visualization, Supervision, Resources, Conceptualization. **Nadia Innocente:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114499.

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