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Bio-based emulsifiers through enzymatic hydrolysis of a soy protein isolate

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ABSTRACT

Protein-based surfactants are gaining interest in several industrial sectors, due to their low-cost and biodegradability. Their properties make them a valid bio-based alternative to petrochemical-based tensides, thus reducing environmental and health concerns. To enhance their techno-functional properties, protein structure must be modified. Compared to chemical methods, enzymatic hydrolysis offers an industrially viable and sustainable approach for this purpose. Particularly, by adjusting enzymatic hydrolysis parameters (type of enzyme, incubation time, temperature and pH), the surfactancy of proteins can be substantially altered, broadening both the application range and the conventional emulsifiers choice. In this study, a commercial soy protein isolate (SPI) was hydrolysed through a biocatalytic approach, using Alcalase® 2.4L FG and Protamex® as enzymatic preparations, at different incubation time (15, 120, 360 min). The resulting Soy Protein Hydrolysates (SPHs) were characterised by ultrafiltration with molecular weight cut-off membranes of 10, 5, and 1 kDa. Then, the emulsifying properties of the prepared SPHs were investigated by interfacial tension (IFT) studies. The two hydrolysates, that achieved the greatest IFT reduction, were tested as emulsifiers in simple peanut oil-in-water (O/W) emulsions, prepared with a sonicator device. Their stability over time was evaluated by turbidimetry, emulsion stability index, confocal microscopy images and dynamic light scattering analyses. Protein secondary structures/emulsifying capability relationship was proposed based on circular dichroism (CD) analyses. Furthermore, for potential industrial applications, more complex O/W emulsions (including rheological modifier, solubilizing agent, antioxidants, and buffers) were also formulated using a rotor-stator. Finally, the stability and rheological behavior of these complex emulsions were thoroughly examined.

1. Introduction

The global surfactants market, recently valued at USD 42 billion, is driven by the extensive use of emulsifiers across various industrial sectors as daily chemicals, agrochemicals, pharmaceuticals and food production. This market is projected to grow from 2020 to 2025, reaching 52 billion USD by 2025 (Markets and Markets,)https://www.MarketsandMarkets.com/Market-Reports/Biosurfactants-Market-493.html. Surfactants are chemical compounds capable of altering surface or interfacial properties, which make them valuable in many industrial processes, as wetting agents, detergents, and stabilizers of disperse systems, particularly as emulsifiers (Schramm, 2005). However, the widespread application of petroleum-based surfactants poses significant risks to aquatic ecosystems (Guo et al., 2022). When surface-active agents are continuously released in wastewaters, they tend to bioaccumulate, resulting in environmental toxicity across various compartments (Traverso-Soto et al., 2013).

Given the environmental and biological issues linked to petrochemical-based surfactants, natural alternatives are attracting increasing attention (Xia, 2001). Protein-based surfactants (PBS) represent a promising alternative due to their low-cost and biodegradability. Interest in PBS has grown not only for their potential as renewable, bio-derived products but also as a solution for repurposing waste from animal and plant protein by-products (De Schouwer et al., 2019; Goldberg et al., 1991). Among plants protein, soybean stands out as a globally significant source, recognized for its rich and balanced amino acid profile (Sangiorgio, Vidović, et al., 2022b). In recent decades, soy proteins (mainly consisting of 11S and 7S globulins) (F. Liu & Tang, 2013) have been incorporated into many food products, valued for their excellent nutritional value, functional properties and potential health benefits.

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It is well known that proteins tend to accumulate at both air-water and oil-water interfaces, forming an interfacial layer, making them highly effective for stabilizing foams and emulsions (Hailing, 1981). Indeed, their amphipathic nature classifies proteins as zwitterionic surfactants (S. Magdassi, 1996). But to function effectively as surfactants, proteins must diffuse and adsorb quickly to new interfaces during homogenization, unfold, and form highly viscoelastic films through intermolecular interactions. However, low solubility both in water and in oil, high molecular weight, compact tertiary structure and limited molecular flexibility caused by the presence of the buried hydrophobic groups, frequently inhibit the emulsifying abilities of proteins. Thus, to improve their surfactancy, modifications may be required (Tamm et al., 2016). Enzymatic hydrolysis effectively produces protein hydrolysates (PHs), *i.e.*, mixtures of peptides and amino acids, and thus more functional groups and enhanced access to hydrophobic regions, previously hidden within the protein core (Scarabattoli et al., 2023; Tamm et al., 2016). These structural modifications boost the functionality of protein hydrolysates, with outcomes strongly dependent on the specific hydrolysis conditions such as pH, temperature, and enzyme concentration (Wouters et al., 2016). Moreover, enzymatic methods are also preferred over chemical ones as they operate under mild conditions, reducing side reactions and preserving the protein's nutritional value (Y. Q. Liu et al., 2019). By selecting enzyme specificity and the degree of hydrolysis (DH), hydrolysates with distinct functional, biological, and nutritional properties can be generated (Islam et al., 2022).

In this study, SPI was hydrolysed using two commercially available enzymatic formulations, Alcalase® 2.4L FG and Protamex®, over different incubation times to produce soy protein hydrolysates (SPHs). Both formulations contain Subtilisin A (E.C. 3.4.21.62), a serine endopeptidase with broad specificity for peptide bonds and a preference for large uncharged residues, resulting in peptides with generally hydrophobic characteristics (Tacias-Pascacio et al., 2020). Protamex®, being an enzyme mixture, also includes bacillolysin, a metalloendopeptidase (E.C. 3.4.24.28), with specificity similar to termolysin, preferentially releasing C-terminal leucine and phenylalanine residues (Kechaou et al., 2009; (Brenda,)).

Despite previous studies (Ding et al., 2021; Horax et al., 2017; Islam et al., 2022; Jung et al., 2005; Meinlschmidt et al., 2016; Y. Wang et al., 2022) examined the influence of both enzyme type and DH on soy protein hydrolysate properties, to the best of our knowledge, comprehensive studies on the surfactant properties of SPHs across different incubation times remain limited.

Therefore, in this paper, we investigated the surfactant capabilities of several SPHs focusing on the effect of hydrolysis time. Specifically, *i)* SPI was hydrolysed using Alcalase® 2.4L FG and Protamex® over varying incubation time, and the resulting SPHs were characterised by ultrafiltration with different molecular weight cut-off membranes (10, 5 and 1 kDa); *ii)* the physico-chemical properties of SPHs were evaluated by measuring peanut oil/water (Milli-Q) interfacial tension; *iii)* simple oil-in-water (O/W) emulsions were prepared and their stability over time was assessed, also by evaluating the Circular Dichroism (CD) behaviours; *iv)* additionally, complex emulsion formulations were created using the two most effective samples and their stability was analysed by confocal microscopy, ζ-potential measurements and rheology.

2. Experimental section

2.1. Materials and methods

All solvents, chemicals and reagents were purchased from Sigma-Aldrich and used without further purification. Xantham gum was purchased from Special Ingredients Ltd (Foxwood Industrial Park, Chesterfield, UK). Soy protein isolate (SPI) type 90 was kindly supplied by ABS FOOD s.r.l. (Peraga di Vigonza, Italy). The chemical and amino acids composition of SPI was reported in Fig. S1 (**Supplementary materials**). Proteolytic enzymes were provided by Novozymes® (Bagsværd, Denmark), including the endopeptidase Alcalase® 2.4 L FG (enzyme activity: 2.4 AU g^{-1}, Anson Units) derived from *Bacillus licheniformis* and the *Bacillus* sp. protease complex Protamex® (enzyme activity: ≥1.5 AU g^{-1}).

^{1}H NMR spectra were recorded at 400.13 Hz on a Bruker AVANCE NEO 400 spectrometer equipped with TOPSPIN software (Bruker, Karlsruhe, Germany) and a BBI probe (Double Resonance Broadband Probe) at 300 K. NMR samples were prepared by dissolving SPHs in H_2O/D_2O (9:1). ^{1}H chemical shifts (δ) are given in parts per million and were referenced to the solvent signal (δ^{H} 4.71 ppm from tetramethylsilane (TMS) for D_2O). Solvent suppression was achieved by including the Excitation Sculpting. Fourier-Transform Infrared (FT-IR) spectra (between 4000 and 400 cm^{-1}) were acquired on a PerkinElmer Spectrum 100 spectrophotometer operating in attenuated total reflection (ATR) mode equipped with a single-bounce diamond crystal (incident angle of 45°). Nitrogen content in soy protein hydrolysates (SPHs) was evaluated by elemental analysis (PerkinElmer, Series II CHNS/O analyser, PerkinElmer, Massachusetts, USA).

2.2. Hydrolysis of SPI with proteases

Soy protein isolate (SPI) was suspended in HPLC grade H_2O (5 % w_{SPI}/v_{H20}) and the resulting mixture was treated with proteolytic enzymes under magnetic stirring. Reaction conditions were set up according to optimal pH and temperature for each enzyme, see Table 1. An enzyme/substrate ratio of 1 % (w/w) was selected for Alcalase® 2.4 L FG and for Protamex®, following the methodology of Sangiorgio et al. (Sangiorgio, Vidović, et al., 2022b). The enzymes were inactivated by heat treatment at 90 °C for 15 min, at desired incubation time (15, 120 and 360 min). Reaction mixtures were then centrifuged at 5886 g (9000 rpm) for 15 min, and the supernatants (soy protein hydrolysates, SPHs) were collected, freeze-dried and stored at −20 °C. The SPH yields for each enzymatic hydrolytic treatment are reported in Table S1 (**Supplementary materials**). For the preparation of complex emulsions, the protease-mediated hydrolysis reaction was scaled-up. SPI (10 g) was suspended in HPLC grade H_2O (2 % w_{SPI}/v_{H20}) and treated with proteolytic enzyme(s) under mechanical stirring, following the same protocol above-mentioned.

2.3. Degree of hydrolysis

The degree of hydrolysis (DH) of SPHs was determined using the OPA-NAC method (Nielsen et al., 2001), with some modifications (see Degree of Hydrolysis in **Supplementary materials**).

2.4. Ultrafiltration

SPHs were dissolved in HPLC grade H_2O (*ca* 1 % w/v) and filtered sequentially through 10, 5 and 1 kDa cut-off membranes by using a 400 mL ultrafiltration cell system (Amicon® stirred cells, model 8400, 400 mL capacity, Merck Millipore Corporation). The filtration was carried out under nitrogen gas pressure at 60 psi. Four different fractions of ultrafiltered hydrolysate with molecular weights *i*) > 10 kDa, *ii*) from 10 to 5 kDa, *iii*) from 5 to 1 kDa and *iv*) < 1 kDa were recovered separately and freeze-dried. Ultrafiltration yields (*w/w*, %, referring to the weight of the starting dry hydrolysate) are reported in Fig. 1a,b. and are referred to the fractions: >10 kDa, 5–10 kDa, and <5 kDa.

Table 1
List of optimal pH and temperature conditions for proteases used.

Enzymatic Formulation	pH	Temperature (°C)	References
Alcalase® 2.4 L FG	8.0	50	Rostammiry et al. (2017)
Protamex®	7.5	50	Islam et al. (2022)

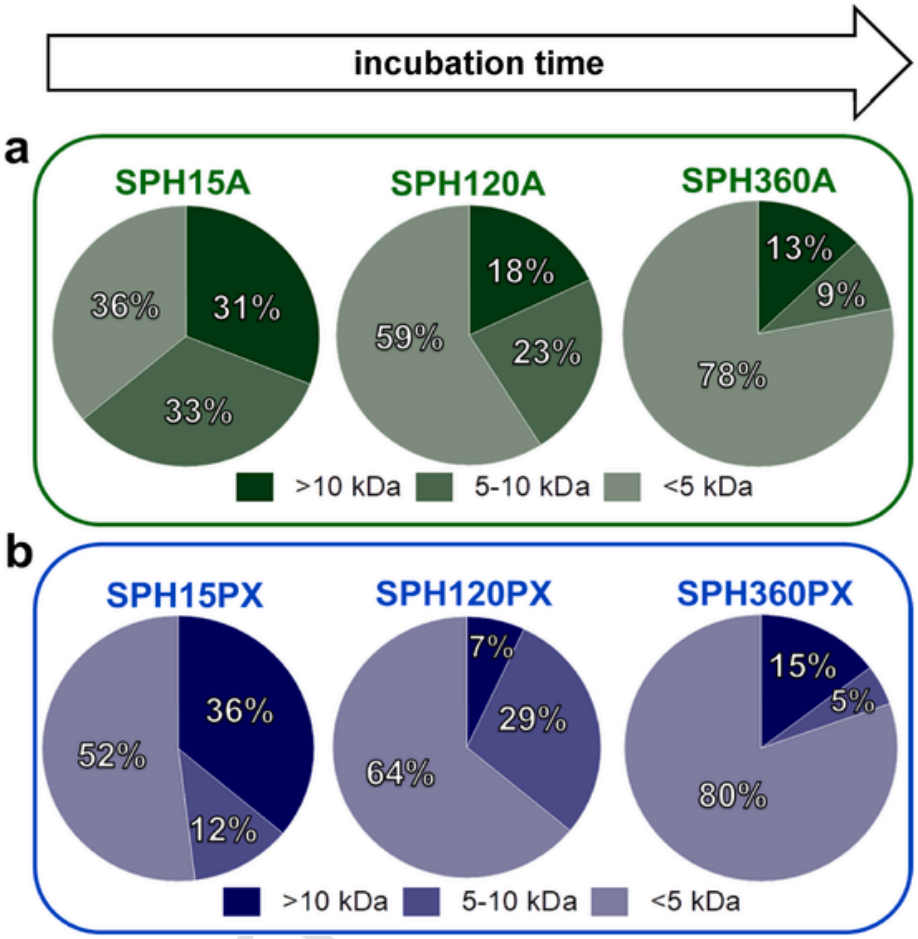

Fig. 1. Peptide molecular weight distribution by ultrafiltration of SPHs at increasing incubation time using ***a***) Alcalase® 2.4L FG and ***b***) Protamex®.

2.5. Interfacial tension measurements

The interfacial tension (IFT) between peanut oil and water values was measured at increasing SPHs concentrations (3, 6, 9 and 12 g L^{-1}), at 25 ± 1 °C by means of a Gibertini tensiometer, according to the du Noüy ring method (B.P.L. du Noüy, 1925). The method was corrected based on the Harkins-Jordan equation, with necessary parameters (*e.g.*, platinum ring radius, liquid density) inputted into the software. IFT values were measured at equilibrium after 45 min and are presented as average values from three replicates.

2.6. Simple emulsions preparation

SPHs were dissolved in water (Milli-Q) at room temperature and mixed with peanut oil in a ratio of 8:2 (v/v). Peanut oil-in-water (Milli-Q) (O/W) emulsions were prepared by using a Thermo Fisher Q700 sonicator (3 mm-titanium alloy microtip) at a fixed SPHs concentration of 12 g L^{-1} and phase volume (ϕ_v) of 0.2. The operative conditions contemplate a frequency of 20 kHz in pulsed mode (3 s on and 3 s off) at 50 % amplitude for 90 s.

2.7. Turbidimetric analysis and emulsion stability

Emulsion stability was assessed over 7–24 h by turbidimetric analysis (Song et al., 2000), measuring the absorbance at 550 nm using a Shimadzu UV–Vis spectrophotometer (UV-2600). Turbidity (τ) was calculated (Aizawa, 2014) as:

$$\tau = \ln(10)A$$

Normalized turbidity values (τ/τ_0 where τ_0 is the turbidity value at time 0 min) were analysed as a function of time. Moreover, the emulsion stability was calculated at different times according to the following equation (Zhang et al., 2014):

$$\textit{Emulsion stability}\ (\%) = \frac{H_2}{H_1} * 100$$

where H_1 is the initial emulsion layer height (cm) and H_2 is the height after the desired time.

2.8. Dynamic light scattering (DLS) analysis

Dynamic light scattering (DLS) measurements were performed by means of a Zetasizer Lab-Blue Instrument (Malvern Panalytical Ltd, Malvern, UK) equipped with an Avalanche Photodiode detector and a He–Ne laser (633 nm, nominal power 4 mW). All analyses were carried out at 25 °C in a side scattering mode at a 90° detection angle. Data were analysed by ZS Xplorer Software (Malvern Panalytical Ltd, Malvern, UK). Samples were diluted to 1:20 before analysis.

2.9. Confocal microscopy images

The droplets size distribution of fresh and aged O/W emulsions was evaluated by processing (ImageJ software) the images (average droplets number around 150) acquired by Nikon A1 laser scanning con-

focal microscope (LSCM) working in oil immersion (NA1.4) equipped with a 60 × objective. Before each analysis, emulsions were stained with Rhodamine B and images were acquired at an excitation wavelength of 561 nm, with emitted light detected between 620 and 770 nm.

2.10. Circular dichroism (CD) analyses

CD spectra were recorded on a Jasco J815SE instrument using a Suprasil quartz cuvette with 200 μm pathlength to minimize scattering phenomena (Castiglioni et al., 2007). UV spectra were recorded using the same apparatus, ensuring the photomultiplier HT voltage stayed below 600 V. The scanning speed was set to 100 nm/min and 32 scans were collected. The concentration of both hydrolysates in water (SPH15PX and SPH360A) was fixed at 0.1 g L^{-1}. The corresponding oil-in-water emulsions were tested after a 1:100 dilution. The resulting CD spectra were normalized by dividing the original curves by a factor of 5 for comparison with the spectra of SPHs in pure water.

2.11. Complex emulsions formulation

Complex peanut oil-in-water (Milli-Q) (O/W) emulsions were prepared by means of a Silverson Rotor-stator L5M-A (square hole head) at a phase volume (Φ_v) of 0.08. Hydrolysates were dissolved in water (c_{SPHs} 12 g L^{-1}) at room temperature, followed by the addition of xanthan gum (0.7 %, w/w; as rheological modifier), glycerol (4.3 %, w/w), EDTA (0.1 %, w/w), citric acid (0.05 %, w/w) and 2,6-di-*tert*-butil-4-methylphenol (BHT, 0.2 %, w/w). Then, peanut oil was added to the water phase and homogenized at 4000 rpm for 15 min.

The emulsion stability was tested by accelerated stress ageing, consisting of five consecutive hot (60 °C)-cold (4 °C) cycles every 24 h. The emulsion stability % was calculated according to the formula mentioned before and the ζ-potential was determined by DLS after dilution (1:50). Moreover, droplets size distribution of fresh, 10-day aged, and stress-tested emulsions was also assessed by confocal microscopy.

2.12. Rheological features

Rheological measurements of complex emulsions were performed on an Anton Paar Modular Compact rheometer with a P-PTD200/AIR SN82424770 cell and a CP50-1 cone plate (diameter: 49.981 mm; cone angle: 1.004; cone truncation: 103 μm) arrangement. Frequency sweep experiments were performed from 1 to 100 Hz at 25 °C with a constant strain of 5 %.

2.13. Statistical analysis

IFT and DLS experiments were performed in triplicate ($n = 3$). Data were analysed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test to assess statistically significant differences between samples at a 95 % confidence level ($p < 0.05$). Statistical analysis was performed using GraphPad Prism 9 (San Diego, CA, USA). Results are presented as means ± standard deviation (S.D.).

3. Results and discussions

3.1. Preparation of soy protein hydrolysates and relative characterizations

The functional properties of peptides produced by enzymatic hydrolysis are strongly dependent on multiple factors, including the enzyme specificity and the degree of hydrolysis. To investigate the influence of these two important aspects on the surfactancy of SPHs, Alcalase® 2.4L FG and Protamex® (A and PX in the following, respectively) were chosen as proteolytic enzymes and the hydrolysis reactions were conducted at different incubation time. Other studies examined the enzymatic hydrolysis of SPI at various hydrolysis times, specifically from 10 to 120 min (Meinlschmidt et al., 2016) and between 60 and 480 min (Islam et al., 2022). On this base, incubation times of 15, 120 and 360 min were selected for this work. The resulting soy protein hydrolysates were labelled as SPH followed by the incubation time and the enzyme (*e.g.*, SPH15A, SPH120PX, etc).

All SPHs were obtained in satisfactory yields, ranging from 53 to 64 % (Table S1 and 4th**column** in **Supplementary materials**). No significant differences in terms of N content among the hydrolysates produced with the two enzymes were observed (Table S1 and 5th column in **Supplementary materials**). FT-IR and ^{1}H NMR spectra were recorded for all SPHs. In the FT-IR spectra, the characteristic vibrational modes associated with the polypeptide repeat unit, such as amide I and amide II bands, can be identified (Fig. S2 in **Supplementary Materials**). In the ^{1}H NMR spectra, signals corresponding to the amide moiety appear in the range 6.5–8.5 ppm (see Fig. S3 in **Supplementary materials**).

The degree of hydrolysis (DH), evaluated by OPA-NAC method, increased with the longer incubation times for both enzymes, which is consistent with previous studies (Meinlschmidt et al., 2016; Islam et al., 2022, (Huang et al., 2024). After 15 min, **SPH15A** and **SPH15PX** showed DH values of (9.8 ± 0.2) % and (7.7 ± 0.1) %, respectively. At 120 min, these values rose to (11.4 ± 0.6) % for A and (10.4 ± 0.1) % for PX, respectively, and after 360 min of hydrolysis, the DH values were similar for both enzymes, around 12.7 %. Such DH values suggest the potential for SPHs to exhibit surfactancy. Indeed, recent literature indicates that a DH between 1 % and 10 % is favourable for functional properties in emulsions (Tamm & Drusch, 2017). However, prolonged hydrolysis, resulting in higher DH values, can be detrimental, as excessively small peptides may struggle to effectively stabilize fluid interfaces, which is crucial for the formation and stability of dispersed systems (Martínez-Maqueda et al., 2012).

The peptide size distribution of the SPHs was evaluated through ultrafiltration with membranes of decreasing molecular weight cut-offs (10, 5, and 1 kDa), as shown in Fig. 1a and ***b***. With both enzymes, longer incubation times resulted in an increased proportion of peptides with molecular weights below 5 kDa. Notably, Tamm and coworkers reported that Subtilisin A, which is present in both formulations, and it is known for its low specificity, tends to generate hydrolysates rich in small peptides (Tamm & Drusch, 2017). By looking at the fraction with MW > 10 kDa, Protamex® exhibited an irregular trend: as incubation time increased, the percentage of peptides over 10 kDa initially dropped from 36 % to 7 % (w/w), before rising again to 15 % (w/w). In contrast, hydrolysis with Alcalase® 2.4L FG, consistently showed a decline in the fraction with MW above 10 kDa over time. Yolandani et al. found similar molecular weight distributions, *via* SEC-HPLC analysis, for soy protein hydrolysates obtained with A and PX at varying degrees of hydrolysis (DH), indicating that Protamex® yields a slightly higher number of low molecular weight peptides (<5 kDa) than Alcalase® 2.4L FG at lower DH values (Yolandani et al., 2024).

3.2. Interfacial properties and evaluation of emulsions stability

Since proteolytic enzymes possess specific cleavage sites along the protein chain, hydrolysates from different proteases may exhibit distinct physico-chemical properties (Y. Y. Wang et al., 2021). Therefore, the ability of SPHs obtained with the Alcalase® 2.4L FG and Protamex® protocols to reduce the peanut oil/Milli-Q water interfacial tension (IFT) was evaluated using the du Noüy ring method (Ballabio et al., 2024; Sangiorgio et al., 2022a, 2024; Semproli et al., 2023). Specifically, measurements were taken at increasing SPHs concentration (from 3 to 12 g L^{-1}). High tenside concentrations are not ideal for industrial applications, so 12 g L^{-1} was chosen as the maximum.

As shown in Fig. 2, IFT decreased with the increasing of SPH concentrations, for both A and PX. Moreover, the hydrolysates obtained af-

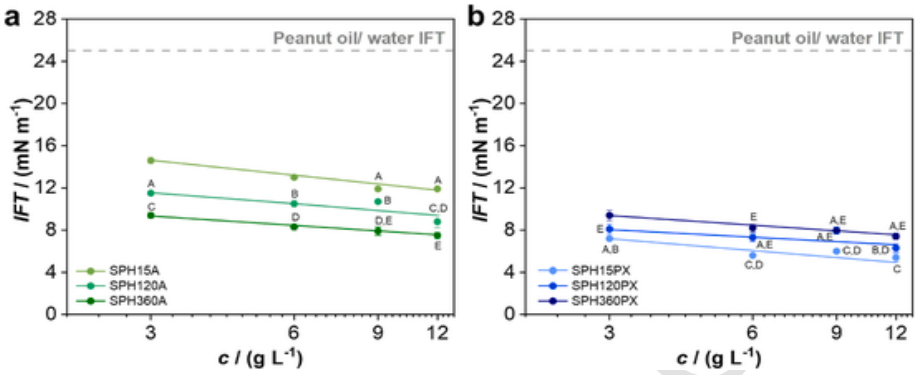

Fig. 2. Peanut oil/water interfacial tension values at increasing concentrations (from 3 to 12 g L^{-1}, logarithmic scale) of SPHs after 15, 120 and 360 min of enzymatic reaction using ***a***) Alcalase® 2.4L FG and ***b***) Protamex®. The grey dotted line represents the starting peanut oil-water IFT value, in the absence of any surfactant. Data are reported as average values on three replicates. Values are mean ± SD; different letters (A, B, …E) correspond to different values ($p < 0.05$).

ter 360 min (**SPH360A** and **SPH360PX**) demonstrated comparable IFT reduction abilities, likely due to their similar molecular weight distribution (Fig. 1a,b) and degree of hydrolysis. However, shorter incubation times, revealed a reduction in the performance for Alcalase® 2.4L FG-derived hydrolysates, whereas a quick hydrolysis (15 min) with Protamex® produced SPH15PX which effectively reduced the IFT.

This contrasting behaviour can be attributed to the different hydrolytic capabilities of Alcalase® 2.4L FG and Protamex®, both classified as endoproteases but characterised by different protease composition. The mechanism of enzyme-substrate interaction and cleavage sites vary significantly between the two (Aluko, 2015; Xu et al., 2011). It has been suggested that Alcalase® 2.4L FG is thought to primarily hydrolyse protein chains near the protein aggregates' surface. Its low selectivity leads to the formation of a large number of small protein fragments, while the core of the aggregates remains largely intact. This unhydrolyzed core exhibits a lower diffusion rate with respect to that of small fragments, which migrate to the interface more rapidly. However, these smaller fragments lack in amphiphilicity, thus resulting in poor interfacial properties (Ding et al., 2021; (Huang et al., 2024)).

In contrast, although Subtilisin A is the main enzyme in Protamex®, it also contains a different protease, *i.e.* a bacillolysin, that preferentially releases C-terminal Leu and Phe. This higher selectivity may allow Protamex® to penetrate deeper into the protein aggregates' core to select suitable peptide bonds for cleavage within just 15 min of hydrolysis (Ding et al., 2021).

In summary, the two enzymatic formulations, show an opposite ability to generate hydrolysates which reduce peanut oil/water IFT as a function of incubation time.

Based on the IFT study, the most effective samples from the Alcalase® 2.4L FG and Protamex® treatments, namely **SPH360A** and **SPH15PX**, were selected to test their emulsifying ability. Therefore, peanut oil-in-water (Milli-Q) (O/W) emulsions were prepared at SPH concentration of 12 g L^{-1}. The stability of these emulsions was assessed by turbidimetry until phase separation occurred, after which the emulsion stability index was calculated (Zhang et al., 2014) (Fig. 3). Regarding the former method, the rapid decrease in the normalized turbidity (τ/τ_0) curve reflects emulsion-breaking processes (Frenkel et al., 1982), leading to phase separation. **SPH15PX**-stabilized emulsion exhibited superior surfactancy over 10 days, compared to **SPH360A,** which underwent partial phase separation after only 7 h. Notably, the **SPH15PX**-based emulsion remained stable for approximately 7 days (emulsion stability of 89 %), as detailed in Fig. 3b. Moreover, the yellowish colour of **SPH360A**-stabilized emulsions may be due to emulsion-breaking processes. Specifically, it is reported that Ostwald ripening is responsible for colour changes (Weiss & McClements, 2001). According to the equation for the Ostwald ripening rate calculation (see "The rate of Ostwald ripening", in **Supplementary materials**), a higher IFT can accelerate this destabilization process (Kim et al., 2024). **SPH360A** exhibits an IFT of 7.4 ± 0.3 mN m^{-1}, while **SPH15PX** has a lower IFT of 5.4 ± 0.4 mN m^{-1}. This implies that the Ostwald ripening rate is lower in **SPH15PX** compared to **SPH360A**.

The droplets size distribution of fresh and 48h aged emulsions was evaluated by dynamic light scattering and confocal microscopy images (see Figure S4a,b,c,d in **Supplementary materials**). No significant changes in the average diameter were observed (d ~ 50 nm) for both **SPH360A**- and **SPH15PX**-based emulsions after 48h of ageing.

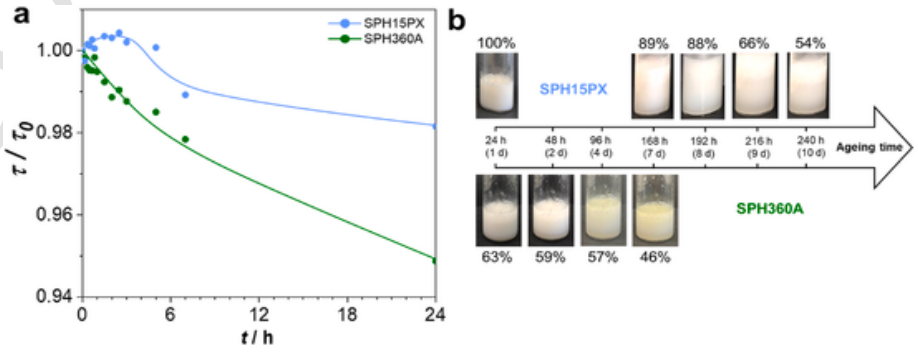

Fig. 3. a) Normalized turbidity (τ/τ_0) of O/W emulsions stabilized by **SPH15PX** and **SPH360A** at 12 g L^{-1}. **b)** Emulsion stability values and relative pictures at different ageing times for both emulsions stabilized by the two selected surfactants.

3.3. Circular dichroism (CD) measurements

SPH15PX and **SPH360A** were analysed using Circular Dichroism (CD) in the far-UV region (Woody, 2012) to determine if this technique could differentiate them in water and after emulsion preparation. In all cases, a negative band centered just below 200 nm was observed. This signal is diagnostic of "random coil" (unordered) secondary structure of peptides resulting from the enzymatic hydrolysis, as previously reported by Rahmani-Manglano et al. (Rahmani-Manglano et al., 2022).

Notably, no significant differences were observed between the **SPH15PX** and **SPH360A** spectra in water (Fig. 4a), except for a shoulder at about 230 nm, which may indicate a residual secondary structure in the **SPH15PX** (Rahmani-Manglano et al., 2022). Interestingly, the shape of the two spectra for both hydrolysates remained similar after emulsions preparation (Fig. 4b and Fig. 4c), with a slightly more pronounced shoulder for **SPH15PX**. This suggests that the residual secondary structure of **SPH15PX** persists and may be further stabilized at the peanut oil-water interface.

These results are fully in agreement with those obtained by Manglano and co-authors (Rahmani-Manglano et al., 2022), who investigated the adsorption of Whey Protein Concentrate Hydrolysates (WPCH) at the oil/water interface by using Synchrotron Radiation Circular Dichroism (SRCD).

Their study revealed a correlation between peptide conformations in WPCHs at the tricaprylin oil/water interphase and thermal stability of the resulting emulsions. They proposed that hydrophobic interactions – arising from the reorientation and solvation of the proteins/peptides towards the oil phase – played a pivotal role (over electrostatic interactions) in driving conformational changes, upon adsorption at the O/W interface (Bañuelos & Muga, 1996). Specifically, a slight transition from an unordered to α-helix conformations was found to enhance the thermal stability of the oil-in-water emulsion. This improvement was attributed to increased peptide–peptide interactions in more ordered conformations, leading to the formation of a stronger interfacial layer. Furthermore, α-helical structures have been suggested to improve emulsification due to their superior solubility in aqueous environment (Ricardo et al., 2021). In our case, **SPH15PX**, which contains a higher proportion of peptides with molecular weights greater than 10 kDa, appears to better stabilize its corresponding emulsion compared to **SPH360A**.

3.4. Complex emulsions formulation

The results discussed so far were derived from a simplified system suitable to assess the emulsifying capacity of the synthesized hydrolysates. For potential industrial applications of SPHs, it is essential to explore more realistic scenarios where additional components contribute to system stabilization (*i.e.*, antioxidants, preservatives, buffers, *etc*). Thus, two complex emulsion formulations were designed with **SPH15PX** and **SPH360A**, along with xanthan gum (as rheological modifier), glycerol (to help the solubilization of xanthan gum in water), EDTA and BHT (as antioxidants) and citric acid (to buffer the pH at 5). This recipe can serve as a starting point for cosmetic formulations (Amari et al., 2023).

The high turbidity of emulsions, even after 10 days of ageing, made it challenging to detect emulsion-breaking processes. Therefore, zeta potential measurements were employed to evaluate the stability of these systems. Analyses were performed on fresh, 10 days aged emulsions and on those subjected to accelerated stress tests (hot-cold cycles), see Fig. S5 in **Supplementary materials**. Both fresh emulsions exhibited ζ-values below −50 mV. Moreover, no significant reduction in zeta potential was observed after 10 days of ageing or stress testing, highlighting the high stability of the systems.

The droplets size distribution was evaluated using confocal microscopy images (Fig. 5a,*b*). Both **SPH360A-** and **SPH15PX**-stabilized emulsions showed a narrow droplet size distribution, with **SPH15PX** yielding smaller droplets. Notably, the hydrolysate from the PX protocol resulted in emulsions with a higher number of droplets having a diameter lower than 1 μm (15 % vs 2 % for **SPH15PX** and **SPH360A**, respectively). A subtle increase in the droplet dimensions was observed in both samples after 10 days of ageing and after the stress test. Furthermore, Fig. 5b indicates a greater tendency for droplets stabilized by **SPH360A** to aggregate. In simple emulsions, where only SPHs were used for stabilization, the droplet sizes were smaller (30–100 nm) compared to those in complex ones (1–10 μm), which is attributed to the homogenization method employed, *i.e.*, sonicator for the former and rotor-stator for the latter. To evaluate how the emulsification technique affected droplets formation, the same complex O/W emulsion formulation was prepared using a sonicator device, with **SPH15PX** as the representative stabilizing agent. The rotor-stator produced larger droplets than those obtained by sonication (see Figure S6a,b in **Supplementary materials**), a difference ascribable to the energy input of the emulsification method, fully in agreement with data reported by El-Jaby et al. (El-Jaby et al., 2009). Emulsions prepared using both the rotor-stator and sonicator devices, exhibited a ζ value of approximately - 45 mV (see Figure S6c in **Supplementary materials**). These values support, once again, the high stability of SPHs-stabilized emulsions.

Finally, the frequency sweep experiments were performed progressing from low to high frequencies (Fig. 6), which reflects a transition from a low-energy state to a high-energy one. Each sample underwent a two-step analyses, in order to evaluate potential phase separation or intrinsic instability caused by the high shear rates applied. Results indicate that "fresh" **SPH360A** samples exhibit a shear-thickening behaviour with possible phase separation occurring at higher frequencies, leading to a sudden decrease in both Storage (G′) and Loss (G′′) Moduli. After 10 days of ageing, the sample appears even more homogeneous, as evidenced by the Loss Modulus, which maintains shear thickening behaviour up to 100 Hz. However, the stress test somehow altered the

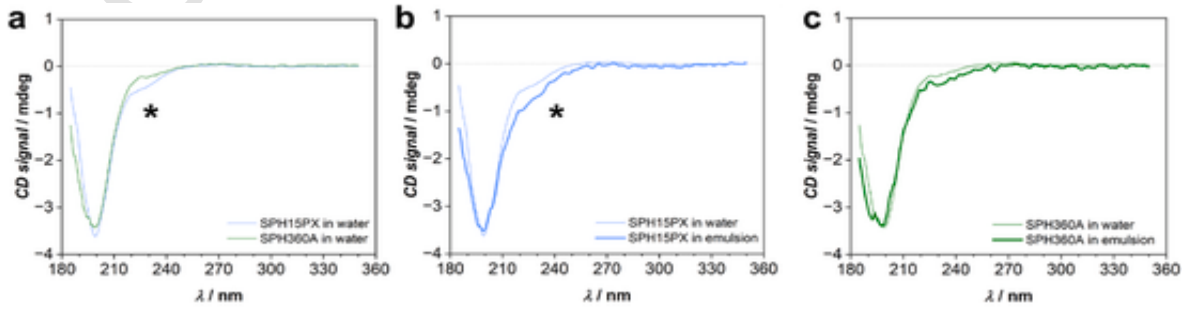

Fig. 4. a) CD spectra of SPH15PX and SPH360A in water; **b)** and **c)** comparison of CD spectra of SPH15PX and SPH360A in solution and in diluted peanut oil-in-water emulsion, respectively.

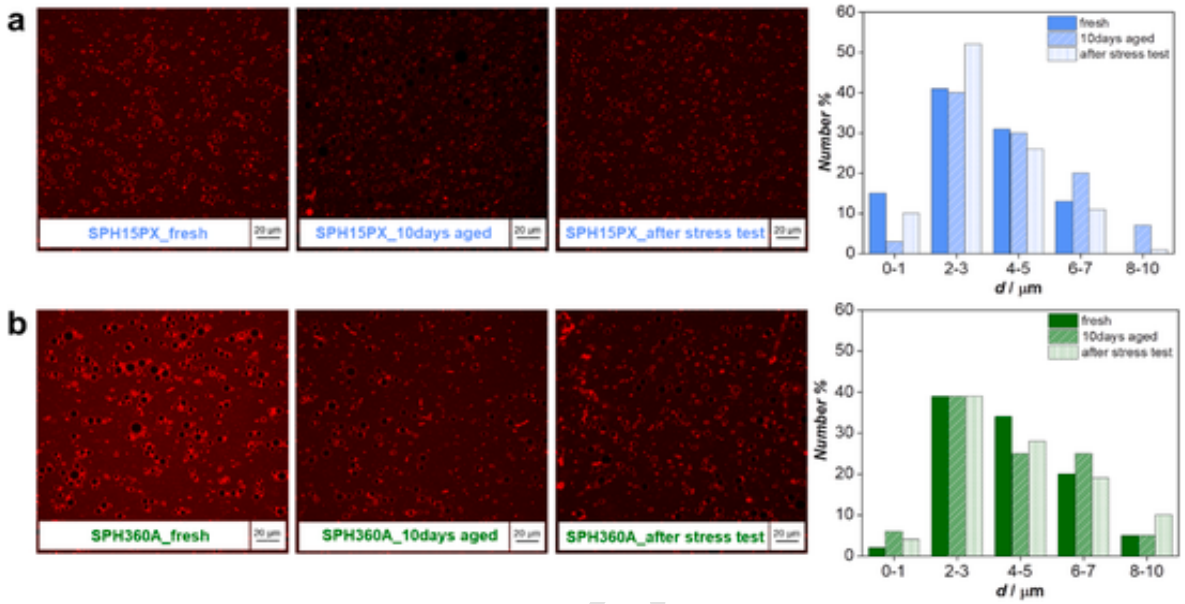

Fig. 5. Confocal microscopy images and relative droplets size distribution of fresh, 10 days-aged and after stress test emulsions stabilized by ***a***) **SPH15PX** and ***b***) **SPH360A**.

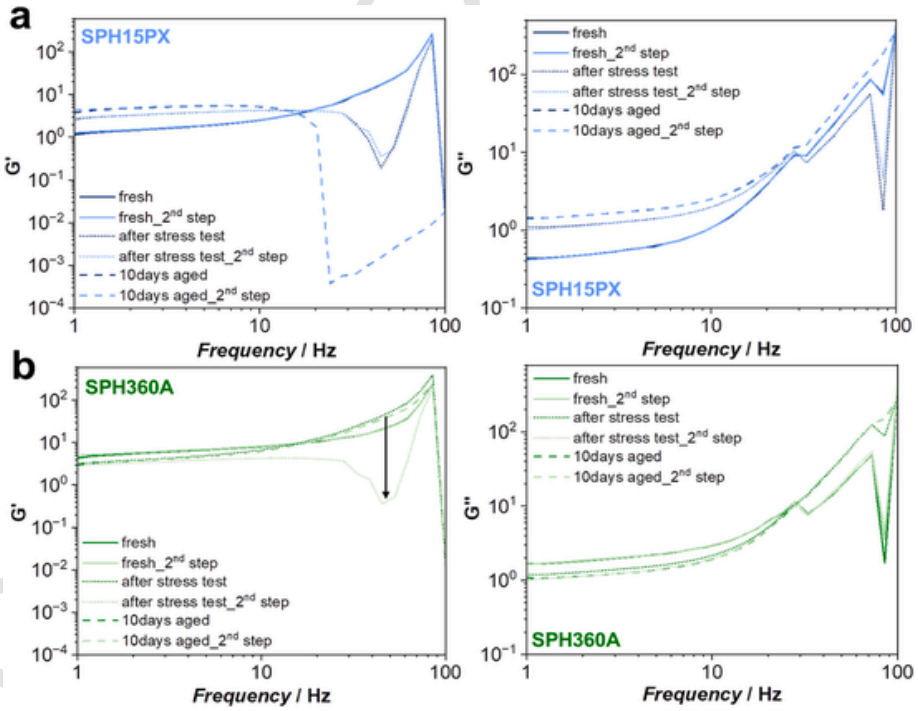

Fig. 6. Storage Modulus (G′) and Loss Modulus (G″) by two-step frequency sweep rheological measurements for emulsions stabilized by **a) SPH15PX** and **b) SPH360A**.

homogeneity of the sample: during the first 1–100 Hz step, G′ and G" behaved similarly to the fresh and aged samples, but in the second 1–100 Hz step, there was a significant drop in G' below 10 Hz (see the arrow in Fig. 6b), which then recovers at higher frequencies.

Then, the fresh **SPH15PX** sample shows behaviour comparable to that of **SPH360A**, but stress test and ageing seem to have a more pronounced effect. After 10 days, the ageing process likely introduced some phase separation, becoming evident in G′ at higher frequencies, while after stress test a sharp decline in G' between 10 and 100 Hz during both steps was observable. The trend in Loss Modulus for the fresh sample is somewhat more uniform than that of **SPH360A** at high frequencies; G" did not exhibit a sudden drop until influenced by the stress test. As in **SPH360A**, 10 days of ageing led to more homogeneous Loss Modulus behavior across the tested frequency range.

Notably, at low frequencies, G' and G" for **SPH360A** are higher than those for **SPH15PX**. Although pinpointing a single reason for this be-

havior is challenging, also given the complexity of the formulation, we can hypothesize that the presence of high molecular weight aggregates may increase the overall viscosity of the system. This idea is further supported by the general trend of the curves: while G′ and G″ exhibited similar maxima, the shear-thickening effect was more pronounced in the **SPH15PX** sample, suggesting a more aggregation degree under high shear rates.

4. Conclusions

Traditional emulsifiers remain cost-effective and reliable for many applications, but the trend toward sustainable and eco-friendly ingredients is likely to continue driving the adoption of bio-based alternatives. Enzymatic hydrolysis of protein-rich biomass usually releases peptides with enhanced surface properties compared with those of parent proteins.

In this paper, a commercially available soy protein isolate (SPI) was hydrolysed with two enzymatic formulations, Alcalase® 2.4L FG and Protamex®, at different incubation times (15, 120 and 360 min) and the resulting soy protein hydrolysates (SPHs) were fractionated by ultrafiltration. Extending the incubation time from 15 to 360 min led to a higher proportion of peptides with molecular weights below 5 kDa in both Alcalase- and Protamex-derived hydrolysates. The surfactant properties of SPHs were throughly investigated, revealing that interfacial behaviour was strongly influenced by both the type of enzyme and the incubation time. SPH15PX and SPH360A were the most effective samples in lowering peanut oil-water interfacial tension, due to the different cleavage site preferences of proteases. Simple peanut oil-in-water (O/W) emulsions stabilized by SPH360A and SPH15PX were prepared using a sonicator and their stability was evaluated by turbidimetry analyses. The SPH360A-based emulsion underwent phase separation after 7 h, whereas the one stabilized by SPH15PX remained fully stable for 7 days. This behaviour can be attributed to a higher content of peptides with more ordered conformations in peanut oil-in-water emulsions, as observed by CD analyses.

In order to investigate SPHs as potentially industrial surfactants, two complex formulations were also prepared incorporating additional ingredients (*i.e.* rheological modifier, solubilizing agent, antioxidants, and buffers). Fresh, after 10 days of storage and accelerated stress test emulsions showed ζ-values below −50 mV. Moreover, both SPH-based emulsions maintained a narrow droplets size distribution, with only a slight increase in the droplet diameter after a spontaneous (10 days) or an accelerated (stress test) ageing. Finally, the frequency sweep experiments (1–100 Hz) demonstrated a shear-thickening behaviour of SPHs emulsions. These findings confirm the ability of enzymatic hydrolysis to tune the techno-functional properties of protein-derived hydrolysates, thereby enhancing their applications as emulsifiers in many industrial sectors.

Uncited reference

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CRediT authorship contribution statement

Giorgia Ballabio: Writing – original draft, Methodology, Investigation, Data curation. **Letizia Scarabattoli:** Writing – original draft, Methodology, Investigation, Data curation. **Martina Rozzoni:** Methodology, Investigation. **Marco Aldo Ortenzi:** Methodology, Investigation. **Giovanna Longhi:** Investigation, Methodology. **Eleonora Pargoletti:** Writing – review & editing, Investigation. **Marco Rabuffetti:** Methodology. **Carlo F. Morelli:** Methodology. **Giovanna Speranza:** Writing – review & editing, Supervision, Conceptualization. **Giuseppe Cappelletti:** Writing – review & editing, Writing – original draft, Supervision, 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.

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Data availability

Data will be made available on request.

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2025.111472.

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